intervals over a 16-day period. In the laboratory, 25 large (~3 mm in diameter) and 25 small (~0.5 mm in diameter) propagules were each gently released into a cylinder of ambient seawater (27°C) and the sinking rate clocked over the lowermost vertical depth interval of 28 cm.
15. Duplicate samples of all species of Corallinaceae encountered were collected along with three species (two genera) of the closely related family Squamariaceae, whereas representatives of each species were left in situ as field controls. The taxa collected included 12 genera (16 species) with paired thalli (experimental + control) of each species placed in a flowing-seawater system exposed to indirect sunlight. After several days of acclimation, during which no signs of mortality were observed, four CLOD propagules were dropped on the upper surface of one of each experimental pair of the 16 species, the remaining replicate pair controls had ambient seawater pipetted onto their upper surfaces. The experiment was monitored twice daily for 10 days, during which time signs of infection (bleaching behind band migration) were recorded.
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The Neuron-Restrictive Silencer Factor (NRSF): A Coordinate Repressor of Multiple Neuron-Specific Genes

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The neuron-restrictive silencer factor (NRSF) binds a DNA sequence element, called the neuron-restrictive silencer element (NRSE), that represses neuronal gene transcription in nonneuronal cells. Consensus NRSEs have been identified in 18 neuron-specific genes. Complementary DNA clones encoding a functional fragment of NRSF were isolated and found to encode a novel protein containing eight noncanonical zinc fingers. Expression of NRSF mRNA was detected in most nonneuronal tissues at several developmental stages. In the nervous system, NRSF mRNA was detected in undifferentiated neuronal progenitors, but not in differentiated neurons. NRSF represents the first example of a vertebrate silencer protein that potentially regulates a large battery of cell type-specific genes, and therefore may function as a master negative regulator of neurogenesis.

The molecular basis of vertebrate neurogenesis is not well understood. To identify transcriptional regulators of neurogenesis we previously analyzed the transcriptional regulation of a neuron-specific gene, *SCG10* (*1*). The SCG10 5′ regulatory region can be dissected into two functional domains: a proximal region that is active in many cell lines and tissues, and a distal region that represses this transcription in nonneuronal cells (*2*, *3*). This distal region satisfies the criteria for a silencer: a sequence analogous to an enhancer but with an opposite effect on transcription (*4*).

A 24-bp (approximately) element is necessary and sufficient for silencing of SCG10 (*5*). Similar sequence elements with functional silencing activity have been identified in other neuron-specific genes: the rat type II sodium (NaII) channel, human synapsin I (*5*–*8*), and neuronal Na,K-ATPase subunit (*9*) genes. These data suggest that a common cis-acting silencer element may mediate the transcriptional repression of multiple neuron-specific genes. We have therefore named this element the neuron-restrictive silencer element (NRSE) (*5*); in the context of the NaII channel gene, it has been called repressor element 1 (RE1) (*7*). The NRSEs in the SCG10, NaII channel, and synapsin I genes all form complexes with a protein, the neuron-restrictive silencer factor (NRSF), present in nonneuronal cell extracts, but absent in neuronal cell extracts (*5*, *7*, *8*).

To isolate a complementary DNA (cDNA) clone encoding NRSF, we screened a HeLa cell λgt11 cDNA expression library (*10*, *11*) with a probe containing three copies of the NaII NRSE (*12*). One phage was identified, λH1, that like native NRSF bound both the S36 and the Na33 probes but not the control Sm36 probe (*5*, *12*). Competition experiments with unlabeled probes in an electrophoretic mobility shift assay (EMSA) confirmed that the sequence specificity of the λH1-encoded protein (*13*) was similar to that of native NRSF in HeLa cell nuclear extracts (Fig. 1, compare lanes 2 through 7 and 10 through 15). Further evidence for a relationship between native and recombinant NRSF was obtained with a mouse polyclonal antibody to recombinant NRSF (anti-NRSF) (*14*). This antibody specifically supershifted a portion of the λH1-encoded protein-DNA complex (Fig. 2B, lanes 1 to 4), as well as a portion of the native NRSF complex (Fig. 2A, lanes 1 to 4). No supershifts were seen with a control ascites (Fig. 2, A and B, lanes 6 to 8). The antigenic similarity of the recombinant and native NRSF proteins provides independent evidence that the cDNA clone encodes a portion of NRSF.

