THE ROLE OF THE NEURON-RESTRICTIVE SILENCER FACTOR DURING VERTEBRATE EMBRYOGENESIS

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Alice Jean Paquette

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ABSTRACT

The gene-expression profile of a cell in large part determines what functions that cell can perform. Cell-type specific gene expression is set up over the course of development as different tissues and cell types arise. By studying the mechanisms of cell-type specific gene expression, one may uncover processes involved in the generation of different cell types.

The neuron-restrictive silencer factor (NRSF) was isolated in an effort to discover mechanisms involved in the generation of neurons during vertebrate embryogenesis. NRSF is a zinc-finger transcriptional repressor that is known to have many, primarily neuron-specific genes as putative direct targets. It is expressed widely outside of the nervous system, and in the nervous system it is expressed in neural progenitors. NRSF is downregulated in differentiated neurons. Given its action as a repressor, its expression pattern, and its list of potential target genes, NRSF was likely to play an important role in neural development.

We chose to test this idea by both inhibiting NRSF function and overexpressing NRSF in chicken embryos. In order to inhibit NRSF function in vivo we infected embryos with a retrovirus encoding a dominant-negative form of NRSF. Ectopic expression of three neuronal target genes was observed in non-neural tissue. Within the nervous system, two of these genes were derepressed in neural progenitors. Premature neurogenesis, however, was not seen, and the low-level expression of neuron-specific genes was insufficient to convert neural progenitors into ectopic neurons.

Overexpression of NRSF was performed by electroporating expression constructs for NRSF into one side of the neural tube of developing chicken embryos. One target gene that is normally expressed at low levels in neural progenitors was repressed in this

cell type. Another target gene was repressed in the differentiated neurons. This gene, Ng-CAM, is important for axon pathfinding of spinal-cord commissural neurons. Some axons of NRSF-overexpressing commissural neurons showed pathfinding errors. The extent of neurogenesis and the expression of some NRSF target genes, however, was apparently unaffected by this manipulation. NRSF, therefore, is important for proper gene regulation during neural development, but is not a direct regulator of the process of neurogenesis itself.

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Chapter 1

INTRODUCTION

The gene-expression profile of a cell in large part determines what functions that cell can perform. Specific cell types in the mature organism, therefore, have distinct patterns of gene expression. This cell-type specific gene expression is established over the course of development. In a mature cell gene expression levels can be further modified by physiological stimuli. A gene's regulatory region, therefore, must contain elements that allow the gene to be responsive to changes in both the developmental and physiological state of each cell.

One particularly interesting and complex cell type is the neuron. Neurons are a major component of the nervous system – the organ system, consisting of brain, spinal cord, and peripheral nerves and ganglia, that receives signals from the external and internal environment, processes them, and provides signals to effector organs such as muscles and glands to respond appropriately. In addition to the neuron, which carries signals in the form of nerve impulses, the other major cell type of the nervous system is the glial cell, which provides support functions for neuronal structure and activity.

Although there are an extremely large number of different neuronal subtypes, defined according to their location within the nervous system, their morphology, connectivity, etc., there are many aspects that all neurons have in common. Functioning as a neuron requires having specialized cellular processes, adhering to and forming synapses with appropriate cells, synthesizing a neurotransmitter and packaging it in synaptic vesicles, and having neurotransmitter receptors and ion channels. (This is not an exhaustive list.) All neurons, therefore, must express the genes that act to fulfill these requirements, such as genes encoding specialized cytoskeletal components and cell-adhesion molecules, neurotransmitter-synthesizing enzymes, synaptic vesicle proteins, and subunits of neurotransmitter receptors and ion channels.

As mentioned above, cell-type specific gene expression is established over the course of development. For example, at early stages of development, the genes that underlie the function of mature neurons tend not to be expressed. Instead, there is expression of genes that are involved in promoting cell division, enabling morphogenetic movements, patterning undifferentiated tissue, specifying progenitor cells, etc. It is only during the process of differentiation of neural progenitor cells into neurons that neuron-specific genes begin to be expressed. Neural progenitors in a wide range of animals can give rise to both neurons and glia, i.e., cell types that do and do not express neuron-specific genes. This may in part explain why in general it is only after a neuronal derivative of a neural progenitor ceases to divide that it begins to express the genes characteristic of a mature neuron. Obviously, over the course of development and especially during neurogenesis the expression of neuron-specific genes is tightly regulated.

Regulating the process of neurogenesis

Little is actually known about the factors that directly regulate the expression of neuron-specific genes during neurogenesis. A number of transcription factors are known, however, that regulate the process of neurogenesis. These were initially identified in *Drosophila*, and relatively recently homologues performing similar functions in vertebrates have been isolated and characterized (Jan and Jan, 1993; Kageyama and Nakanishi, 1997; Lee, 1997; Lewis, 1996). In *Drosophila* proneural genes such as *atonal* and members of the *achaete-scute* (*ac-sc*) complex positively regulate neural fate. On the other hand, *hairy* and *Enhancer of split* (*E[spl]*) antagonize the action of these genes

and inhibit neuronal differentiation. Another negative regulator of neurogenesis in *Drosophila* is *extramacrochaetae* (*emc*).

All of these genes encode basic helix-loop-helix (bHLH) transcription factors, except *emc*, which has no basic domain. A bHLH transcription factor binds to DNA via its basic domain and dimerizes with itself or other HLH proteins via its HLH domain. The first two genes mentioned above encode transcriptional activators. The second two encode transcriptional repressors that both repress the transcription of the first two genes and inhibit the function of their products by heterodimerizing with them. The last one encodes a transcription factor that can only act by inhibiting the ability of a dimerization partner to bind to DNA.

The vertebrate homologues of these *Drosophila* genes isolated so far include the *ac-sc* homologues: Mash1, Cash1 and 4, and Xash1 and 3; the *atonal* homologues: Math1, Math2/NEX-1, Xath3, neurogenin (ngn) 1 and 2, and NeuroD; the *hairy* and *E[spl]* homologues: Hes1 through 5; and the *emc* homologues: Id1 through 4 (Kageyama and Nakanishi, 1997; Lee, 1997). A number of these genes have been shown to be important regulators of different stages of vertebrate neural development. The following examples are not an exhaustive review, but give an idea of the kinds of roles these *Drosophila* homologues can have in the context of vertebrate embryogenesis.

Vertebrate neural development begins when the neural plate and a set of placodes are formed by the transformation of ectoderm into neural ectoderm. Xash3 has been shown to be expressed in the *Xenopus* neural plate and to have a role in determining the amount of ectoderm that is converted to neural ectoderm, i.e., in positioning the borders of the neural plate (Ferreiro et al., 1994). In birds and mammals, the neural plate then begins to roll up, forming crests at each edge from which neural

crest cells begin to migrate. Id2 has been shown to be highly expressed in these crests at the cranial level in chicken and to be involved in regulating the amount of ectoderm that contributes to the cranial neural crest cell population (Martinsen and Bronner-Fraser, 1998). The neural plate eventually forms the neural tube, and neural crest cells continue to migrate from the dorsal part. Among other structures, neural crest cells form sensory, or dorsal root, ganglia and sympathetic ganglia. Ngn1 and 2 have been shown to be expressed in sensory ganglia (Sommer et al., 1996) and to be involved in determining sensory fate (Perez et al., 1999). Mash1, on the other hand, has been shown to be expressed in sympathetic ganglia and to be required for sympathetic differentiation (Guillemot et al., 1993; Sommer et al., 1995).

When the neural tube first forms, it consists entirely of neural progenitors, which are mitotically active. Later, however, neurons begin to be generated by progenitor cells giving rise to daughters that first undergo a terminal cell division then migrate laterally to form the marginal zone. It is in this region of the developing neural tube where these newly born neurons upregulate neuron-specific genes. The various HLH genes tend to be expressed in either the mitotically inactive marginal zone or the mitotically active ventricular zone (where the progenitors reside). This expression pattern is related to the functional classification of each of these genes as either a neuronal determination gene or a neuronal differentiation gene (Lee, 1997; Ma et al., 1996). Neuronal differentiation genes, such as NeuroD, begin to be expressed in post-mitotic neurons as they migrate into the marginal zone, and gain-of-function experiments have shown that they can promote neurogenesis (Lee et al., 1995). Neuronal determination genes, such as the neurogenins, are expressed in neural progenitors, and gain-of-function experiments have shown that they upregulate neuronal differentiation genes and, likely in doing so,

promote neurogenesis (Ma et al., 1996). Conversely, loss-of-function experiments have shown that neuronal determination genes are required both for the expression of the downstream, neuronal differentiation genes and for neuronal differentiation itself (Cau et al., 1997; Ma et al., 1999).

Neurogenesis regulated solely by the neuronal determination and differentiation genes described so far would likely result in a very small number of mature neurons, because progenitor cells would be promoted into differentiating before sufficient numbers of progenitor cells had been generated. Neurogenesis, therefore, as in the case of the differentiation of many different cell types, requires the effective regulation of the antagonistic processes of cell division and cell differentiation (Kageyama and Nakanishi, 1997). As mentioned above, a number of negative regulators of neurogenesis have been identified in *Drosophila*. Vertebrate homologues of these genes, therefore, have the potential of being negative regulators of vertebrate neurogenesis, but their function cannot be assumed a priori. The *emc* homologues Id1–4, for example, do indeed lack a basic domain, and as such have the ability to inhibit the transcriptional regulatory ability of bHLH proteins, but Id2 has been found to promote neuronal differentiation (Martinsen and Bronner-Fraser, 1998), and Id4 is known to be first expressed in newly born neurons (Jen et al., 1997). Id1 and Id3, however, are expressed in neural progenitors as would be expected for a potential inhibitor of neurogenesis (Jen et al., 1997).

Of the vertebrate homologues of the *hairy* and *E[spl]* genes, the Hes1 gene has been most convincingly shown to be a true inhibitor of neurogenesis, or "anti-neuronal HLH gene" (Kageyama and Nakanishi, 1997). Hes1 is expressed in neural progenitors (Sasai et al., 1992), and it has been shown to be able to directly repress the

transcription of the neuronal determination gene, Mash1 (Chen et al., 1997). Targeted disruption of Hes1 in mouse results in upregulation of Mash1, and premature neuronal differentiation in both the telencephalon and retina (Ishibashi et al., 1995; Tomita et al., 1996). In the eye, the increased rate of neuronal differentiation resulted in smaller retinas, as would be expected if Hes1 were required to prevent differentiation sufficiently for enough progenitor cells to be generated (Tomita et al., 1996). Maintaining the expression of Hes1 in neural progenitor cells via retroviral transduction prevented the cells from differentiating into neurons in both the cerebral cortex and retina (Ishibashi et al., 1994; Tomita et al., 1996). Hes1, therefore, has essentially all the characteristics of a negative regulator of neurogenesis.

