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Identification of potential target genes for the neuron-restrictive silencer factor

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ABSTRACT **The neuron-restrictive silencer factor (NRSF) represses transcription of several neuronal genes in nonneuronal cells by binding to a 21-bp element called the neuron-restrictive silencer element (NRSE). We have performed data base searches with a composite NRSE to identify additional candidate NRSF target genes. Twenty-two more genes, 17 of which are expressed mainly in neurons, were found to contain NRSE-like sequences. Many of these putative NRSEs bound NRSF *in vitro* and repressed transcription *in vivo*. Most of the neuronal genes identified contribute to the basic structural or functional properties of neurons. However, two neuronal transcription factor genes contain NRSEs, suggesting that NRSF may repress neuronal differentiation both directly and indirectly. Functional NRSEs were also found in several nonneuronal genes, implying that NRSF may play a broader role than originally anticipated.**

Transcriptional regulation of gene expression is an important mechanism in the development of neurons. Genes encoding transcriptional regulatory proteins have been revealed by many of the mutations affecting neuronal development in organisms such as *Drosophila* and *Caenorhabditis elegans* (1, 2). Overexpression of transcription factors in *Xenopus* oocytes and targeted mutagenesis of transcription factor genes in mice have also shown the importance of transcriptional regulation during vertebrate neuronal determination and differentiation (3–7). In parallel to these genetic studies, molecular analyses have identified transcription factors common to subsets of neurons that may be important for establishing and maintaining a particular neuronal phenotype (8–12).

To understand the biological role of a transcription factor, it is important to determine the complement of genes it regulates. For example, in pituitary cells, Pit-1 is essential for proper pituitary development and is known to activate its own gene as well as other pituitary-specific genes, such as prolactin (8, 13). Similarly, a target for mec-3 and unc-86, two proteins necessary for neurogenesis in *C. elegans*, is the *mec-3* gene itself (1, 14) as well as the *mec-7* tubulin gene (15). For the most part, however, target genes for other transcriptional regulators implicated in neural development have not yet been identified.

While most transcription factors thought to be involved in neurogenesis act positively, the importance of negative regulation in this process is becoming increasingly clear (16, 17). One of the first negative-acting transcriptional regulators to be implicated in vertebrate neuronal development is the neuron-restrictive silencer factor (NRSF; also known as REST). NRSF was originally defined as a silencer-binding protein for the neuron-specific gene, SCG10 (18). NRSF also negatively regulates the type II sodium channel and synapsin I genes (19, 20). NRSF is a zinc-finger transcription factor (21, 22) that binds to a conserved element known as the neuron-restrictive silencer element (NRSE) or repressor element-1 (18, 19). In contrast to most positive regulators of neuronal genes, NRSF activity, protein, and mRNA are not present in the majority of neuronal cells but are instead found in most nonneuronal cell types (18, 19, 21, 22). Thus, NRSF appears to prevent expression of certain neuronal genes in nonneuronal cells. In addition, NRSF mRNA is expressed in undifferentiated neural precursors (21, 22), suggesting that it may prevent precocious expression of the neuronal phenotype during neurogenesis.

A further understanding of NRSF function would be aided by the identification of additional target genes. In this report, we provide evidence that there are many neuronal genes that contain NRSE-like sequences and that these sequences are likely to act as NRSF-mediated silencer elements. Furthermore, these genes are involved in a wide range of neuronal functions and include two transcription factors that may be positive regulators of neuronal differentiation. Potential NRSEs are also found in some nonneuronal genes, suggesting that NRSF may regulate nonneuronal as well as neuronal genes.