We performed parallel EMSAs with probes containing potential NRSEs from

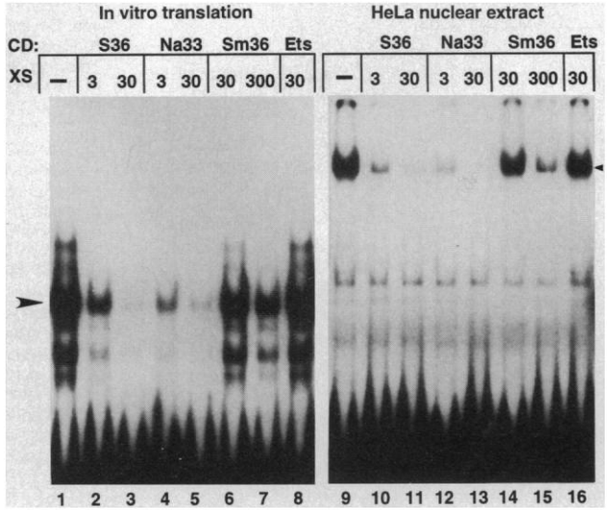

Fig. 1. λH1 encoded protein has the same DNA-binding specificity as native NRSF. EMSAs were performed using a HeLa cell nuclear extract or in vitro translated NRSF (*13*). The probe was a restriction fragment containing two copies of S36. Competitors used were the S36, Na33, and Sm36 oligonucleotides (*12*) and an Ets binding site oligonucleotide (Ets) (*30*). XS indicates molar excess of competitor DNA (CD). The large arrowhead marks the λH1-encoded protein-DNA complex (lane 1), the small arrowhead marks the NRSF-DNA complex (lane 16). The λH1 cDNA does not encode the full-length protein.

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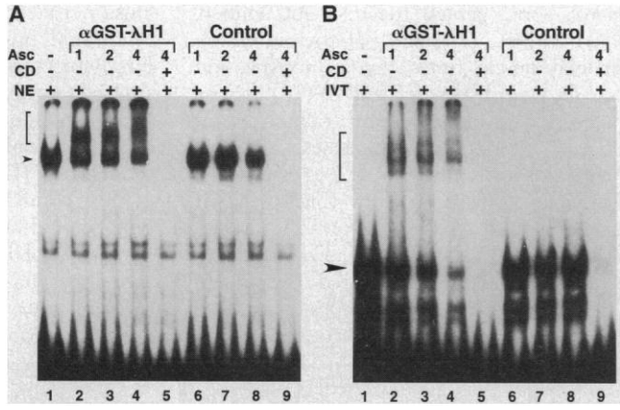

Fig. 2. Antibodies against GST-λH1 recognize the native NRSF-DNA complex. (**A**) The indicated amounts (in μl) of αGST-λH1 (*14*) or a control ascites (Asc) were added to an EMSA containing HeLa nuclear extract (NE). The competitor DNA (CD) was the S36 oligonucleotide present at 300-fold molar excess. (**B**) An EMSA with in vitro translated (IVT) NRSF (*13*). The EMSAs were performed as in (*A*), except that the acrylamide gel used for analysis had an 80:1 acrylamide to bis ratio. Brackets indicate the antibody-supershifted protein-DNA complexes, and the arrowheads the unperturbed complexes. No complexes were formed in a reaction containing Asc alone (*16*).

Table 1. Recombinant NRSF has repressor activity. PC12 cells were cotransfected with reporter plasmids and an expression plasmid containing λHZ4 (*22*). The pCAT3 reporter contains the SCG10 promoter (*2*) fused to the bacterial CAT enzyme; pCAT3-S36++ is pCAT3 with two tandem S36 NRSEs inserted upstream of the SCG10 sequences. The activity of each reporter plasmid in the absence of pCMV-HZ4 was normalized to 100%. The numbers represent the mean ± the standard deviation of two independent experiments performed in duplicate.