Interestingly, Hes1 is expressed not only within the nervous system during embryogenesis, but also in developing muscle (Sasai et al., 1992). One might predict, therefore, that in a Hes1 null mutant embryo presumptive muscle cells might express neuronal determination genes and begin to differentiate into neurons. Indeed, when the neuronal determination gene, ngn1 or 2, is ectopically expressed in dermomyotomal cells, ectopic sensory neurons arise (Perez et al., 1999). Loss of Hes1 function in the developing muscle does not, however, lead to ectopic neurogenesis (Ishibashi et al., 1995). This is likely because outside of the context of the nervous system loss of repression of neuronal determination genes by Hes1 is insufficient for these target genes to be adequately upregulated to begin the process of neurogenesis. Hes1 may, in fact, play an anti-myogenic role during muscle development, because, as a bHLH transcription factor, through heterodimerization it can inhibit the function of other bHLH factors that are involved in the differentiation of other cell types. MyoD is a bHLH transcription factor that can induce fibroblasts to differentiate into muscle. Co-

expression of Hes1 along with MyoD can prevent this transformation (Sasai et al., 1992). Thus, Hes1 may not only serve to negatively regulate the process of neurogenesis during embryonic development, but of myogenesis as well. This illustrates the fact that when cascades of transcriptional regulatory factors are involved in generating specific cell types, the farther upstream the factor is in the regulatory cascade, the less tightly it is associated with the final phenotype.

Regulation of neuron-specific gene expression during neurogenesis

Identification and characterization of the transcription factors described above has gone a long way towards elucidating the mechanisms involved in regulating neural development. The proper balance between the number of neural progenitors and differentiated neurons over the course of neural development is maintained by the opposing actions of the anti-neuronal HLH genes and the neuronal determination genes. As the daughters of neural progenitors drop out of the cell cycle, the neuronal determination genes succeed in activating the transcription of the neuronal differentiation genes. This event is soon followed by initiation of expression of the neuron-specific genes that are characteristic of the mature state.

Although attempts have been made to identify the direct targets of the neuronal differentiation genes, establishing a gene as a true, in vivo direct target is difficult. This is because most transcription factors recognize short DNA elements that are frequently found throughout the genome. The recognition element of all bHLH factors that activate transcription is the E-box: 5' CANNTG 3'. The presence of this sequence in the regulatory region of a gene is obviously insufficient to indicate that the gene is a target

of a particular bHLH factor. Only with corroborating in vivo expression data, in vitro DNA binding and transcriptional activation data, etc., can a gene be considered a candidate for direct regulation by a particular bHLH protein. This problem has left almost completely unanswered the question of how neuron-specific gene expression is regulated during neurogenesis. The following section describes an attempt at addressing this question.

Identification of the Neuron-Restrictive Silencer Element

A sequence element that mediates the transcriptional regulation of neuron-specific gene expression is the Neuron-Restrictive Silencer Element (NRSE), also known as Repressor Element-1 (RE-1) (Maue et al., 1990; Mori et al., 1990). This sequence element was first discovered in the regulatory regions of two different genes whose expression is restricted to the nervous system: Superior Cervical Ganglion 10 (SCG10) and the type II sodium channel. Coarse deletion analyses of the promoters and 5' flanking regions of these two genes revealed that in each case removal of a particular segment of the regulatory region resulted in upregulation of reporter gene expression in non-neuronal cell lines, while expression in neuronal cell lines was unaffected. This result was also replicated in vivo, where the regulatory region of the SCG10 gene with the deletion directed ectopic reporter gene expression in many organs such as kidney, lung, and spleen, while the intact construct gave expression only in neural tissue (Wuenschell et al., 1990). Detailed deletion analyses combined with sequence comparison of the relevant regions of the two genes later identified a 21 base-pair (bp) element as responsible for part of the repressive activity of the deleted sequences (Kraner et al.,

1992; Mori et al., 1992). This element was termed the NRSE by one group and RE-1 by the other.

The mechanism of the tissue-specific nature of the transcriptional repression mediated by this element was investigated by gel-shift analysis (Kraner et al., 1992; Mori et al., 1992). A shifted band was found to be present when an NRSE probe was incubated with extracts of non-neuronal cell lines, but not of neuronal cell lines. The tissue-specificity of the repression, therefore, was regulated at the level of NRSE binding. Either the protein that bound to the NRSE was not present in the neuronal cell lines, or the protein was unable to bind to the NRSE in these cells. To further characterize this NRSE-binding activity, mutations were made in the NRSE that significantly reduced binding. The mutated NRSE was then tested to determine whether diminished binding of the factor identified in the gel-shift would correlate with diminished ability to mediate repression of a reporter construct in non-neuronal cells. The mutated NRSE was, indeed, much less effective than the wild-type sequence. These results provided the data and tools necessary for the identification of a specific NRSE-binding factor.

Identification of the Neuron-Restrictive Silencer Factor

The human clone of the transcription factor that binds to the NRSE was identified by three different groups using different criteria and different techniques. Anderson and colleagues used phage display with radioactive wild-type and mutant NRSE sequences as probes. They identified a phage clone that expressed a protein fragment that recognized the wild-type but not mutant NRSE (Schoenherr and Anderson, 1995). This group

termed the protein the Neuron-Restrictive Silencer Factor (NRSF). Mandel and colleagues used the yeast one-hybrid method with wild-type and mutant NRSE sequences inserted upstream of reporter genes (Chong et al., 1995). They identified a colony that expressed an activation-domain fusion protein that activated reporter-gene expression only when it was under the control of the wild-type NRSE. This group named the protein the RE-1 Silencing Transcription Factor (REST). In contrast to the other two groups, Strominger and colleagues did not use binding to the NRSE as the criterion for identification, yet they identified a segment of the same protein as did the previous groups (Scholl et al., 1996). This group used phage display with a different regulatory element, the X2 box of the MHC class II gene, DPA, to identify a plaque expressing an X2 box-binding protein fragment. The portion of the protein identified in this way was completely different from the portion identified according to its ability to bind the NRSE. This would indicate that the intact protein contains two distinct DNA-binding domains; however, X2 box binding has not been replicated by other groups (Thiel et al., 1998; G. Mandel, personal communication).

NRSF was found to have a single type of recognizable structural motif: the Krupple-type, C_2H_2 zinc finger. The protein contains nine of these zinc fingers, eight of which are clustered together and form the DNA-binding domain isolated in the two NRSE-based screens (Chong et al., 1995; Schoenherr and Anderson, 1995). The ninth is at the extreme C-terminus, and structure-function analysis has shown that this is one of two domains involved in the repressive activity of NRSF (Tapia-Ramirez et al., 1997; Thiel et al., 1998). This zinc finger has been found to interact with a corepressor termed CoREST (Andres et al., 1999). The second repression domain is at the extreme N-terminus of the protein (Tapia-Ramirez et al., 1997; Thiel et al., 1998), and has been

found to interact with Sin3A and histone deacetylase, factors involved in chromatin remodeling (Huang et al., 1999). The significance of these protein-protein interactions will be discussed further below.

When NRSF was originally cloned, it was confirmed in a number of different ways to be at least a component of the non-neuronal NRSE-binding activity demonstrated by gel-shift analysis (Chong et al., 1995; Schoenherr and Anderson, 1995). First, protein synthesized from the cloned cDNA was shown to specifically recognize the NRSE in gel-shift experiments. Second, antibodies were generated against the cloned protein and shown to be able to supershift a gel-shift complex from the appropriate cell extracts. Third, a cDNA expression construct was cotransfected into a neuronal cell line along with various reporter constructs. In this way the cloned protein was shown to be able to repress the reporter gene in an NRSE-specific manner. Fourth, expression of the NRSF gene was assessed in the various cell lines where the NRSE-binding activity was and was not detected. A correlation between NRSF expression and the presence of NRSE-binding activity in a particular cell line would not have been found, however, if binding were regulated post-transcriptionally. Nonetheless, a perfect correlation was found, with neuronal cell lines showing very low or undetectable levels of NRSF expression and non-neural cell lines expressing NRSF at high levels. (Interestingly, glial cell lines were shown to express low-to-moderate levels of NRSF; however, their NRSE-binding activity was not assessed by gel-shift.) The correlation of NRSF expression with NRSE-binding activity indicated that tissue-specific regulation of NRSE-containing target genes is primarily at the level of transcription of NRSF.

NRSF expression was also analyzed by in situ hybridization in mouse and rat embryos at stages when both neural progenitors and mature neurons are present

(Chong et al., 1995; Schoenherr and Anderson, 1995). NRSF was found to be expressed in almost all tissues outside of the nervous system. This was consistent with the proposed role of NRSF of repressing neuron-specific genes in non-neural cells. Interestingly, NRSF expression was also detected at low levels in neural progenitors, while regions composed exclusively of mature neurons showed almost undetectable levels of NRSF. This suggested that NRSF may also play a role in the process of neurogenesis. The question of the expression of NRSF in glial cells in vivo was not addressed by these studies, because for the most part, glia have not begun to differentiate at the stages analyzed. In addition, the peripheral ganglia examined, which may have contained differentiating glia at these time points, contained a mixture of undifferentiated neural progenitors and differentiating neurons and glia, such that double-labeling would have been required to determine the expression pattern at the cellular level.

A later study, however, did partially address this question of whether NRSF was expressed in glia in situ (Palm et al., 1998). Timmusk and colleagues performed a detailed and sensitive in situ hybridization analysis of NRSF expression in adult rat brain. This yielded many important results: (1) NRSF expression is undetectable in almost all glia of the adult central nervous system (CNS). (2) Expression of NRSF is actually maintained, at very low levels, in most mature neurons. (3) NRSF is expressed at high levels in the non-neural tissues of the brain, including the choroid plexus, ependymal layer, and meninges. These data facilitate the interpretation of a number of in vivo studies of various genes, where the transcriptional activity of regulatory regions, including or deleted for the NRSE, are tested in transgenic mice.

Transcriptional regulation of many neuronal genes by NRSE/NRSF

I contributed to a study that analyzed the functionality of a large number of NRSE-like sequences found via a database search in a wide variety of different genes (Schoenherr et al., 1996; see Chapter 2). This investigation, as well as generating rules for predicting the functionality of a particular sequence, provided evidence that a large number of predominantly neuron-specific genes were direct targets of regulation by NRSF. Many other groups, however, have independently identified NRSE-like sequences in the regulatory regions of their genes of interest and have established a role for NRSE/NRSF in the transcriptional regulation of these genes. To date, the list of genes investigated by these other groups includes synapsin I, brain-derived neurotrophic factor (BDNF), choline acetyltransferase (ChAT), the neuron-glial cell adhesion molecule (Ng-CAM), L1 cell adhesion molecule, the m4 subtype of muscarinic acetylcholine receptor (mAChR), the β2-subunit of the neuronal nicotinic acetylcholine receptor (nAChR), the AMPA receptor subunit GluR2, the *N*-methyl-D-aspartate receptor 1 (NMDAR1), and the γ2 subunit of the type A receptor for γ-aminobutyric acid ($GABA_A$ receptor) (Bai et al., 1998; Bessis et al., 1997; Bessis et al., 1995; Kallunki et al., 1997; Kallunki et al., 1998; Kallunki et al., 1995; Li et al., 1993; Lonnerberg et al., 1996; Mieda et al., 1997; Mu and Burt, 1999; Myers et al., 1998; Schoch et al., 1996; Timmusk et al., 1999; Timmusk et al., 1993; Wood et al., 1996). All of these genes are expressed predominantly in neurons and encode proteins that are directly involved in neuronal functioning. While an in-depth review of each of these investigations would be excessive, a number of specific results are particularly informative and have implications for future experiments.