MATERIALS AND METHODS

DNA Data Base Searching. A composite NRSE was derived from the NRSE sequences in the rat SCG10, rat type II sodium channel, human synapsin I, and rat BDNF genes, all of which have been shown to bind NRSF (22). A single nucleotide was assigned to a given position if it occurred in at least three of these four NRSE sequences, and more variable positions were assigned an N. This sequence (TTCAGCACCNCGGACAGNGCC) was used to search the GenBank DNA sequence data base using the FASTA search program (23). The parameters used were: word size, 1 and 3; gap penalty, 12.0; and gap extension penalty, 4.0. Sequences in GenBank are divided into four different sections (rodents, primates, other mammals, and other vertebrates), and each of these sections was searched separately. The top 300 sequences were retrieved from each search. Alignments were not considered further if they contained gaps or two mismatches at the adjacent G residues known to be necessary for NRSF binding (18). Sequences were also removed from consideration if they were located in genes of unknown expression pattern or function (such as sequence tagged sites or pseudogenes). In determining the number of distinct genes identified, homologous genes from different species were counted as one. Similarly, members of closely related multigene families, such as olfactory receptors and cytochrome P450s, were counted as single genes, even if several members contained potential NRSEs.

Electrophoretic Mobility-Shift Assays (EMSAs) and Transient Transfections. EMSAs were performed using native

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Abbreviations: NRSF, neuron-restrictive silencer factor; NRSE, neuron-restrictive silencer element; EMSA, electrophoretic mobility-shift assay.

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NRSF and *in vitro*-translated, truncated human NRSF (λH1) essentially as described (18, 22), except that, in the experiments shown, Klenow-labeled, double-stranded oligonucleotides were used as probes. Oligonucleotides were inserted into the *Hin*dIII site of the SCG10 promoter reporter construct, CAT3 (26). Cell culture, transient transfections of 10T1/2 cells, and chloramphenicol acetyltransferase assays were performed as described (18, 22).

NRSE Oligonucleotides. Oligonucleotides were generated from the NRSE-like sequences listed in Table 1. In addition to the core NRSE-like sequence, the oligos contained an additional 5 bp from the endogenous gene on either side and *Hin*dIII-compatible ends to facilitate subcloning. Precise sequences are available on request.

RESULTS

Potential NRSF Target Genes. A search of the GenBank DNA sequence data base using a 21-bp composite NRSE (see *Materials and Methods*) revealed a large set of genes containing sequences with substantial similarity to the composite. This set included 25 distinct genes containing presumptive NRSEs with two or fewer mismatches to the test sequence. As expected, the three original genes with functionally defined NRSEs (SCG10, type II sodium channel, and synapsin I; refs. 18–20) were among them. To assess the specificity of this data base search, control searches were performed with five randomly shuffled versions of the composite NRSE, as well as the composite NRSE entered backwards. Only two genes with three or fewer mismatches from their respective test sequences were identi-

Table 1. Genes with NRSE-like sequences

Gene	Sequence comparison	Binding activity*	Silencing activity*	Location†
Consensus‡	TTCAGCACCACGGACAGCGCC			
Neuronal genes				
Rat SCG10	----------G--T---	+	+	5′-Reg
Rat type II sodium channel§	-----A----------A--	+		5′-Reg
Human synapsin§	---------G-------T---	+	+	5′-Reg
Rat BDNF§	---------TT------A---	+		Intron
Rat NMDA rec. 1	---------T--------AT-	+	+	5′-UTR
Human nicotinic ACh rec. β2¶	----------	+	+	5′-UTR
Chicken β4-tubulin	---------G----------	+	+	Intron
Chicken middle neurofilament	----------T--	+		5′-Reg
Human glycine rec.	----------G----T-	+	+	5′-UTR
Rat glycine rec.	---------T-A------T--	+		5′-UTR
Rat synaptophysin	-C-------GT------A---	+	+	Intron
Human L1¶	----------G--------AA	+		Intron
Rat atrial natriuretic peptide	----------ACG-	+		3′-UTR
Mouse calbindin¶	AG-------G----------	+		5′-UTR
Rat GABA-A rec. δ subunit	---------GA----G---GA	+	+	Intron
Rat nicotinic ACh rec. α7	AG----G--G---C---A---	−	−	5′-UTR
Mouse P-Lim	---------G----------			5′-UTR
Mouse Hes-3	GG----------			Coding
Human CRF	---------G----------			Intron
Human olfactory rec.	G--------CA----------			Coding
Mouse synaptotagmin IV	---------T-------A--A			5′-UTR
Mouse AMPA rec.	--T------G----------T			5′-Reg
Rat VGF	--------GCT----------			5′-UTR
Rat proenkephalin	--------AC----G--G---			Intron
Nonneuronal genes				
Rat APRT	G--------C-------G---	+		Intron
Sheep keratin	A----------G-	+	+	5′-Reg
Mouse skeletal actin	GG----------C-------	+		3′-Reg
Bovine P450-11β	---------TA------A-G-	+	+	Jxn
Human P450-11β	---------TA------AAG-	+		Jxn
Human T-cell rec. β	G------A-CT------A---	−	−	Coding
Human myosin light chain	C--------CAT-----T---	−	−	Coding
Mouse macrophage protein	CC-------T-A-C-------	−	−	Coding
Rat troponin T	------C--TA------C---			Intron
Rat somatostatin trans. factor	---------T-------A--A			5′-UTR