Reporter plasmid	pCMV-HZ4 (μg)	CAT activity (%)	Repression
pCAT3.S36++	0	100	1
	1	8.3 ± 0.6	11.4
	4	3.1 ± 0.3	32
pCAT3	0	100	1
	1	77 ± 0.8	1.3
	4	67.5 ± 4	1.5

*Repression is calculated as 100 ÷ percent CAT activity at a given plasmid concentration.

the synapsin I and brain-derived neurotrophic factor (BDNF) (*8*, *15*) as well as the SCG10 and NaII channel genes. Native NRSF yielded similarly sized complexes with all four probes (Fig. 3, lanes 1 to 4). A portion of these four complexes could be supershifted by the antibody to NRSF (*16*), and all four probes bound recombinant NRSF (Fig. 3, lanes 5 to 8). Thus both native and recombinant NRSF were able to interact with putative NRSEs in multiple neuron-specific genes. Additional consensus NRSEs were identified in at least 14 other neuronal genes by a nucleotide database search (*17*).

To isolate longer NRSF cDNA clones, multiple cDNA libraries were screened using a λH1 probe (*18*). Although Northern blots indicated that the NRSF mRNA is 7 to 8 kb (*16*), we were unable to isolate NRSF cDNAs <2 kb, perhaps reflecting a strong stop to reverse-transcription. The sequence of the longest human clone obtained, λHZ4 (2.04 kb), has a continuous open reading frame (*19*) that encodes a novel protein containing eight zinc fingers of the C_2H_2 class with interfinger sequences that place NRSF in the GLI-Krüppel family of zinc finger proteins (Fig. 4, A and B) (*20*, *21*). However, these zinc fingers contain a conserved tyrosine residue absent from the canonical finger sequence (Fig. 4B, dashed box). COOH-terminal to the zinc fingers is a 174–amino acid domain rich in lysine (26%; 46 of 174) and serine or threonine (21 percent; 37 of 174; Fig. 4A).

To determine whether the longest NRSF cDNA encoded a protein with transcriptional repressing activity, we transfected an expression vector containing λHZ4 (pCMV-HZ4) into PC12 cells (which do not contain NRSF activity) together with various target plasmids (*22*). Increasing amounts of pCMV-HZ4 repressed transcription from an NRSE-containing target plasmid from 11 to 32 times (Table 1). In control transfections performed with a target plasmid lacking an NRSE or containing a mutated (5) NRSE, the repression was only 1.5 times at the maximal pCMV-HZ4 concentration (Table 1) (*16*). These results indicated that the λHZ4 clone contains at least a portion of the transcriptional repression domain and that this repression requires NRSE-binding.

The absence of NRSF activity in neuronal cells (*2*, *5*–*7*) could reflect a lack of NRSF gene expression or an inactivation of NRSF. To distinguish between these possibilities, we performed RNase protection assays (*23*) on several neuronal and nonneuronal cell lines. No NRSF transcripts were detectable in two neuronal cell lines, MAH and PC12 (Fig. 5, lanes 4 and 5; rNRSF). In contrast several glial and two fibroblast cell lines expressed NRSF mRNA (Fig. 5, lanes 6 to 9). These data indicated that the absence of NRSF activity in neuronal cells is due to a lack of NRSF expression, not to its functional inactivation.

Using a mouse NRSF cDNA clone (*16*)

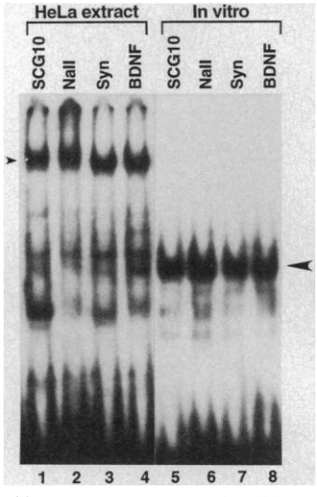

Fig. 3. Native and recombinant NRSF recognize NRSEs in four different neuron-specific genes. EMSAs were performed using either HeLa nuclear extract (lanes 1 to 4) or in vitro synthesized NRSF (lanes 5 to 8). The probes contained NRSEs from the SCG10 (lanes 1 and 5); NaII channel (lanes 2 and 6); synapsin I (lanes 3 and 7); or the BDNF (lanes 4 and 8) genes. The large and small arrowheads indicate the specific complexes obtained with recombinant and native NRSFs, respectively.