These studies begin with a common set of steps that implicate the NRSE-like sequence of a particular gene as being important for its cell-type specific expression: (1)

A segment of the regulatory region of the gene is selected for analysis of its ability to direct transcription of a reporter gene. This region can consist of any combination of the 5' flanking region, promoter, 5' untranslated region (UTR), introns, and 3' UTR. In some cases the endogenous promoter is replaced by a heterologous one, because of a complicated pattern of transcription initiation sites, for example. (2) The reporter construct is transfected into various cell types in order to ascertain whether its expression is cell-type appropriate. These cell types can be cell lines or primary cells recently isolated from embryonic or adult tissue. Some categories of cell type are "neuronal," "glial," and "non-neural," meaning neither neuronal nor glial. (3) Either the region containing the NRSE or the NRSE itself is deleted or mutated, and its effect on cell-type specific expression is assessed. If the NRSE is involved in the proper repression of the gene, then ectopic or increased expression is detected in an inappropriate cell type. Analysis of the NMDAR1 gene has only been pursued to this extent (Bai et al., 1998). (4) A few studies have gone on to examine the expression of intact vs. NRSE-deleted/mutated reporter constructs in vivo in transgenic mice.

So far NRSF mRNA has been found in all glial cell lines tested and in a preparation of primary CNS glia (Palm et al., 1998; Schoenherr and Anderson, 1995). This is in agreement with the derepression that was observed in primary glia but not primary neurons with an NRSE-deleted GluR2 reporter construct (Myers et al., 1998). It is also consistent with the selective expression in neurons but not glia of adenoviruses carrying multiple copies of the NRSE upstream of various promoters (Millecamps et al., 1999; Miyaguchi et al., 1999; Tabuchi et al., 1999). These results are in sharp contrast, however, to those derived from reporter constructs analyzed in transgenic mice, where the lack of derepression in CNS glia upon NRSE deletion/mutation correlates well to the

lack of NRSF expression shown in almost all glia of adult rat brain (Palm et al., 1998). No derepression in any non-neuronal cells of the brain was detected for NRSE-mutated reporter transgenes containing either regulatory sequences of BDNF or of the β2-subunit of the neuronal nAChR (Bessis et al., 1997; Timmusk et al., 1999). Interestingly, however, in the case of an NRSE-deleted reporter construct based on the L1 gene (Kallunki et al., 1998), derepression in non-neuronal cells of the brain was detected in exactly those non-neuronal cells found to express NRSF: the glia of the olfactory bulb that ensheathe the sensory afferents, the meninges, and the choroid plexus (Palm et al., 1998). A precise study has not yet been made of NRSF expression in peripheral glia. One would predict, however, that either NRSF or an alternative NRSE-binding protein is expressed in this cell type, because derepression of the NRSE-deleted L1 reporter gene was found in embryonic peripheral nerve (Kallunki et al., 1997).

Aside from the question of the influence of the NRSE in non-neuronal cells of the nervous system, in vivo analysis of intact vs. NRSE-deleted/mutated reporter constructs in transgenic mice has shed light on the role of the NRSE both in non-neural tissues and in neurons themselves. It was somewhat unexpected that in the cases of all three genes examined, deletion/mutation of the NRSE resulted in derepression within neurons of the adult brain. In the case of reporter genes both for the β2-subunit of the neuronal nAChR (Bessis et al., 1997) and for BDNF (Timmusk et al., 1999) neuronal derepression was widespread, whereas for L1 derepression was limited to the neurons of the thalamus (Kallunki et al., 1998). Given the, albeit low-level, expression of NRSF found in neurons of the adult brain, however, this neuronal derepression might have been anticipated.

Two of these studies showed in addition, however, a very surprising result, which indicated for the first time that the NRSE might be able to act as an enhancer in some

neuronal contexts (Bessis et al., 1997; Kallunki et al., 1998). The NRSE-deleted transgene based on L1 was expressed at lower levels than the intact transgene in the neurons of the adult striatum, cortex, and hippocampus. In the case of the β2 nAChR subunit, expression of the intact transgene began during embryogenesis in a widespread pattern, including almost all differentiating neurons of the developing central and peripheral nervous systems, while by adulthood expression was limited to a small subset of neurons in the hypothalamus. The NRSE-deleted transgene had the opposite type of expression pattern, where almost all embryonic expression was missing and adult expression was promiscuous, as mentioned above. The fact that the expression of the deleted transgene was lower than the intact transgene during embryogenesis indicated that, depending on the context, the NRSE in the β2 nAChR subunit gene can act as an enhancer.

The mechanism for this new function of the NRSE has not been established. Based on data derived from chimeric reporter constructs, it has been proposed that an NRSE acts as an enhancer in neurons when it is located either within 50 bp of the transcription start site or within the 5'UTR (Bessis et al., 1997). This is not supported, however, by the facts that (1) both the NRSE of the β2 nAChR subunit, in the context of its own regulatory region, and the NRSE of the NMDAR1 gene show no enhancer function in neuronal cell types, even though they are located in the 5'UTR (Bai et al., 1998; Bessis et al., 1995); and (2) the NRSE of the L1 gene does show enhancer function in certain neurons, even though it is located greater than 10 kb upstream of the transcription initiation site (Kallunki et al., 1998).

What do the analyses of regulatory regions in transgenic mice indicate about the role of the NRSE in cell-type specific expression outside of the nervous system? The first

study of this kind was done with the SCG10 gene, before the specific sequence of the NRSE had been identified (Wuenschell et al., 1990). In this investigation, the expression pattern of the intact reporter gene construct was compared to one in which a region of the regulatory region consisting of many kilobases was deleted. This large region was later confirmed to contain the NRSE. Nonetheless, numerous other regulatory elements were likely deleted along with it. This study showed that, while the intact construct showed nervous system-specific expression, the "NRSE-deleted" construct gave expression not only in the brain but also in a wide range of non-neural tissues. Later studies of other genes, where solely the 21 bp NRSE was either deleted or mutated, have only partially replicated this result.

The three genes studied each gave different results. In the case of the β2 nAChR subunit, no ectopic expression was detected outside of the nervous system (Bessis et al., 1997). In the case of BDNF, which is normally expressed at low levels in the thymus and lung, the NRSE-mutated reporter gene was derepressed in these two non-neural tissues, but in no others (Timmusk et al., 1999). Finally, in the case of the L1 gene, significant ectopic expression was seen in non-neural tissues (Kallunki et al., 1997; Kallunki et al., 1998). Most of these tissues, however, derive from the neural crest. The neural crest generates not only the neurons and glia of the peripheral nervous system but many non-neural tissues as well, including the bones of the head, the teeth, part of the cornea and heart, and melanocytes. Ectopic expression was found at all of these sites. This was explained by an analysis at embryonic stages that demonstrated that deleting the NRSE resulted in premature expression of the L1 reporter gene in the neural crest (Kallunki et al., 1997). Thus, all neural crest derivatives showed reporter activity, instead of only the derivatives that became neural. Non-neural expression of the NRSE-

deleted reporter gene was not only restricted to neural-crest derivatives, however. During embryonic and postnatal development ectopic expression was detected in the apical ectodermal ridge and dorsal ectoderm of the forelimb; the mesoderm of the abdominal body wall, genital tubercle, and hindlimb; and the metanephric tubules and the ureter of the kidney.

This range of results for the three different genes analyzed in vivo so far has two major implications for the results of inhibiting NRSF function by blocking its ability to bind to the NRSE: (1) Only select non-neural tissues may show derepression of the endogenous target gene. (2) The set of tissues where derepression occurs may be different for each particular target gene.

Although deletion/mutation analysis of a regulatory region can show that the NRSE is involved in the cell-type specific expression of a gene, additional experiments are required to suggest that it is NRSF that mediates the NRSE-dependent regulation. One way to implicate NRSF is to use gel-shift analysis. The putative NRSE of the gene of interest is compared to a known NRSE in its ability to bind to a factor that is similar to NRSF in (1) its pattern of activity in extracts of the cell types analyzed, (2) its mobility and binding specificity, and (3) its antigenicity. Another approach to demonstrating that NRSF may be responsible for the decrease of expression of an NRSE-containing reporter gene in certain cell lines is to ectopically express NRSF. The hypothesis is that cell lines that are insensitive to the presence or absence of an NRSE are so because they lack NRSF. Therefore, if NRSF is expressed in these cell lines, then an NRSE-containing reporter construct should be repressed relative to one lacking the NRSE.

This has frequently been the case. There are two significant exceptions, however, that have important implications for the potential results of ectopic expression studies

of NRSF in vivo. The regulatory region of the ChAT gene has been found to contain both an NRSE that reduces its expression in non-neural cell types and an enhancer element that increases its expression specifically in the cholinergic neuronal cell line, SN6 (Lonnerberg et al., 1996). When this enhancer is deleted from the reporter construct, expression of NRSF in SN6 cells results in an NRSE-dependent reduction of reporter gene activity. When this enhancer is included in the reporter construct, however, ectopic NRSF is no longer able to significantly repress reporter-gene expression. This indicates that the presence of strong transcriptional activators present in some tissues may be able to overcome repression by ectopic NRSF in vivo. In the case of the other reporter genes that were repressed in neuronal cell types by ectopic NRSF expression, this may be explained by the particular regulatory region included in the reporter construct. In vivo ectopic expression experiments will assess the expression of endogenous target genes, which will have their full complement of regulatory elements.

A similar example of this phenomenon was found in the case of a reporter construct containing regulatory regions of the m4 mAChR gene (Mieda et al., 1997). Ectopic expression of NRSF in the neuroblastoma/glioma hybrid cell line, NG108-15, was only able to partially repress this reporter gene (two-fold). When a type II sodium channel-based reporter construct was cotransfected into this cell line with the NRSF expression construct, however, reporter gene expression was highly repressed (ten-fold). Swapping the specific NRSE sequences between these two reporter constructs did not change their response to NRSF expression, indicating that the m4 NRSE was not simply weaker than that of the type II sodium channel. The regulatory regions of these two genes were simply differentially susceptible to repression by NRSF. This suggests

that in vivo some target genes may be repressed by ectopic NRSF expression, and others may not.

Mechanism of transcriptional repression by NRSF

The mechanism whereby NRSF represses the transcription of NRSE-containing genes has remained relatively obscure for many years since the identification of NRSF. Very recently, however, significant strides have been made toward addressing this question. It has long been known that the NRSE functions in a position- and orientation- independent manner (Maue et al., 1990; Mori et al., 1990). This was the basis for terming the repressive sequences a silencer element, according to the classical definition (Brand et al., 1985). Since these preliminary tests of NRSE function, numerous groups have tested the limits of the flexibility of the NRSE. Not only does the NRSE function in all positions tested, including downstream of the reporter gene (Kallunki et al., 1997; Mu and Burt, 1999; Thiel et al., 1998), but it is also capable of mediating repression of the following heterologous promoters/enhancers: the thymidine kinase promoter, the SV40 promoter, and the enhancer of the glucose-regulated protein 78 gene (Bessis et al., 1997; Lonnerberg et al., 1996; Thiel et al., 1998). This ability to repress reporter-gene expression independent of context suggests that NRSF is an active repressor that functions either by directly inhibiting the basal transcription machinery or by recruiting a corepressor that performs the transcriptional repression (Leichter and Thiel, 1999).

A number of corepressors have recently been identified (Leichter and Thiel, 1999 and references therein). A corepressor binds to the repressor domain of a transcriptional repressor and is required for the ability of the repressor protein to inhibit transcription.