All genes with two or fewer mismatches to the composite NRSE used in the data base search are listed. Selected genes with three or greater mismatches, for which NRSF binding was experimentally determined, are listed as well. The NRSE-like sequences are compared to the revised consensus NRSE (see also Fig. 3). Deviations shown are derived from comparison to this revised consensus and not to the composite NRSE used in the original data base search. Rec., Receptor; BDNF, brain-derived neurotrophic factor; NMDA, *N*-methyl-D-aspartate; ACh, acetylcholine; GABA, γ-aminobutyric acid; CRF, corticotropin-releasing factor; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; VGF, vascular endothelial growth factor; APRT, adenine phosphoribosyltransferase; trans., transactivating.

*A blank space indicates that the sequence has not been tested for the activity.

†Intragenic location: Coding, Intron, 5′-regulatory region (Reg), 5′- or 3′-untranslated region (UTR), or intron/exon junction (Jxn).

‡New NRSE consensus derived from results of search (not original test composite NRSE).

§Previously tested for binding activity (22).

¶Independently identified in other studies (24, 25).

fied from all six control searches. This demonstrates that NRSE-like sequences are present in the data base much more frequently than random sequences of identical length and base composition.

As NRSF was identified as a negative regulator of neuronal genes, the genes identified by the composite NRSE search were classified as either neuronal or nonneuronal according to their expression pattern. Neuronal genes are defined as those expressed in neurons, with no or limited expression elsewhere. Nonneuronal genes are defined as those expressed in many cell types, which may include neurons. If only the genes containing potential NRSEs with two or fewer mismatches from the composite are considered, then neuronal genes outnumber nonneuronal genes by 4:1 (20:5). Notably, no bias toward neuronal genes was found in any of the control data base searches, even among genes with greater than two mismatches from the composite test sequence. The bias toward neuronal genes in the search performed using the composite NRSE is consistent with the proposed function of NRSF, although the presence of nonneuronal genes suggests an additional role or roles for this protein. A listing of the 25 neuronal and nonneuronal genes with two or fewer mismatches from the composite, as well as selected genes with a greater number of mismatches that were tested for NRSF binding (see below), is given in Table 1.

Many Potential NRSEs Can Bind NRSF and Repress Transcription. Which of the NRSEs in Table 1 are likely to regulate the transcription of their respective genes? To address this question, we tested 24 of the sequences for their ability to bind NRSF *in vitro*. We then examined 13 of these potential NRSEs for silencing activity in transient transfection assays. From these data, we are able to (*i*) refine the NRSE core consensus, thereby facilitating identification of functional NRSEs by sequence inspection; (*ii*) predict NRSF-mediated repression for 17 additional genes whose NRSEs were not directly tested by a transfection assay; and (*iii*) demonstrate NRSF-mediated repression for NRSEs from seven new potential NRSF target genes.

Of the 24 sequences tested for NRSF binding activity, 16 were from neuronal genes and 8 were from nonneuronal genes. The selection of these potential NRSEs for functional assays was not unbiased. Most were chosen on the basis of their high degree of similarity to the composite NRSE, and a few were selected for their divergence to define the limits of NRSF binding (Table 1). Double-stranded oligonucleotide probes representing these 24 potential NRSEs were tested for their ability to bind *in vitro*-translated human NRSF in an EMSA. A truncated form of the protein, encoded by λH1, was used in this assay (22) because the full-length form was found to require nonspecific flanking sequences to bind efficiently (data not shown).