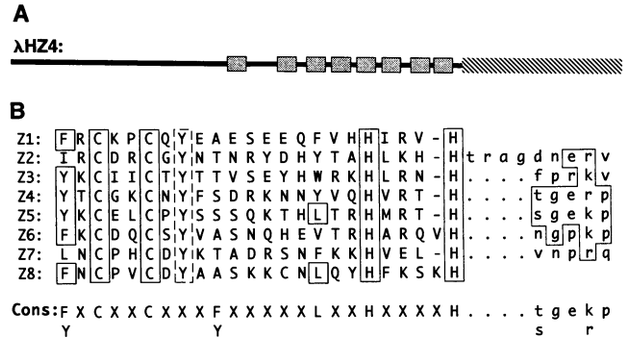

Fig. 4. (**A**) Schematic diagram of the predicted amino acid sequence (*19*) from the NRSF λHZ4 cDNA clone. Stippled boxes indicate the position of zinc fingers, cross-hatched region a domain rich in basic amino acids. (**B**) Alignment of NRSF zinc finger and interfinger sequences. The eight zinc fingers of human NRSF were aligned beginning with the conserved aromatic residue and including the interfinger sequences of fingers z2-7. The consensus (Cons) for GLI-Krüppel zinc fingers and interfinger sequences is shown for comparison.

as a probe, we next performed in situ hybridization experiments on mouse embryos (*24*). At E12.5, NRSF mRNA was detected in the ventricular zone of the neural tube (Fig. 6A, arrow), a region containing multipotential progenitors of neurons and glia (*25*), which do not express SCG10 mRNA (compare Fig. 6B, arrow). In contrast, the adjacent marginal zone of the neural tube which contains SCG10 positive neurons (Fig. 6B, open arrow) was largely devoid of NRSF expression (Fig. 6A, open arrow) NRSF mRNA was also detected in the ventricular zone of the brain (Fig. 6E, arrowhead). In the peripheral nervous system, NRSF mRNA was absent or expressed at low levels in sympathetic and dorsal root sensory ganglia (DRG) at E13.5 (Fig. 6C, small and large arrowheads), whereas these ganglia expressed SCG10 mRNA (Fig. 6D, small and large arrowheads). Thus, these data suggest that NRSF is expressed by undifferentiated neuronal progenitors but not by differentiated neurons in vivo.

The SCG10 NRSE is required to prevent expression in multiple nonneural tissues throughout development (*3*). This broad requirement for the NRSE was reflected in a broad expression of NRSF mRNA. The NRSF mRNA was detected in many embryonic nonneural tissues such as the adrenal gland, aorta, genital tubercle, gut, kidney, lung, ovaries, pancreas, parathyroid gland, skeletal muscle, testes, thymus, tongue, and umbilical cord (Fig. 6, E and F) (*16*). RNase protection revealed NRSF transcripts in many adult nonneuronal tissues, including heart and liver (*16*), which expressed little NRSF mRNA in embryos (Fig. 6). This broad expression pattern is consistent with a role for NRSF as a near-ubiquitous negative regulator of neuron-specific gene expression.

Four lines of evidence support the conclusion that our cDNA clones encode a functional fragment of authentic NRSF. First, recombinant and native NRSFs showed similar in vitro DNA binding specificities. Second, antibodies generated against recombinant NRSF bound to native NRSF. Third, the presence or absence of NRSF mRNA in cell lines paralleled both NRSE-dependent silencing activity and NRSF DNA-binding activity in nuclear extracts. Fourth, the longest NRSF cDNA clone repressed transcription in