KAP-1/KRIP-1 is a corepressor that interacts with the KRAB domain – a repressor domain found in many zinc-finger proteins – and is necessary for the repressor function mediated by that domain. N-CoR (nuclear corepressor) is a corepressor that interacts with the C-terminal region of the unliganded thyroid hormone receptor (TR). This region of TR is a repressor domain, and N-CoR is required for ligand-independent inhibition of transcription by TR. N-CoR can recruit the histone deacetylase HD1. N-CoR-mediated repression may, therefore, involve chromatin remodeling. An additional corepressor, silencing mediator for retinoid and thyroid receptors (SMRT), contains a conserved nuclear receptor interaction domain found also in N-CoR.

As mentioned above, NRSF contains two separate repressor domains – one at each end of the protein. Each of these domains can be fused to the heterologous DNA-binding domain of GAL4 and as such repress transcription of reporter genes containing UAS elements (the element to which GAL4 normally binds). It has recently been shown that these two NRSF repressor domains function by binding distinct nuclear factors (Leichter and Thiel, 1999). This was done using a squelching assay. Each repressor domain was fused to each of two proteins – the GAL4 DNA-binding domain and a nuclear-localized version of glutathione S-transferase (GST). It was tested whether co-expression of a particular GST-fusion protein could inhibit repression by a particular GAL4 fusion protein. Transcriptional repression by the N-terminal repression domain could be overcome by expression of the N-terminal GST-fusion protein but not the C-terminal GST-fusion protein, and vice versa. This indicated that each repressor domain of NRSF required a titratable nuclear factor for its repressive function, but that the factor was not the same for the N-terminal domain as for the C-terminal domain.

Two other GST-fusion proteins were also tested in this assay. These were the repressor domain of the thyroid hormone receptor α (known to interact with N-CoR), and the repressor domain of NK10 (a KRAB domain known to interact with KAP-1/KRIP-1). If the ability of either repressor domain of NRSF to repress transcription was inhibited by the expression of one of these GST-repressor domain fusions, then one could propose that N-CoR or KAP-1/KRIP-1 was a corepressor for NRSF. This was not found to be the case. However, a novel protein related to N-CoR and SMRT, termed CoREST, has since been found to act as a corepressor for the C-terminal repressor domain of NRSF (Andres et al., 1999). (Recall, NRSF is also known as REST (Chong et al., 1995).)

CoREST was identified by a yeast two-hybrid screen, using the C-terminal domain of NRSF as bait (Andres et al., 1999). This domain includes a single zinc finger, which must be intact for CoREST to bind to NRSF. This correlates well to the reduced ability of NRSF to repress a reporter gene when this zinc finger is mutated (Tapia-Ramirez et al., 1997). The interaction of CoREST with NRSF was demonstrated by both yeast two-hybrid and GST pull-down assays. In addition, coimmunoprecipitation of endogenous NRSF by an anti-CoREST antibody showed that this interaction occurs in vivo. When CoREST was enabled to bind DNA by fusing it to the GAL4 DNA binding domain, it was found to be able to mediate repression even in the absence of NRSF, indicating that recruiting CoREST to a transcriptional unit is sufficient to inhibit transcription. CoREST was further shown to mediate repression by NRSF by using a squelching assay similar to that described above. In this case, the CoREST-binding domain of NRSF (the N-terminal repressor domain) was coexpressed with full-length NRSF and shown to be able to inhibit reporter-gene repression. When extra CoREST was expressed along with these proteins, it was able to rescue the inhibition of repression. Unlike NRSF, CoREST appears to be

expressed ubiquitously, both in non-neuronal and neuronal cell lines and tissues, indicating that it likely does not contribute to the tissue-specific regulation of NRSF target genes and that it may be involved in the function of additional transcriptional repressors.

A distinct mechanism of transcriptional repression by NRSF has recently been reported that involves the N-terminal repressor domain. This domain has been shown to be able to bind to a complex including the corepressor Sin3A and histone deacetylase (Huang et al., 1999). The acetylation state of histones is important for transcriptional activity, because it determines in part the accessibility of a promoter to binding by the transcription apparatus (Huang et al., 1999, and references therein). When the lysines of the N-terminal tails of histones are acetylated, their positive charge is neutralized, decreasing their affinity for the negatively-charged DNA. The subsequent unfolding of the nucleosome results in greater access of the DNA to transcription factors and other transcriptional machinery. The opposite process has been shown to be involved in the repression of transcription by the repressors RB and MAD. In these cases, the repressors bind to the corepressor Sin3A or Sin3B, which recruit histone deacetylases. These enzymes deacetylate the lysines of the histone tails, stabilizing the chromatin structure and masking nearby promoters from the transcription apparatus.

A potential role of histone deacetylases in repression by NRSF was first established by assessing the effect of the histone deacetylase inhibitor, trichostatin A (TSA) on the expression of both endogenous NRSF target genes and transiently transfected reporter constructs (Huang et al., 1999). The ability of NRSF to inhibit transcription was found to be reduced in the presence of TSA, and this effect was mapped to the N-terminal repressor domain. This domain was then shown to be able to

associate with both Sin3A and histone deacetylase 1 (HDAC1) by coimmunoprecipitation using both transfected and endogenous proteins. Finally, the acetylation state of the histones associated with the promoter of the neuronally expressed, NRSF target gene, GluR2, was assessed. This was done in both the C6 glioma cell line and primary cortical neurons with and without TSA treatment. First, this experiment showed that the promoter of this endogenous gene was associated with acetylated histones in neurons but deacetylated histones in glia, as would be predicted. Second, upon treatment with TSA, acetylation of the histones associated with the GluR2 promoter dramatically increased in glia, but was unaffected in neurons. This, combined with the earlier result of TSA treatment of glia increasing expression of the endogenous GluR2 gene, confirmed the idea that transcriptional repression of GluR2 in glia is dependent on histone deacetylation. The association of the N-terminal repression domain of NRSF with Sin3A and HDAC1, along with the ability of TSA to inhibit the repression of a reporter gene by this domain of NRSF, indicates that NRSF is likely to repress endogenous GluR2 expression by recruiting HDAC1 to deacetylate the histones surrounding the GluR2 promoter.

The fact that NRSF may be able to affect transcriptional repression by modifying chromatin conformation may have implications for studies that attempt to inhibit NRSF function. Binding of NRSF to its target genes may begin a process that results in those genes being "trapped" in a repressive chromatin conformation that is not easily reversed. If an inhibitor of NRSF function is introduced after this process is completed, full derepression of NRSF target genes may not be seen. An extreme version of this scenario is that NRSF may only be required to initiate repression of a target gene. Displacing NRSF from its binding site at a later point in time, therefore, may have little

consequence for the expression level of that gene. Clearly a transcription factor can have an initiation rather than a maintenance function, however, without being involved in chromatin remodeling, but the ability of NRSF to affect chromatin structure may make this type of function more likely.

Theme of the thesis

The experiments described herein aimed to analyze the role of NRSF during vertebrate embryogenesis in regulating neuron-specific gene expression both in non-neural tissues and in neural cell types during neural development. The three data chapters and the appendix describe the efforts made toward achieving that aim.

Chapter 2 consists of a paper published in 1996 in the *Proceedings of the National Academy of Sciences* and describes the identification of a large set of putative direct targets of NRSF transcriptional regulation (Schoenherr et al., 1996). This study is important for an investigation of NRSF function, because it defines the potential realm of influence for NRSF. The most direct potential consequence of perturbing NRSF function in vivo is a change in the expression levels of NRSF target genes in various tissues. I am middle author on this paper. The work for which I am responsible consists of (1) preparing all the materials for and conducting the experiments that generated the two data figures: Fig. 1 – binding of NRSF to the potential NRSE sequences, and Fig. 2 – repression of reporter constructs containing the potential NRSEs; (2) actively participating in the editing of the first draft of the paper, composed by C.J. Schoenherr.

Chapter 3 consists of a manuscript that I prepared for submission to the journal *Development*. It describes the effects of inhibiting NRSF function in developing chick

embryos in both neural and non-neural tissues. This data was later combined with data describing the phenotype of mouse embryos homozygous for a targeted mutation of NRSF, generated by a fellow lab member, Z.-F. Chen. These two data sets were published together in a single paper in *Nature Genetics* in 1998 (Chen et al., 1998).

Chapter 4 describes the generation, characterization, and use of monoclonal antibodies against NRSF. These antibodies were generated in order to more accurately assess the timing of NRSF expression and extinction during neurogenesis. Unfortunately, they were not sensitive enough to detect the low endogenous levels of NRSF in neural progenitors. These antibodies have been useful, however, in gel-shift analysis, Western analysis, and in immunocytochemistry on cells expressing moderate to high levels of NRSF. This chapter describes the characterization of these antibodies and their use in both this and collaborating labs. The expression of NRSF is carefully examined during the differentiation of the mouse embryonal carcinoma cell line, P19 – a model system of neuro- and gliogenesis – to gain insight into potential NRSF function during these processes. This data has not been published except for the use of these antibodies by collaborators (Shimojo et al., 1999; Xu et al., 1999).

Chapter 5 describes preliminary data from NRSF-overexpression experiments in the nervous system of chick embryos in vivo. NRSF expression constructs were electroporated into the neural tube of early embryos before neurons of the spinal cord begin to differentiate. These initial experiments have indicated a role for NRSF in the proper regulation of neuronal target genes in both neural progenitors and differentiating neurons. Axon targeting appears to be affected in a subset of the NRSF-overexpressing neurons as well.

The Appendix describes an attempt at identifying proteins in addition to NRSF that can bind to the NRSE. A number of different results lead to the suspicion that an alternative NRSE-binding protein might exist. A yeast one-hybrid screen was set up in order to search for such a protein. The set of yeast strains generated by this effort will be useful for future library screening.

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Chapter 2

IDENTIFICATION OF POTENTIAL TARGET GENES FOR THE NEURON-RESTRICTIVE SILENCER FACTOR

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

CHRISTOPHER J. SCHOENHERR* , ALICE J. PAQUETTE*, AND DAVID J. ANDERSON* §

*Division of Biology 216-76 and Howard Hughes Medical Institute, California Institute of Technology, Pasadena, CA 91125

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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.

Present address: Department of Molecular Biology, Princeton University, Princeton, NJ 08544.
§To whom reprint requests should be addressed. e-mail: andersond@starbase1.caltech.edu.