Fig. 1*A* shows that 15 of 16 probes derived from neuronal genes could bind NRSF in a manner similar to the NRSE originally defined in SCG10 (arrow). Three of these sequences (from the type II sodium channel, synapsin I, and BDNF genes) have previously been shown to bind NRSF (22). Two of the probes derive from the same gene (glycine receptor inhibitory subunit) in different species. The one probe that did not bind NRSF derived from the nicotinic acetylcholine receptor α7 subunit gene (Fig. 1*A*, lane 17). Furthermore, the probe derived from the GABA-A receptor δ subunit gene yielded a complex with a different mobility from the control NRSE (Fig. 1*A*, lanes 1 and 13), suggesting that this probe either binds NRSF in a different conformation or with a different affinity. All of the complexes detected contain NRSF, as EMSAs performed with these probes on control *in vitro* translation reactions yielded no specific complexes (data not shown). Of the eight probes selected from the nonneuronal genes, five bound NRSF (Fig. 1*B*), although the cytochrome

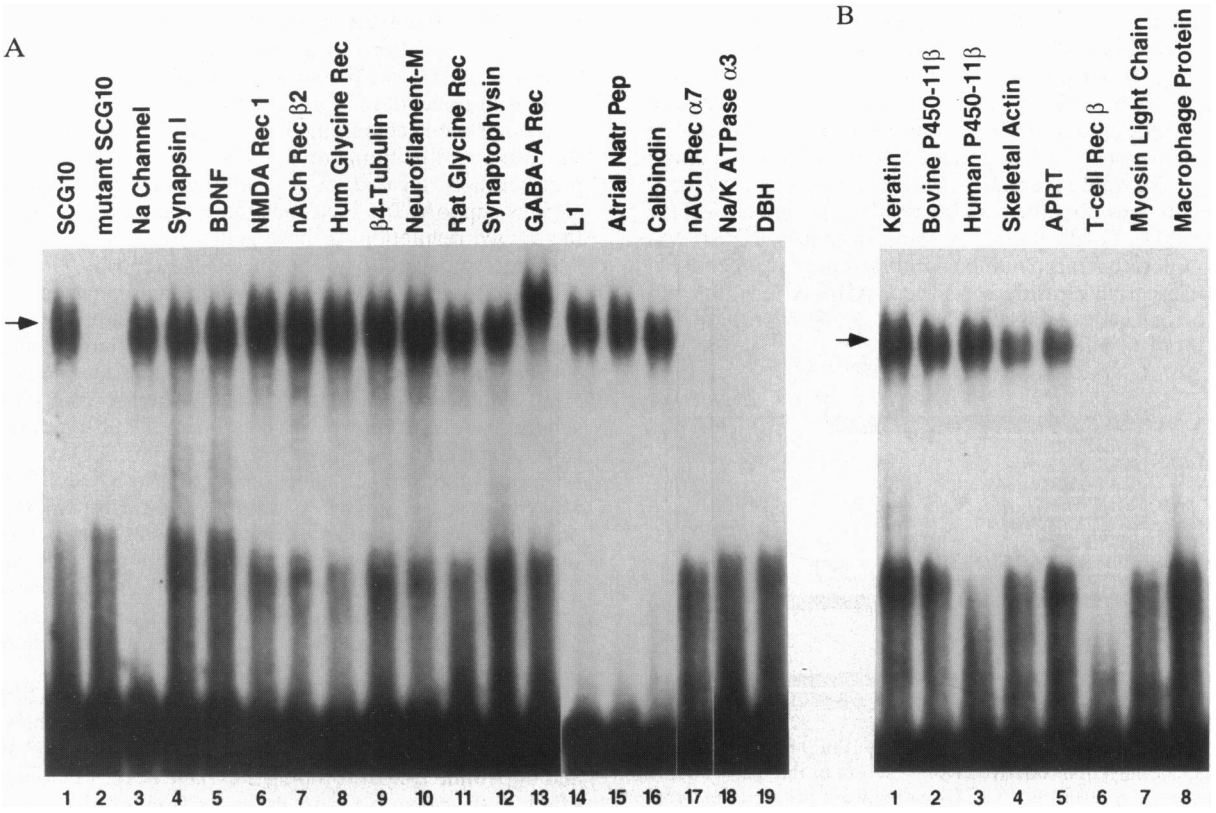

FIG. 1. (*A*) Recombinant NRSF recognizes NRSEs in 15 different neuron-specific genes. EMSAs were performed using *in vitro*-synthesized human NRSF encoded by the λH1 cDNA (22). The arrow indicates the the specific complex. The probes consisted of radiolabeled double-stranded oligonucleotides derived from the genes listed above the lanes. (*B*) Recombinant NRSF recognizes NRSEs in five different nonneuronal genes. EMSAs were performed as in *A*. The arrow indicates the specific complex. The labeled probes were derived from the genes listed above the lanes. See Table 1 for full gene names and NRSE sequences.

P450-11β probes derive from the same gene in different species. The results from the EMSAs performed with labeled probes from each gene were confirmed using each oligonucleotide as an unlabeled competitor against a single, labeled SCG10 NRSE probe. In addition, these competition EMSAs were performed using NRSF from HeLa nuclear extracts, confirming that native as well as recombinant NRSF can bind these sequences (A.J.P. and C.J.S., unpublished data).