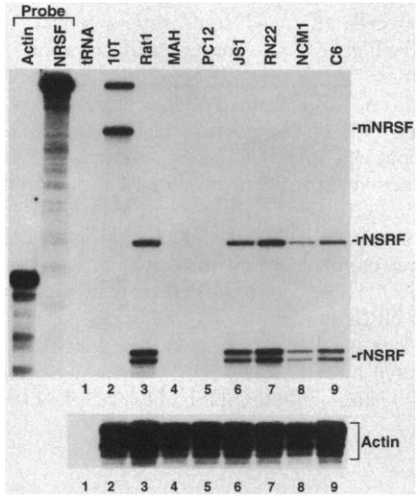

Fig. 5. Analysis of NRSF message in neuronal and nonneuronal cell lines. RNase protection assays (*23*) were performed on total RNA from various cell lines. The two neuronal cell lines were MAH (*31*) and PC12 (*32*). The glial lines were: RN22 (*33*), JS-1 (*34*), NCM-1 (*35*), and C6 (*36*); the fibroblast lines were Rat1 and mouse C3H10T 1/2 (10T). "tRNA" indicates a negative control. The probes were derived from mouse NRSF and rat β-actin cDNAs. rNRSF and mNRSF indicate the protected products obtained with RNA from rat or mouse cell lines, respectively. The size difference between mNRSF and rNRSF most likely reflects an incomplete protection of the mouse probe by the rat transcript.