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

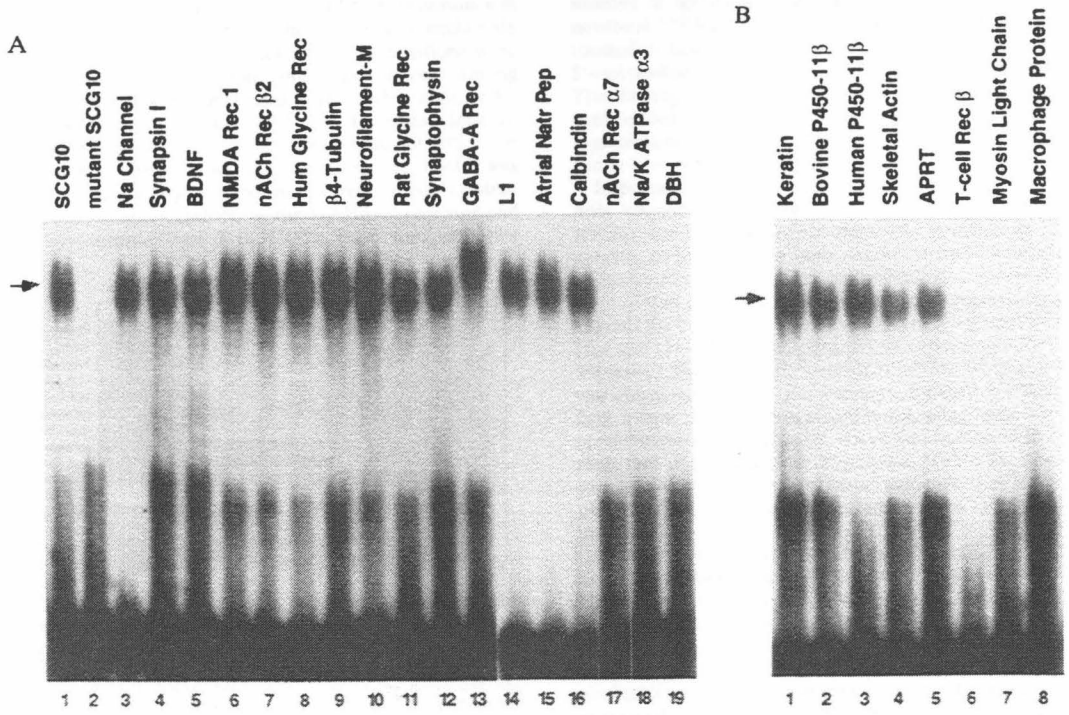

FIG. 1. (*A*) Recombinant NRSF recognizes NRSEs in 15 different neuron-specific genes. EMSAs were performed using *in vitro*-synthesized human NRSF encoded by the λH1 cDNA (22). The arrow indicates the the specific complex. The probes consisted of radiolabeled double-stranded oligonucleotides derived 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

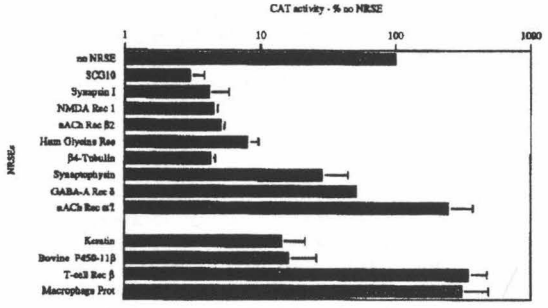

FIG. 2. Sequences that can bind NRSF are able to mediate silencing. Oligonucleotides derived from a subset of the neuronal and nonneuronal genes tested for NRSF binding were inserted upstream of the SCG10 promotor fusion construct, CAT3, which lacks the endogenous SCG10 NRSE (26), and transiently transfected 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.

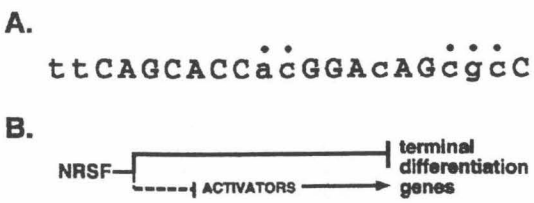

FIG. 3. (*A*). Consensus NRSE derived from 19 sequences for which NRSF binding was experimentally determined. Nucleotides in uppercase letters represent positions that are either invariant, or differed only twice, among the 19 functional NRSEs. Lowercase letters represent residues that varied more frequently. 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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Chapter 3

INHIBITION OF NRSF IN VIVO RESULTS IN THE DEREPRESSION OF MULTIPLE NEURON-SPECIFIC TARGET GENES IN BOTH NEURAL AND NON-NEURAL TISSUES DURING VERTEBRATE EMBRYOGENESIS

SUMMARY

The appropriate expression of neuronal genes during embryogenesis is often thought to be accomplished by neuron-specific transcriptional activators. Recently, however, an important role for negative regulation is emerging. One sequence element known to mediate negative regulation of many different neuronal genes is the NRSE (or RE1). The protein that binds to this element, NRSF (also known as REST and XBR), has been shown to be a transcriptional repressor and is expressed during embryogenesis both widely outside of the nervous system and in neural progenitors. In this study we have attempted to assess the importance of NRSF in vivo for the proper repression of endogenous target genes in their natural chromosomal context. This was done by introducing a dominant-negative form of NRSF (dnNRSF) into early chicken embryos via replication-competent retrovirus. We provide support that NRSF is, indeed, required outside of the nervous system for repression of NRSE-containing, neural genes. We also show a requirement of NRSF function within the nervous system, both for repression of neural target genes in glial progenitors and for prevention of premature expression of these genes in neural progenitors.

INTRODUCTION

The proper expression of genes that are characteristic of the terminally differentiated state of a cell results in large part from a combination of positive and negative transcriptional regulation (Arnone and Davidson, 1997) that is implemented over the

course of a cell's developmental history. The largest body of evidence currently exists for the role of transcriptional activators, which guide tissue-specific expression according to the subsets of tissues in which they themselves are expressed. Comparatively, the action of transcriptional repressors is poorly understood, especially in vertebrates (Gray and Levine, 1996). Although promoter analysis has identified repressor elements in numerous vertebrate cell-type specific genes (Arnone and Davidson, 1997), relatively few of the factors that interact with these elements have been isolated and characterized in vivo.

Transcriptional repression is beginning to emerge as an important determinant of neuron-specific gene expression (Mandel and McKinnon, 1993; Schoenherr and Anderson, 1995b). Many neuronal genes have been shown to contain different sequence elements that can mediate repression of promoter-reporter constructs in non-neuronal cell lines. One of these elements, the neuron-restrictive silencer element (NRSE), or repressor element 1 (RE1), was originally identified in the pan-neuronal genes, SCG10 and Type II sodium channel, respectively (Kraner et al., 1992; Mori et al., 1992). This 21 base-pair sequence has since been found in tens of neuronal genes, including those encoding enzymes required for neurotransmitter synthesis, neuropeptides, synaptic vesicle components, neurotransmitter receptors, neuron-specific cytoskeletal proteins, and neural cell adhesion molecules (Schoenherr et al., 1996, and references therein). The factor that binds to the NRSE, therefore, may be important in coordinating the expression of these genes that together make up the essence of neuronal structure and function.

Recently, a number of groups identified this protein and named it the neuron-restrictive silencer factor (NRSF), RE1-binding silencing factor (REST), and X-box

repressor (XBR) (Chong et al., 1995; Schoenherr and Anderson, 1995b; Scholl et al., 1996). It contains nine zinc fingers of the C_2H_2 type, with one at the extreme C-terminus and the other eight grouped together in the N-terminal half of the protein. The group of zinc fingers alone can bind to the NRSE (Chong et al., 1995; Schoenherr and Anderson, 1995b), and in cotransfection assays the full-length protein mediates repression of NRSE-containing reporter genes (Chong et al., 1995). The protein contains two known repressor domains: One is at the extreme N-terminus, and the other is the ninth zinc finger itself (Tapia-Ramirez et al., 1997).

In situ hybridization analysis has showed two very interesting aspects of the NRSF expression pattern. First, signal was found in almost all tissues outside of the nervous system. This agreed with the transient transfection studies and with a previous analysis of NRSE function within the SCG10 promoter that was conducted in transgenic mice. In that investigation, deletion of a large region containing the NRSE resulted in ectopic expression of the reporter transgene in most non-neural tissues (Wuenschell et al., 1990). Second, NRSF transcript was found in the ventricular zone of the neural tube, where neural progenitors reside. This indicated that NRSF might have an additional role, one within the nervous system during neurogenesis to prevent premature expression of genes characteristic of terminal differentiation, before events such as cessation of cell division and migration have occurred.

Up to this point, in vivo studies that have attempted to assess the importance of NRSF in neuron-specific gene regulation during embryogenesis have been limited to transgenic mouse analysis of the promoters of individual NRSE-containing putative target genes. These studies have demonstrated inappropriate expression of a randomly integrated promoter-reporter transgene, when the ability of the NRSF to repress

transgene transcription has been eliminated through deletion of the NRSE (Bessis et al., 1995; Kallunki et al., 1997). Although this approach has been fruitful, it has clear limitations: First, only single genes can be examined at a time. Second, it is unable to assess the effect of loss of NRSF function on intact, endogenous genes in their natural chromosomal context. Last, it cannot reveal the in vivo consequences of derepression of the multiple target genes, because it is only the expression of a reporter gene that is affected.

The cloning of NRSF has made possible two ways of carrying out a loss-of-function analysis. One is to generate NRSF-mutant mice through homologous recombination (Chen et al., 1998). The other, employed here, is to introduce into developing embryos a dominant-negative form of the protein, dnNRSF, as designed by Mandel and colleagues (Chong et al., 1995; Tapia-Ramirez et al., 1997). This approach has the advantage of inhibiting NRSF function in a mosaic way such that uninfected tissues can support the normal growth of the embryo. We have used the replication-competent avian retrovirus system to express this protein early in chicken embryogenesis. We report here the resulting evidence that NRSF function is required both for repression of endogenous neuronal target genes in non-neuronal cells and for the prevention of precocious expression of these genes in neuronal progenitors.

MATERIALS AND METHODS

Retroviral construction

Two retroviruses were constructed in RCASBP(B) (Fekete and Cepko, 1993; Homburger and Fekete, 1996; Petropoulos and Hughes, 1991). The experimental virus, dnNRSF, expresses the zinc-finger domain of mouse NRSF flanked by the 20 amino acids (aa)

preceding and the 33 aa following it (C.J. Schoenherr and D.J. Anderson, unpublished; Genbank accession #U13878) with a myc epitope tag at the amino terminus. This protein fragment is similar to p73, also referred to as the "dominant negative form of REST" (Chong et al., 1995; Tapia-Ramirez et al., 1997). The control retrovirus, nLuc, expresses luciferase (luc^+ of pGL3-Basic, Promega) with a nuclear localization signal and five myc epitope tags at the amino terminus.

The dnNRSF retrovirus was constructed as follows. Mouse NRSF cDNA was isolated from a C3H10T 1/2 cell cDNA library by low-stringency hybridization using a human NRSF fragment as probe (C.J. Schoenherr and D.J. Anderson, unpublished). A fragment containing the zinc-finger domain was obtained by the polymerase chain reaction (PCR) using primers containing exogenous EcoR I sites along with a stop codon in the reverse primer: forward - 5' C A C A G A A T T C C G C T C C A G A A A C A C C C G T G 3'; reverse - 5' C A C A G A A T T C T T A C T C G T T G C T G A C G G C G T T 3'. This fragment was cloned into the EcoR I site of the expression vector, pCS2+MT (Rupp et al., 1994; Turner and Weintraub, 1994). The Nco I - Xba I/blunted fragment of this plasmid was then cloned into the Nco I - Eco RI/blunted shuttle vector, SLAX 12 NCO (Morgan and Fekete, 1996). Finally, the Cla I fragment from this plasmid was cloned into the Cla I site of RCASBP(B).

The nuclear luciferase (nLuc) retrovirus was cloned as follows. A three-way ligation was performed with the Nco I fragment of pCS2+NLS/MT (Rupp et al., 1994; Turner and Weintraub, 1994), the Nco I - Xba I/blunted fragment from pGL3-Basic (Promega), and the SLAX 12 NCO vector cut with Nco I and Sma I. Then the Cla I fragment from this plasmid was cloned into the Cla I site of RCASBP(B).

Isolation of chicken NRSF fragment

A fragment of chicken NRSF containing most of the zinc fingers was cloned by first reverse-transcribing poly A+-enriched RNA from embryonic day 3 (E3) chick embryos, then using PCR with degenerate primers corresponding to the second and ninth zinc fingers of human and mouse NRSF: forward - 5' A C I G C I C A T/C C/T T N A A A/G C A T/C C A 3'; reverse - 5' G/C A/T I C G/T G/A T C G/A C A G/A A A T/G/A A T G/A C A 3' (where "I" designates inosine and "N" indicates all four nucleotides). This fragment was sequenced to compare it to the mouse and human NRSF zinc-finger domain and then was used to generate probe for in situ hybridization analysis of chicken NRSF expression.