To test their ability to repress transcription, 13 of the sequences assayed for NRSF binding were placed upstream of the SCG10 promoter reporter construct, CAT3, which lacks the endogenous SCG10 NRSE (26), and introduced into CH310T1/2 cells. These experiments revealed a parallel between a sequence's ability to bind NRSF *in vitro* and its ability to repress transcription in the transient transfection assay (Fig. 2). Taken with the binding data, the results of this transcriptional repression assay identify five new neuronal genes as strong candidates for NRSF regulation: [*N*-methyl-D-aspartate (NMDA) receptor 1, glycine receptor, nAChR β2, β4-tubulin, and synaptophysin]. Independent studies have shown that the nAChR β2 subunit NRSE is necessary for silencing in nonneuronal cells, in the context of an 1163-bp upstream regulatory region (24). On the other hand, the NRSE from the γ-aminobutyric acid (GABA)-A receptor δ subunit gene, which gave a complex of aberrant mobility in the EMSA, only weakly represses transcription. Our data also indicate that at least two nonneuronal genes may be regulated by NRSF as well (keratin and P450-11β). In addition, promoter analyses have defined functional NRSEs in the rat choline acetyltransferase (P. Lönnerberg, C.J.S., D.J.A., and C. F. Ibáñez, unpublished data) and chicken NgCAM (25) genes. These sequences were not in the data base when we originally performed the searches, but they have been identified in subsequent searches (C.J.S. and A.J.P., unpublished data).

By comparing the 19 different sequences experimentally determined to bind NRSF normally, a refined consensus was derived (Fig. 3), with the most commonly found nucleotide assigned to each position. Thirteen of these positions were either invariant or differed from consensus only once among the 19 sequences (Fig. 3*A*, uppercase letters), while eight of the positions were more variable (Fig. 3*A*, lowercase letters). These results show that the variable residues are not critical for NRSF binding. Furthermore, in some instances, NRSF was shown to tolerate a large number of differences from consensus in these variable residues, as the GABA-A receptor and human cytochrome P450-11β NRSEs have four and five