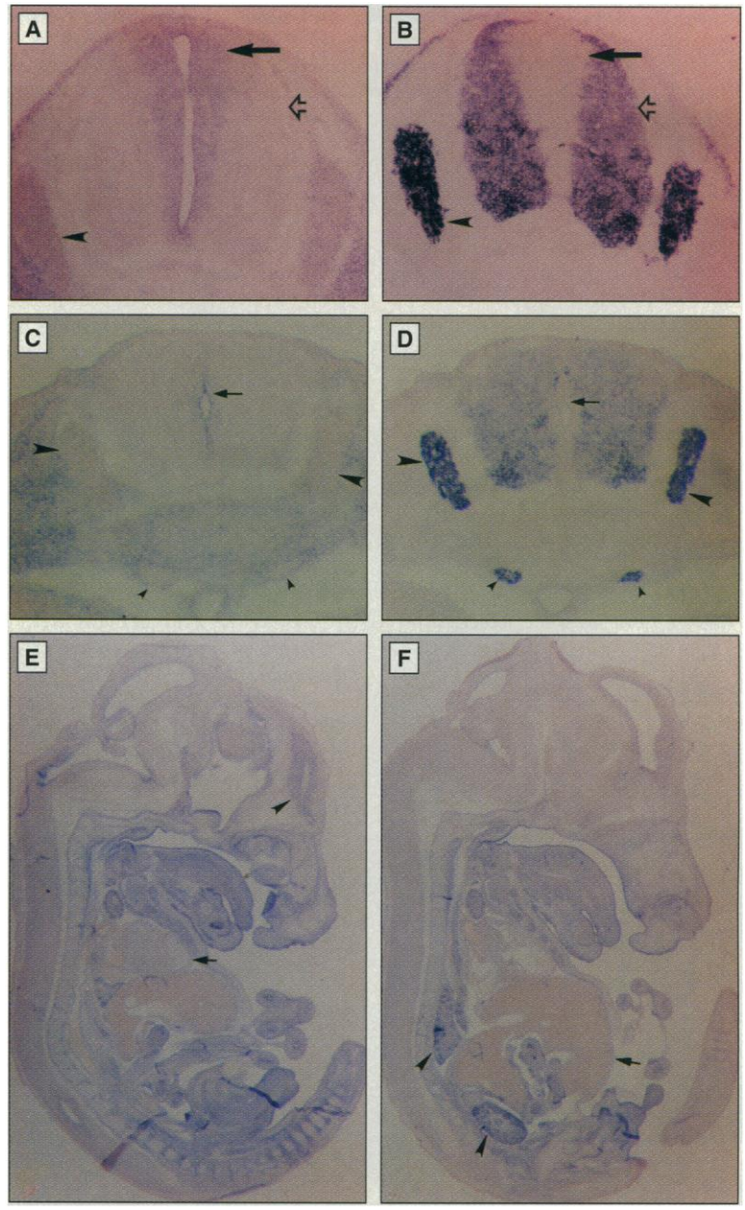

Fig. 6. (**A** to **D**) Comparison of NRSF and SCG10 mRNA expression by nonradioactive in situ hybridization (*24*). Adjacent transverse sections of E12.5 (A and B) and E13.5 (**C** and **D**) mouse embryos were hybridized with NRSF (A and C) or SCG10 (B and D) antisense probes. The solid and open arrows (A to D) indicate the ventricular and marginal zones of the neural tube, respectively. The large and small arrowheads (A to D) indicate the sensory and sympathetic ganglia, respectively. Control hybridizations with NRSF sense probes revealed no specific signal (*16*). (**E** and **F**) Widespread expression of NRSF mRNA in nonneural tissues. In situ hybridization with an NRSF antisense probe was performed on parasaggital sections of an E13.5 mouse embryo. Arrowheads mark several positive tissues, the arrows negative tissues.

vivo in an NRSE-dependent manner.

Functional NRSEs have been identified in four neuron-specific genes: SCG10, NaII channel, synapsin I (*5–8*) and neuronal Na,K-ATPase subunit (*9*), while 14 other neuronal genes contain consensus NRSEs (*17*). Although silencer function has not yet been demonstrated for these potential NRSEs, native and recombinant NRSF bound to six of these sequences (Fig. 3) (*16*), and previous data indicate a strong correlation between NRSF binding and silencing activity (*5*, *7*). We therefore conclude that NRSF may silence at least 18 neuron-specific promoters. Thus NRSF may be the first vertebrate silencer factor that coordinately represses a battery of cell type–specific genes. This would provide experimental support for the idea that the maintenance of the differentiated state involves active negative regulation of gene expression (*26*).

In other systems, positive-acting transcription factors that regulate multiple lineage-specific target genes have been shown to function as master regulators of cell type determination or differentiation (*27–29*). By analogy, NRSF may function as a master negative regulator of the neuronal phenotype. Specifically, the presence of NRSF in neuronal progenitors, together with its proposed coordinate negative regulation of many neuronal genes, suggests that relief from NRSF-imposed repression may be a key event in neurogenesis. The identification of NRSF therefore provides an opportunity to further understand the control of this event.

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12. S36 and Na33 are the NRSE elements present in the SCG10 and NaII channel genes, respectively. The Sm36 sequence contains two point mutations in the S36 sequence and has an approximately 100 times lower affinity for NRSF. For oligonucleotides, the sequence of the top strands of the oligonucleotides used for library screening and EMSAs are given below. The upper case sequences represent actual genomic sequence, the lower case sequences were used for cloning purposes: S36: agctGCAAAGCCATTTCAGCACCACGGAGAGTGCCTCTGC; Na33: agcttATTGGGTTTCAGAACCACGGACAGCACCAGAGTa; Syn: agcttCTGCCAGCTTCAGCACCGCGGACAGTGCCTTCGCa; BDNF: agcttAGAGTCCATTCAGCACCTTGGACAGAGCCAGCGGa; Ets: agcttGCGGAACGGAAGCGGAAACCGa.
13. The λH1 cDNA was subcloned into the Eco RI site of pRSET B (Invitrogen). Recombinant λH1 was produced by coupled in vitro transcription and translation with a rabbit reticulocyte lysate according to manufacturer's protocol (Promega). Mobility shift assays were performed as described [N. Mori, C. Schoenherr, D. J. Vandenbergh, D. J. Anderson, *Neuron* **9**, 45 (1992)], except that 0.5 μg supercoiled plasmid and 10 μg of BSA were included in each reaction.
14. The λH1 cDNA was inserted into the Eco RI site of pGEX-1, a prokaryotic glutathione *S*-transferase fusion expression vector [D. B. Smith and K. S. Johnson, *Gene* **67**, 31 (1988)]. Gel slices containing the fusion protein were used as antigen to produce a polyclonal mouse ascites as described [S. K. H. Ou, J. M. C. Hwang, P. H. Patterson, *J. Immunol. Meth.* **165**, 75 (1993)].
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18. Five different cDNA libraries, derived from human, mouse, and rat tissues were screened by plaque hybridization. The selection of libraries included those made with inserts size-selected for length greater than 4 kb. No cDNA isolated from any library extended beyond the 5′ end of clone λHZ4.
19. The sequence of the λHZ4 human NRSF cDNA has been submitted to Genbank, under accession no. U13879.
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