In situ hybridization

In situ hybridization was carried out using a combination of two protocols. Most of the procedure was done essentially according to (Birren et al., 1993) except that pre-hybridization and hybridization was done at 65°C for most probes (see below). Post-hybridization and post-antibody washes, however, were derived from a modification (Myat et al., 1996) of a standard procedure (Strahle et al., 1994).

Retroviral generation and infection

Primary chick embryonic fibroblasts (CEFs) were generated from Line 0 chicken embryos (U.S. Department of Agriculture Poultry Research Laboratory, Ann Arbor, MI) as described (Hunter, 1979). Retroviral constructs were transfected into these cells and virus grown and collected essentially according to (Morgan and Fekete, 1996) except

that concentration was carried out in polycarbonate UltraBottles (Nalge Company) in a Type 60Ti rotor (Beckman) at 30,000 RPM.

Three different infection protocols were used. CEF infection was performed at a multiplicity of infection of approximately 4 in the presence of 8 micrograms/ml Polybrene™, hexadimethrine bromide (Sigma). After 24 hours the cells were split into multiple dishes and fixed after an additional 24 hours for immunocytochemistry.

Somite infection was carried out as follows. White Leghorn chicken eggs were incubated at 38°C until stages (St.) 14 to 15 of development (Hamburger and Hamilton, 1992), or about 21-26 somites, E2. Embryos were injected with virus at a titer of $\geq 10^8$ infectious units/ml with 80 micrograms/ml hexadimethrine bromide and 0.025% Fast Green (Sigma). Virus was injected unilaterally into the five somites, 16 through 21, which are at the level of the presumptive wing bud. The somites on the other side were left un-injected to serve as an internal negative control. The eggs were then re-incubated for 3 days before fixation.

For neural tube/crest infection, embryos were incubated until 8-13 somites, about St. 10-11, E1.5. The neural tube was filled with virus solution, and eggs were then incubated for an additional 3 days before fixation.

Analysis of gene expression

Immunocytochemistry was performed on equivalent sister dishes of CEFs infected contemporaneously with either the experimental or control retrovirus. Cells were fixed for 10 minutes at room temperature in 3.7% formaldehyde in phosphate-buffered saline (PBS). Anti-c-myc, 9E10 hybridoma supernatant (Developmental Studies Hybridoma Bank) was used to detect infected cells expressing either of the myc epitope-tagged

transgenes. The TuJ1 antibody (BAbCo, 1:1000) was used to detect expression of Class III β-tubulin. HRP-conjugated goat anti-mouse IgG (Chemicon, 1:200) was used as the secondary antibody, and the signal was developed with diaminobenzidine intensified with nickelous sulfate.

Both somite and neural-tube/crest infected embryos were processed as follows. Embryos were fixed overnight in 4% paraformaldehyde in PBS at 4°C. Embryos were cryostat sectioned at the level of the wing bud at 15 microns and distributed over 10 microscope slides, with each slide carrying sections from at least two embryos - both from the same injection day, but one dnNRSF-infected and the other nLuc-infected. All results shown compare embryos processed on the same slide. Immunohistochemistry was performed on sections of somite-infected embryos and was done similarly to that described for cells above. A total of 20 dnNRSF-infected, 11 nLuc-infected, and 2 un-infected embryos were analyzed for TuJ1 staining.

In situ hybridization analysis of infected embryos was carried out using the following probe templates. Probes for retroviral transgenes were synthesized by using subclones generated in the process of making the retroviruses: the SLAX 12 NCO vector containing each gene. SCG10 expression was detected using a plasmid containing chicken SCG10 cDNA (P. Jeffrey, personal communication). To confirm that signal from this probe was specific to SCG10, two test in situs were performed, one at the increased hybridization and wash temperature of 70°C and another with an added RNAse A digestion step. Ng-CAM expression was specifically detected by routinely hybridizing and washing at 70°C and by using a probe containing a region with little conservation among members of the CAM family. The Ng-CAM sequence was isolated by PCR using the plasmid, pSCT-NgCAM (Buchstaller et al., 1996), as template and including 10% DMSO.

Primers were designed to generate a fragment corresponding to aa 258 through 602: forward - 5' C G C G G A T C C G T G T T G G A G T G C A T C G C T G 3'; reverse - 5' C C G G A A T T C G A C T C T G A G C T G C G C T T C G 3'. For somite infections a total of 10 dnNRSF-infected, 10 nLuc-infected, and 2 un-infected embryos were analyzed for SCG10 and Ng-CAM expression. For neural tube/crest infections a total of 10 dnNRSF-infected, 7 nLuc-infected, and 3 un-infected embryos were analyzed for these markers.

RESULTS

In an effort to assess the role of NRSF as a regulator of neuron-specific gene expression during vertebrate embryogenesis, a form of NRSF that inhibits the function of this system was introduced into developing chick embryos. A replication-competent avian retrovirus, RCASBP(B) (Fekete and Cepko, 1993; Homburger and Fekete, 1996; Petropoulos and Hughes, 1991) was engineered to express a myc-epitope tagged fragment of mouse NRSF containing the DNA-binding domain alone - the group of eight zinc fingers in the N-terminal half of the protein. Two identified silencing domains lie outside of this region (Tapia-Ramirez et al., 1997), and it has been shown that a similar fragment of human NRSF, p73, can derepress NRSE-containing reporter genes in cells that contain endogenous human NRSF (Chong et al., 1995). We independently confirmed this function of our construct, dnNRSF, in mouse fibroblasts (data not shown).

Both p73, also referred to as a "dominant-negative form of REST," and dnNRSF presumably act to inhibit transcriptional repression by competing with the functional, endogenous protein for binding to NRSEs (Chong et al., 1995). It has been shown that p73 has no direct effect on transcription itself in two ways (Tapia-Ramirez et al., 1997): (1) In cells that contain very low levels of endogenous NRSF, expression of p73 results in

a negligible increase (0.2 fold) in the activity of an NRSE-containing reporter gene. (2) When made to bind the heterologous DNA element, UAS, by fusion to the GAL4 DNA-binding domain, p73 does not alter the transcriptional activity of a UAS-containing reporter gene. dnNRSF, which is a slightly shorter, mouse version of p73, is also likely to have no ability to regulate transcription itself.

A second RCASBP(B) construct, nLuc, was generated as well. This encodes a luciferase gene with a nuclear-localization signal and myc epitope tags at the 5' end and served as a negative control for retroviral infection and for expression of an exogenous nuclear protein containing a myc epitope tag.

The sequence and expression pattern of avian and mammalian NRSF are highly evolutionarily conserved

Our dnNRSF construct was generated from mouse NRSF, yet we intended to use it to inhibit chicken NRSF function in vivo. In order to determine whether the DNA-binding domain of mouse NRSF would be effective at competing with endogenous chicken NRSF for NRSE binding, we wanted to compare the sequence of the Zinc-finger regions of chick and mouse NRSF. We, therefore, used PCR with degenerate oligonucleotide primers to clone a fragment of chicken NRSF. Comparison of human, mouse, and chicken sequence (Chong et al., 1995; C.J. Schoenherr and D.J. Anderson, unpublished; Schoenherr and Anderson, 1995a) shows that the zinc-finger region of all three species is almost identical (Fig. 1A), with the chick having 92% aa identity and 82% nucleotide identity to the human sequence. We therefore predicted that dnNRSF should be as effective in chicken as in mouse cells in blocking the function of endogenous NRSF. This prediction was confirmed in retrovirally infected chick embryo fibroblasts by

transfecting them with an NRSE-containing reporter construct. The chick cells infected with dnNRSF showed a 6.5-fold derepression in reporter activity relative to those infected with an uninserted control virus (data not shown).

We next wanted to confirm that NRSF was likely to serve a similar function in chick as in mouse by comparing its expression pattern in these two species. We therefore used the cDNA fragment of chicken NRSF generated above as a probe for in situ hybridization analysis at the stage at which infected embryos would be processed. This analysis also allowed us to identify tissues with particularly high levels of NRSF expression, which might be most sensitive to perturbation of NRSF function. At E5 NRSF mRNA is broadly expressed outside of the nervous system. Expression is high in the derivatives of the somite, including the myotome and dermis, with lower levels of expression in condensing sclerotome (Fig. 1B). NRSF message is also seen in the ectoderm, mesonephros, and in mesenchymal tissues. In the central nervous system (CNS) expression is higher within the neuroepithelium of the spinal cord (Fig. 1B, arrowhead), where neural progenitors reside, than in the differentiated, marginal zone. In the peripheral nervous system (PNS) the cells within the spinal nerves appear to have a rather low level of NRSF expression. This expression pattern is similar to that previously reported for mouse NRSF (Chong et al., 1995; Schoenherr and Anderson, 1995a), supporting the ideas that this chicken clone does indeed represent the avian ortholog of NRSF and that NRSF function is likely conserved between avians and mammals.

Infection with dnNRSF retrovirus results in derepression of a candidate target of NRSF transcriptional repression

We sought to test the ability of the dnNRSF virus to derepress endogenous genes that are putatively regulated by NRSEs. As a first candidate we chose the $c\beta_4$ tubulin gene, which encodes the neuron-specific, Class III β-tubulin recognized by the monoclonal antibody, TuJ1 (Lee et al., 1990). βIII-tubulin expression is one of the earliest known markers of neuronal differentiation (Menezes and Luskin, 1994). This gene has previously been found to contain a good consensus NRSE that is able both to bind NRSF in vitro and to mediate silencing of a heterologous reporter construct in NRSF-expressing cells (Schoenherr et al., 1996).

As an initial test we infected the same CEF cells that were originally generated for production of the viruses (Fig. 2). Expression of the dnNRSF protein in infected cells was confirmed by immuno-staining with an antibody to the myc epitope tag of the transgene. The staining pattern was primarily nuclear (Fig. 2B), indicating that regions of NRSF N- and C-terminal to those included in the dnNRSF construct do not contain essential nuclear localization signals. Cultures infected in parallel with the negative-control retrovirus, nLuc, showed similar nuclear expression (with somewhat higher sensitivity of detection due to the presence of more than one myc tag per protein molecule, Fig. 2E). TuJ1 immunocytochemistry revealed expression of βIII-tubulin in dnNRSF- but not in nLuc-infected CEFs (Fig. 2C, F). This result provided the first evidence that dnNRSF was able to inhibit the repression of an endogenous, chromosomal NRSE-containing gene.

Many previous studies have suggested that NRSF represses neural genes in non-neural tissues (see Discussion). A distinct role has been proposed by some investigators

that within the nervous system NRSF negatively regulates the expression of neural genes during neurogenesis. We decided to address the former of these two roles first. Of all non-neural tissues, derivatives of the somite appeared to express among the highest levels of endogenous NRSF. Therefore, we infected early somites with each of the two retroviruses in order to assess in vivo the effect of inhibition of NRSF function outside of the nervous system. Virus was injected on one side of the embryo only, into the five somites at the level of the presumptive wing bud. This was done at a stage just after these somites have formed (St. 14-15).