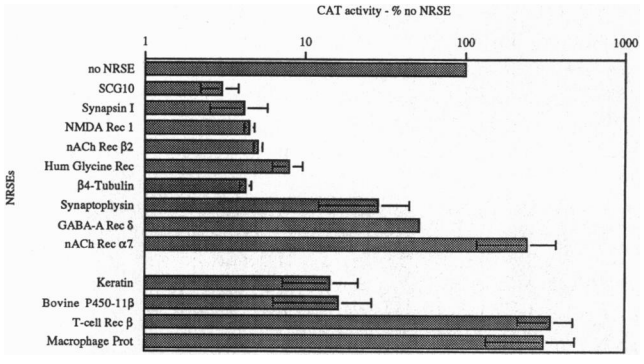

FIG. 2. Sequences that can bind NRSF are able to mediate silencing. Oligonucleotides derived from a subset of the neuronal and nonneuronal genes tested for NRSF binding were inserted upstream of the SCG10 promotor fusion construct, CAT3, which lacks the endogenous SCG10 NRSE (26), and transiently transfected into CH310T1/2 cells, which contain endogenous NRSF. Choramphenicol acetyltransferase activity is presented as a percentage the activity of the non-NRSE containing CAT3 plasmid (no NRSE). Error bars represent the SEMs of two independent experiments.

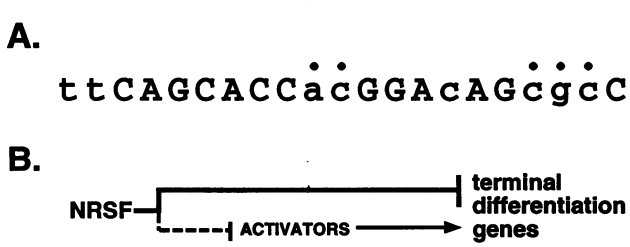

FIG. 3. (*A*). Consensus NRSE derived from 19 sequences for which NRSF binding was experimentally determined. Nucleotides in uppercase letters represent positions that are either invariant, or differed only twice, among the 19 functional NRSEs. Lowercase letters represent residues that varied more frequently. Dotted residues are those most frequently modified in functional NRSEs. (*B*). Potential dual action of NRSF in repressing terminal differentiation genes. Dashed line indicates that the repression of activators is more speculative and is based on the single example of the NRSE in the *P-Lim* gene (12).

mismatches, respectively; yet they bound NRSF (Table 1). While some of these changes may have been compensatory, the relative indifference of NRSF to variations at these residues suggests that a "core" NRSE can be defined (NNCAGCACCNNGCACAGNNNC). All sequences with six or more deviations from the consensus (e.g., nicotinic acetylcholine receptor α7 subunit and below) were found not to bind NRSF.

NRSEs Are Preferentially Located in Noncoding Regions and Are Evolutionarily Conserved. Besides the ability to bind NRSF and repress transcription, two additional criteria were used to evaluate whether a potential NRSE was likely to be functionally relevant *in vivo*: position in the transcription unit and evolutionary conservation. Functionally relevant regulatory sequences tend to be located in nontranscribed or untranslated regions or in introns, rather than in coding regions. Where the information was available, we correlated the positions of NRSEs in their respective transcription units with their number of deviations from the consensus NRSE. Among 21 neuronal NRSEs with four or fewer mismatches, 90% were located in noncoding regions, and of those, 50% were in the 5′-untranslated region; one was in the 3′-untranslated region. The finding that functional NRSEs in neuronal genes are preferentially located in 5′-flanking and other noncoding regions supports the idea that these sequences contribute to the *in vivo* regulation of these genes.

Important regulatory sequences also tend to be evolutionarily conserved. Among eight neuronal genes containing NRSEs for which sequence data was available in different species, most showed a high degree of nucleotide sequence conservation (even between amphibian and mammalian species) (Table 2). In the case of the glycine receptor inhibitory subunit the evolutionary conservation of NRSF-binding ability (for the rat and human genes) was experimentally verified. Moreover, the intragenic location of all the NRSEs in Table 2 was conserved as well. For example, a putative NRSE in the first intron of the corticotrophin-releasing factor was conserved between *Xenopus*, rat, sheep and human, with no more than two deviations from consensus (Table 2). These data provide additional circumstantial evidence that many of the putative NRSEs we have identified are likely to be functionally important *in vivo*.