After three days of re-incubation, embryos were sectioned at the wing-bud level and analyzed for expression of the transgene and of βIII-tubulin. In situ hybridization for the dnNRSF transgene, with a probe specific for mouse NRSF, revealed almost complete infection of one side of the embryo with minor secondary infection on the opposite side (Fig. 3A). Analysis of a near adjacent section with the antibody to the myc epitope tag indicated that dnNRSF protein was indeed synthesized by these infected tissues (Fig. 3B). The apparent difference between the extent of infection indicated by these two methods likely represents a combination of a difference in sensitivity between the two techniques and a time delay between mRNA and protein expression, especially in regions of secondary infection. Infection similar to that with the dnNRSF retrovirus was obtained with the control retrovirus, nLuc (Fig. 3D, E), except that in this particular embryo significant secondary infection had occurred on the uninjected side.

Immuno-staining of dnNRSF-infected embryos with the TuJ1 antibody revealed that βIII-tubulin expression was no longer restricted to the nervous system on the injected side (Fig. 3C). In addition to its normal expression pattern in differentiated neurons and their processes, areas of βIII expression included the myotome, dermis,

ectoderm, mesonephros, and various mesenchymal regions. All of these tissues were retrovirally infected (Fig. 3A), and all of these tissues also express endogenous chick NRSF (Fig. 1B). Notably, however, βIII-tubulin misexpression was not seen in all infected regions of the embryo, e.g., the condensing sclerotome (arrowheads, Fig. 3A, C), where endogenous NRSF expression is relatively low (Fig. 1B). Importantly, TuJ1 staining was always normal when the embryo was infected with the nLuc, control retrovirus (Fig. 3F), indicating that ectopic expression of βIII-tubulin in vivo is specific to the dnNRSF transgene.

NRSF null mouse embryos have been generated and analyzed with the TuJ1 antibody (Chen et al., 1998). Interestingly, these embryos show ectopic βIII-tubulin expression in a number of areas, including the myotome. The fact that the null phenotype is similar to that observed with expression of dnNRSF supports the idea that the dnNRSF protein acts by interfering with endogenous NRSF activity.

Somite injection of dnNRSF retrovirus results in derepression of three different neural target genes in non-neural tissues

βIII-tubulin is only one of a large number of predominantly neural genes thought to be directly regulated by NRSF (Schoenherr et al., 1996). Among these genes are the neuron-glial cell adhesion molecule, Ng-CAM (Kallunki et al., 1995), the axon growth-associated protein, SCG10 (Mori et al., 1992), and middle molecular-weight neurofilament, NF-M (Schoenherr et al., 1996). At the time of analysis Ng-CAM mRNA is normally expressed at high levels in differentiated neurons and at low levels in glial progenitors. SCG10 and NF-M message is normally only present in differentiated neurons.

We examined the expression of these additional candidate targets of NRSF repression in somite-injected embryos.

Three of the four putative target genes (all but NF-M) were found to be derepressed by dnNRSF in the non-neural tissues analyzed (Fig. 4A, B, C, D; and data not shown). Derepression was never seen in response to expression of the nLuc, control protein (data not shown). Interestingly, direct comparisons of the expression pattern of Ng-CAM, βIII-tubulin, and SCG10 within single dnNRSF-infected embryos revealed that regions of derepression were not the same for all of these genes (Fig. 4). As described above, extra-neural TuJ1 staining was found in almost all infected regions of the embryo except the condensing sclerotome (Fig. 4Cl ,Cr ,Cm , arrow). Ng-CAM, however, appears to be derepressed to varying extents in essentially all infected areas including the condensing sclerotome (Fig. 4Bl, Br, Bm, arrow). In contrast to this widespread pattern, ectopic SCG10 expression was limited to the mesenchyme in the shoulder region and the dermis (Fig. 4Dl, Dr) and to the area around the dorsal aorta (Fig. 4Dm). Unlike both βIII-tubulin and Ng-CAM, derepression of SCG10 was not seen in ectoderm, and derepression within the myotome was barely detectable (Fig. 4Dl, Dr). Therefore, each of these genes had their own specific pattern of derepression. However, the fact that three putative direct targets of NRSF repression showed ectopic expression in response to dnNRSF strongly supports the idea that in vivo NRSF is generally required to prevent expression of neural genes outside of the nervous system.

Given the large number of putative targets of NRSF repression, the fact that Ng-CAM, βIII-tubulin, and SCG10 were all misexpressed in dnNRSF-infected embryos suggested that many additional genes not specifically examined were likely to be derepressed in various tissues of these embryos as well. However, our ability to survey a

larger number of putative NRSF targets was limited by the availability of good avian probes for these genes. Nevertheless, our results raised the possibility that the ectopic expression of multiple neural genes might change the fate of the infected non-neural cells. To address this question embryos were injected and re-incubated as previously described, but were allowed to develop for an additional three days, by which time the musculature is well developed. Surprisingly, dnNRSF-infected embryos were found to have histologically normal muscle fibers and normal levels of myosin, as detected with the MF 20 antibody (data not shown; Bader et al., 1982). There were also no obvious defects in other tissues as compared to control-infected embryos (data not shown). This apparent lack of effect on cell fate may reflect the fact that the expression level of a target gene is significantly lower in areas of derepression than at its normal sites of expression.

dnNRSF-infection of neural crest results in the derepression of neural genes in glial progenitors

Neural crest cells migrate out of the neural tube and give rise to a number of different cell types including peripheral neurons and glia. For example, cells that migrate to the peripheral nerves go on to differentiate into Schwann cells. Ng-CAM is normally expressed at low levels in these cells, whereas SCG10, βIII-tubulin, and NF-M are not. Neural crest cells at the level of the limb bud can be infected by injecting retrovirus into the neural tube of early embryos (St. 10-11) at a time when trunk-level neural crest has not yet begun to migrate (Fig. 5A, D). Therefore, we asked whether neural target genes of NRSF were derepressed in dnNRSF-expressing neural crest derivatives. We analyzed embryos three days after injection. Unfortunately, the high level of normal expression by

neurons within ganglia made it impossible to visualize derepression in associated non-neuronal (satellite) cells in these structures. Similarly, the intense axonal staining of the TuJ1 antibody precluded assessment of βIII-tubulin expression in the Schwann-cell progenitors within the spinal nerves. However, we were able to compare levels of Ng-CAM, SCG10 and NF-M mRNA in these cells between dnNRSF- and nLuc-infected embryos.

Both SCG10 and Ng-CAM were found to be derepressed in the glial progenitors within the spinal nerves of dnNRSF-infected embryos (Fig. 5B, C). While SCG10 expression was never seen in this region of nLuc-infected embryos (Fig. 5E), expression was detectable in those infected with dnNRSF (Fig. 5B, arrow). In the case of Ng-CAM, although a low level of expression as expected was found in both nLuc-infected and un-infected embryos (Fig. 5F, and data not shown), expression was slightly but reproducibly increased in embryos infected with dnNRSF (Fig. 5C). The NRSF, therefore, is likely required for repression of neural genes in glial progenitors.

dnNRSF-infection of the neural tube results in premature expression of neural genes in neural progenitors

As shown earlier (Fig. 1B), an area of high NRSF expression within the central nervous system is the neuroepithelium, or ventricular zone, of the neural tube. As neuronal progenitors, which reside in this area, differentiate, they migrate to the marginal zone, where they come to express markers of differentiated neurons. NRSF expression in the marginal zone is relatively low. We were interested, therefore, in asking whether NRSF activity is required to keep neural target genes from being expressed prematurely in the ventricular zone.

In order to test this idea, we injected the dnNRSF and nLuc retroviruses into the neural tube of early embryos (St. 10-11) as above. At this time neurogenesis has not yet begun in the spinal cord. After three days of re-incubation we analyzed the spinal cords of these embryos at the level of the wing bud for expression of the transgene and of the same four neural target genes listed above. As seen in Fig. 4C, a low level of TuJ1 staining is normally detectable within the ventricular zone of the spinal cord (probably from the small fraction of cells that have just completed their last cell division but have not yet migrated away). Because of the limitations of antibody staining, this level of expression of βIII-tubulin precluded an assessment of whether or not the gene was upregulated in the ventricular zone of dnNRSF-infected embryos.

SCG10, Ng-CAM, and NF-M are not normally expressed in the ventricular zone (Fig. 4B, D, and data not shown). Once again NF-M expression was unaffected (data not shown), however, both SCG10 and Ng-CAM were found to be ectopically expressed within the ventricular zone of the spinal cord of dnNRSF- but not nLuc-infected embryos (Fig. 6A-D and E-H, arrows, respectively). This was also true of other levels of the neural tube as well, e.g., the telencephalon (data not shown). The extent of target gene derepression varied with the extent of retroviral infection, but generally SCG10 expression within the ventricular zone occurred within the dorsal half of the spinal cord (Fig. 6B, arrow), whereas Ng-CAM derepression was usually observed more ventrally (Fig. 6F, arrow). In addition, the in situ signal of Ng-CAM within the neuroepithelium took a shorter time to develop and appeared to be stronger than that of SCG10, even though the normal signal of both of these genes, within differentiated neurons, was ostensibly equivalent (Fig. 6B,F). This may reflect the fact that the Ng-CAM gene contains five copies of the NRSE, while SCG10 has only one. These data support the idea that NRSF is

required to prevent premature expression of neural genes in neural progenitors of the central nervous system as well as to repress neural gene expression in progenitors of peripheral glial.

As mentioned earlier, when the dnNRSF retrovirus was injected into the somite, the resulting inappropriate, low-level expression of neural genes in non-neural tissues did not appear to have much effect on the proper further development of those tissues. However, we were interested in finding out whether within the nervous system the precocious expression of neural genes might perturb subsequent development more severely. For this purpose we allowed some neural tube-injected embryos to re-incubate for longer than the usual three days. At six days of re-incubation, patterning of the spinal cord as assessed by a number of different neuronal markers showed no difference between dnNRSF- and nLuc- infected embryos (data not shown).

DISCUSSION

Potential roles of NRSF during embryogenesis

Tissue-specific gene expression is a complex phenomenon that arises over the course of development through the action of a number of different mechanisms. One of these is negative transcriptional regulation. An important example of a negative transcriptional regulator that contributes to neuron-specific gene expression is NRSF. This factor is known to bind to a large number of putative target genes via the sequence element, NRSE. These target genes are predominantly markers of neuronal terminal differentiation, and as such they indicate that NRSF may play a role in coordinating the process of attaining the neuronal phenotype. Another role for NRSF has also been proposed based on the ability of its binding site to mediate repression of reporter gene

expression when transfected into non-neuronal cell lines (Bessis et al., 1995; Kallunki et al., 1995; Kraner et al., 1992; Li et al., 1993; Lonnerberg et al., 1996; Mieda et al., 1997; Mori et al., 1992; Wood et al., 1996). These data suggest that NRSF may act to prevent expression of neural genes outside of the nervous system.

In this study we present evidence for both of these proposed roles for NRSF. We do this in vivo by assessing the effect of inhibiting the ability of endogenous NRSF to interact with the NRSEs present in endogenous chromosomal target genes. First, we show that NRSF function is required in non-neural tissues to prevent the ectopic expression of the three putative target genes, βIII-tubulin, SCG10, and Ng-CAM. Then we show that in response to dnNRSF expression within the nervous system, not only is SCG10 and Ng-CAM derepressed in peripheral glial progenitors, but these genes are also prematurely expressed in neural progenitors of the central nervous system. These observations indicate that NRSF function is an important factor in proper neuronal gene expression during embryogenesis.