Repressor Elements in the Sodium/Potassium ATPase α3 Subunit and Dopamine β-Hydroxylase Genes Are Not NRSEs. Negative regulatory regions in the Na/K ATPase α3 subunit and dopamine β-hydroxylase genes have been previously identified that appear to restrict expression of these genes to neurons (27, 28). Within these regions, a sequence with limited similarity to the NRSE was identified in both genes, and it was suggested that these sequences were binding sites for NRSF (27, 28). The Na/K ATPase sequence, however, has eight deviations from consensus including one deleted position, and

Table 2. Evolutionary conservation of potential NRSEs

Gene	Species	Sequence comparison
Consensus	—	TTCAGCACCACGGACAGCGCC
Atrial natriuretic peptide	Human	----------T------AAGA
	Horse	--------------C--AAAA
	Cow	----------T------AAG-
	Mouse	----------A------ACG-
	Rat	----------------ACG-
	Guinea pig	----------------AAG-
Corticotropin releasing factor	Human	---------G----------
	Sheep	--------T----------
	Rat	---------G---------T-
	Xenopus	------------------AA
Skeletal actin	Cow	GC----------T-------
	Pig	CC----------T----C--
	Rabbit	CC----------T----C--
	Mouse	GG----------C-------
NMDA receptor 1	Human	--------G----------
	Rat	---------T--------AT-
	Duck	----------G---G------
Calbindin	Human	AG-------GA----------
	Mouse	AG-------G----------
	Chicken	G--------G----------
nAChR β-2	Human	--------------------
	Mouse	------------------T
	Rat	------------------T
P450-11β	Human	---------TA------AAG-
	Cow	---------TA------A-G-
Synapsin I	Human	---------G-------T---
	Rat	--T--T---G-------A---
	Mouse	---------G---T---A---
L1	Human	----------G--------AA
	Rat	-C----------------AGA
Glycine R. (IS)	Human	--------------G----T-
	Rat	---------T-A------T--

All of the NRSEs listed in the table have been shown to bind NRSF in at least one species (Table 1), with the exception of the Corticotropin releasing factor gene. Comparisons were included only if at least two of the sequences were from species from different phylogenetic orders. Glycine R. (IS), glycine receptor inhibitory subunit; NMDA, *N*-methyl-D-aspartate.

the dopamine β-hydroxylase sequence has nine deviations. This is well above the four-to-five deviation cutoff in binding found for the other putative NRSEs. To directly determine whether these sequences could bind NRSF, oligonucleotide probes representing the two sequences were tested in an EMSA with *in vitro*-synthesized NRSF. Fig. 1*A* (lanes 18 and 19) shows that these elements do not bind NRSF. Furthermore, these elements were not active in competition assays with native NRSF and did not repress transcription in transient transfections assays (data not shown). Thus, these sequences are unlikely to represent true NRSEs.

DISCUSSION

To further understand the role of NRSF in neuronal differentiation, we have sought to identify additional target genes for this transcriptional repressor. The large size of the composite NRSE (19/21 bp specified) permitted us to identify such targets by a data base search, since the site should be present at random only once every 2.7×10^{11} bp. By contrast, smaller binding sites for other transcription factors, such as the 6-bp E-box recognized by MASH1 (29) and other basic helix-loop-helix proteins (30), are present on average once every 4 kb, too frequently to be useful in data base searches. We exploited the search output in two ways. First, by correlating nucleotide sequence with *in vitro* DNA binding and *in vivo* silencing assays, we have identified NRSE positions not critical for NRSF binding and thus further refined the NRSE consensus. Second, we have identified close to two dozen potential neuronal target genes for NRSF. The majority of these target genes encode neuronal terminal differentiation products, supporting the idea that NRSF functions as a direct, coordinate negative regulator of a battery of "end-state" genes. However, some neural transcription factors were also identified, suggesting that NRSF may also act indirectly by repressing positive activators of neuronal genes. Finally, our data suggest that NRSF may regulate some nonneuronal genes as well.

Defining an NRSE. The results of our functional assays indicated that at least 7 of the 21 residues of the NRSE are not critical for NRSF binding. This leaves a 14-bp core sequence that is likely to contain residues that are relatively more important for function. Defining a core sequence facilitates identification of potential NRSEs by visual sequence inspection, by placing a greater weight on deviations from the core sequence rather than by simply counting the total number of mismatches.

In principle, residues essential for NRSF binding could be identified by systematic mutational analysis of NRSEs. Previously, we demonstrated that mutating two adjacent G residues (positions 12 and 13) to T residues drastically reduced both binding and silencing (18). However, the rat glycine receptor inhibitory subunit NRSE has an A at position 13 (as well as two other deviations) and can bind NRSF much better than the double mutant. In fact, a comparison of the different naturally occurring, functional NRSEs taken together with its relatively large size, suggests that no single residue is critical for binding. This implies that a single point mutational study would be largely uninformative. These results are also consistent with the idea that the eight zinc finger DNA-binding domain of

NRSF may make multiple contacts along the NRSE (31, 32). However, certain residues may be more important than others, as only two of the five potential NRSEs with five deviations were able to bind NRSF (Table 1).

Role of NRSEs in Nonneuronal Genes. Our data support the idea that the primary function of NRSF is to regulate neuronal gene expression. Nevertheless, we identified five nonneuronal genes with functional NRSEs. In the case of the skeletal actin and cytochrome P450-11β genes, moreover, the NRSE is highly conserved in both sequence and intragenic location across species (Table 2), implying that it may be functionally important *in vivo*.

What does the presence of NRSEs in non-neuronal genes say about the potential function of NRSF? It is possible that NRSF could function to repress nonneuronal as well as neuronal genes in certain tissues. Alternatively, NRSF could activate transcription of some of these nonneuronal genes; other transcription factors can act as either activators or repressors (33–35). Finally, it is formally possible that other binding factors exist that interact with the NRSEs present in nonneuronal genes.

The case of the skeletal muscle actin gene is particularly informative, as this gene is expressed in a cell type (muscle) in which NRSF is known to be expressed and to repress at least one neuronal gene, the NaII channel (19, 21, 36). The repressive function of NRSF on the actin gene in muscle cells might be overridden by strong, muscle-specific activators that interact with the actin but not the NaII channel gene; alternatively, NRSF could even function as an activator of the actin gene in a muscle cell context. Irrespective of its role in muscle, NRSF could still function to repress the skeletal actin gene in nonmuscle cells. Whatever the case, the skeletal actin gene data imply that NRSF may not necessarily be the primary determinant of the transcriptional state of every NRSE-containing gene.

Function of NRSF in the Nervous System. The neuronal genes identified by the NRSE data base search encode proteins that contribute to many different aspects of the neuronal phenotype: neurotransmitter receptors, ion channels, neurotransmitter-synthesizing enzymes, neuropeptides, cell adhesion molecules, synaptic vesicle proteins, and cytoskeletal components. This suggests that NRSF-regulated genes are involved in most or all neuron-specific functions. Importantly, some NRSE-containing genes are only expressed in a subset of neurons, indicating that NRSF regulation is not limited to "panneuronal" genes. NRSF may function in nonneuronal tissues to prevent ectopic expression of these genes and/or in neuronal precursors to prevent the premature activation of these genes.

The identification of the neuronal transcription factor-encoding gene, *P-Lim*, which contains a sequence identical to a functional NRSE (Table 1), raises the possibility that NRSF may repress neuronal gene expression by a dual mechanism (Fig. 3*B*). P-Lim is a LIM homeodomain protein that is a known activator of pituitary-specific genes (12). In addition to its expression in developing and adult pituitary neuroendocrine cells (12, 37), P-Lim is also transiently expressed by motor neuron precursors (11). P-Lim interacts with another transcription factor, Pit-1, a POU-domain protein required for pituitary development (12). These facts suggest that relief from NRSF-imposed repression could allow expression of positively acting transcriptional regulators, which in turn activate transcription of terminal differentiation genes (Fig. 3*B*). Thus, NRSF may act indirectly as well as directly to repress the neuronal differentiation program.

Note Added in Proof. The m4 muscarinic acetylcholine receptor gene has recently been shown to contain a functional NRSE (38).

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