One previous study had implicated a role of NRSF as a negative transcriptional regulator within the nervous system during embryogenesis (Kallunki et al., 1997). As a transgenic mouse analysis, it was limited to observing the effects of NRSE deletion on a synthetic transgene, randomly integrated into the genome. However, it did suggest that NRSF is important for the regulation of the gene, L1, in the PNS. Among other neural crest derivatives, immature Schwann cells of the peripheral nerve showed derepression of the L1 transgene. This is similar to our result with Ng-CAM (the closest chicken relative to L1) in the peripheral nerve of dnNRSF-infected embryos. Our study, however, is the first to indicate that NRSF is required as a repressor within the CNS to prevent precocious expression of neuronal genes in neural progenitors.

Interestingly, other investigations have suggested an additional role for NRSF within the nervous system: NRSF may act as a positive regulator of neuronal genes within differentiated neurons. In a transgenic mouse study of the promoter of the β2 subunit of the nicotinic acetylcholine receptor, the expression level of the NRSE-deleted transgene was lower than that of the NRSE-containing transgene in differentiating peripheral ganglia and in neurons of the adult brain (Bessis et al., 1997). Also, in a study of the L1 transgene at post-natal stages, various areas of the brain showed decreased expression of the NRSE-deleted transgene (Kallunki et al., 1998). The recent demonstration of low-level NRSF expression within neurons of the adult brain provides support for this potentially additional role for NRSF (Palm et al., 1998). Our current data do not support or refute this idea. The two roles for NRSF within the nervous system are not necessarily mutually exclusive.

Action of NRSF at the level of the transcriptional unit

Numerous studies have conjectured about the mechanism whereby NRSF represses NRSE-containing genes. Originally the NRSE was termed a silencer because of its ability to mediate repression independent of its orientation and position in the transcriptional unit (Kraner et al., 1992; Mori et al., 1990). Many groups have confirmed this finding (Kallunki et al., 1995; Li et al., 1993; Lonnerberg et al., 1996; Mieda et al., 1997; Wood et al., 1996). More recently, however, investigators have questioned calling NRSF a silencing factor based on an apparent lack of evidence that it acts by influencing chromatin structure (Tapia-Ramirez et al., 1997). However, two reports have implicated a change in chromatin structure associated with NRSE binding (Tanaka et al., 1998; Vandenbergh et al., 1989). Also, an argument was made that since NRSF can negatively

regulate plasmid-borne sequences, NRSF must not normally act through chromatin structure. However, a negative transcriptional regulator may use more than one mechanism to influence transcriptional activity. Although it may be able to function to a degree on non-chromosomal promoters, it may show more efficient transcriptional repression of a chromosomal form of the gene. Therefore, the actual mechanism(s) of transcriptional repression of NRSF via the NRSE still remains to be elucidated, and referring to the factor as a silencer or not depends on one's definition. (We have used the term, "transcriptional repression" rather than "silencing" throughout this report simply because an NRSE-containing reporter gene is about one order of magnitude less active than one without an NRSE. The gene is not completely turned off, as the term "silenced" may imply.)

What can the data presented here reveal about the action of NRSF at the level of the transcriptional unit? The patterns of derepression of different target genes in different non-neural tissues can provide some insight. When somites were injected with the dnNRSF retrovirus, we found that three of the four putative direct target genes, SCG10, βIII-tubulin, Ng-CAM, and NF-M, were derepressed in infected regions. However, each of the three responsive genes was derepressed to different extents in different tissues. Why weren't all target genes derepressed in the same sets of tissues?

The transcriptional activity of any gene is a function of (1) all of its cis-acting regulatory elements, (2) all of the trans-acting regulatory factors present in a particular cell, and (3) the state of the chromatin structure within which the gene is embedded. In the case of the NF-M gene, for example, which was not found to be derepressed in any of the dnNRSF-infected tissues, perhaps by the time of injection it had already been packaged into heterochromatin so that the NRSE was inaccessible to exchange of

dnNRSF for endogenous NRSF. Or perhaps this exchange was insufficient to modify the existing structure. Differences in gene derepression accounted for solely by cis- and trans-acting regulators are depicted in Figure 7. In the case where some genes are derepressed in a given tissue and others are not, it may result from differences in their complements of cis-acting elements (Fig. 7A). For example, a gene with a binding site for an additional repressor may still not be expressed at detectable levels when repression by NRSF has been relieved by dnNRSF. In the case where a gene is derepressed in some tissues and not others, it may result from differences in the distribution of trans-acting factors among these tissues (Fig. 7B). For example, a tissue that lacks an activator for a particular gene may still not exhibit detectable levels of expression when repression by NRSF has been lifted by dnNRSF.

Whatever the specific explanation for the patterns of derepression observed in this study, the results suggest that there is not a simple, unitary mechanism that governs the neuron-specific expression of these pan-neuronal genes. Consistent with this, no single pan-neuronal enhancer element has thus far been identified in transgenic analyses of multiple vertebrate neuronal promoters. Additionally, in *Drosophila* multiple distinct enhancers are necessary for pan-CNS expression of β1 tubulin (Kohler et al., 1996).

Dominant-negative approach as compared to targeted deletion of NRSF

The current study is an attempted loss-of-function analysis of NRSF. However, a more straightforward approach for conducting such a study is to remove the NRSF gene itself through homologous recombination rather than removing, or reducing, its functionality through introduction of a dominant-negative mutant. Targeted deletion of NRSF in

mouse embryos has been achieved (Chen et al., 1998). How do the results of these two investigations support and compliment each other?

First, the phenotype of the NRSF null embryos reinforces the idea that dnNRSF acts as an inhibitor of endogenous NRSF function. The design of the dnNRSF protein was derived from a previous construct that was shown to be able to derepress NRSE-containing reporter genes in NRSF-expressing cell lines (Chong et al., 1995). This fragment of NRSF was later shown to have no transcriptional activity of its own (Tapia-Ramirez et al., 1997). We therefore expected our construct to have similar properties. One of the results of injecting the dnNRSF retrovirus into the somites of early chick embryos was that βIII-tubulin was later found to be derepressed in the myotome, according to immunohistochemistry with the TuJ1 antibody. Aberrant TuJ1 staining was also found in the myotome of mouse embryos homozygous null for NRSF. This observation supports the idea that dnNRSF indeed acts by inhibiting endogenous NRSF.

Second, expressing dnNRSF via a retrovirus makes infected embryos mosaic for NRSF function. This allows analysis of different tissues at different stages, in the context of an otherwise wild-type embryo. NRSF null mutant embryos die during mid-embryogenesis from E10.5 to E11.5 and begin to show extensive cell death by E9.5. Therefore, in order to assess the effects of loss of NRSF function before major secondary effects appear, analysis of gene expression was carried out at E9.0. Unlike in our experiment, where we saw derepression of SCG10 in certain infected tissues of the chick at E5, SCG10 derepression was not detected in the mutant mouse embryos. This lack of derepression may simply reflect that the relevant tissue had not yet developed. An E9 mouse embryo is generally less mature than in an E5 chicken embryo. Without the gain of additional transcriptional activators or the loss of additional repressors that

accompany the maturation of a tissue, SCG10 transcription, though relieved from NRSF repression, may not be detectable (as described above). The current study, therefore, as a mosaic analysis has advantages over the targeted deletion approach similar to those achieved by time- and tissue-specific knockouts.

Third, by introducing a form of NRSF that masks NRSEs, the dnNRSF approach has the potential to interfere with the function of all proteins that bind to NRSEs. Although currently there is no evidence for other NRSE-binding proteins or other NRSF family members, this provides an alternative explanation for differences between the targeted-deletion and dominant-negative results. Redundancy is a common problem for knockout studies. Although a gene may play a key role in a particular process, a second gene can often compensate to some degree for the loss of the deleted gene. This may explain why no additional putative target genes were found to be derepressed in null mutant mouse embryos. In the case of the dominant-negative approach, however, the ability of the protein to potentially interfere with the function of a related gene can be an advantage, allowing an important phenotype to surface. The current study, therefore, while being unable to formally claim that the observed derepression is due to an inhibition of NRSF activity specifically, has been able to reveal processes in which NRSF is likely to be important.

In summary, the current investigation has validated the use of a dominant-negative form of NRSF to inhibit the repression of multiple, endogenous, chromosomal NRSE-containing genes in vivo. We have shown that the function of NRSF (and/or another NRSF-like protein) is required for repression of three different neural target genes in non-neural tissues during embryogenesis. We have also shown that NRSF function is required for repression of two of these genes in peripheral glial progenitors.

Finally, we have shown that NRSF is required to prevent premature expression of these genes within neural progenitors of the central nervous system.

In none of the infected tissues was cell fate altered by inhibition of NRSF function and the resulting change in neuronal gene expression levels. It has been argued, on the basis of studies of early development, that transcriptional repressors may be particularly important in the initial specification of cell lineages and developmental territories, whereas transcriptional activators are sufficient to achieve proper expression of terminal differentiation genes once cell fate has been specified (Arnone and Davidson, 1997). Our data suggest that NRSF is not involved in specification, but rather functions to maintain the repression of cell-type specific, end-state genes in non-expressing cells. Therefore, at least for neuronal genes, the specificity of terminal differentiation gene expression is, like that of regulatory genes in early development (Gray and Levine, 1996), under the control of negatively as well as positively acting transcription factors.

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FIGURE LEGENDS

Fig. 1. Sequence and expression pattern of chicken NRSF. (A) Sequence comparison of human (H), mouse (M), and chicken (C) NRSF in the zinc-finger region. Chicken sequence was derived by RT-PCR using degenerate oligonucleotide primers. The mouse sequence (C.J. Schoenherr and D.J. Anderson, unpublished data) is deposited in Genbank (accession number U13878). Lines and numbers above the sequence indicate individual zinc fingers. Identical amino acids are boxed. (B) In situ hybridization using antisense probe for chicken NRSF. Shown is a transverse section of an E5 chick embryo at the level of the wing bud. NRSF expression is broad with particularly high levels in somite derivatives and in the ventricular zone of the spinal cord (arrowhead) and very low levels in the spinal nerves.

A

```
   2          3                    4
H: LKHHTRAGDNERVYKCIICTYTTVSEYHWRKHLRNHFPRKVYTCGKCNYFSDRKNNYVQHVRTH
M: LKHHLRAGENERIYKCIICTYTTVSEYHWRKHLRNHFPRKVYTCSKCNYFSDRKNNYVQHVRTH
C: LKHHNKAGENERVYKCTICTYTTVSEYHWKKHLRNHFPRKVYTCSQCSYFSDRKNNYIQHIRTH

        5                6
TGERPYKCELCPYSSSQKTHLTRHMRTHSGEKPFKCDQCSYVASNQHEVTRHARQVH
TGERPYKCELCPYSSSQKTHLTRHMRTHSGEKPFKCDECNYVASNQHEVTRHARQVH
TGERPYQCAMCPYSSSQKTHLTRHMRTHSGEKPFKCDQCSYVASNQHEVTRHARQVH

        7                8
NGPKPLNCPHCDYKTADRSNFKKHVELHVNPRQFNCPVCDYAASKKCNLQYHFKSKH
NGPKPLNCPHCDYKTADRSNFKKHVELHVNPRQFNCPVCDYAASKKCNLQYHFKSKH
NGPKPLTCPHCDYKTADRSNFKKHVELHVNPRQFLCPVCDYAASKKCNLQYHIKSRH
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B

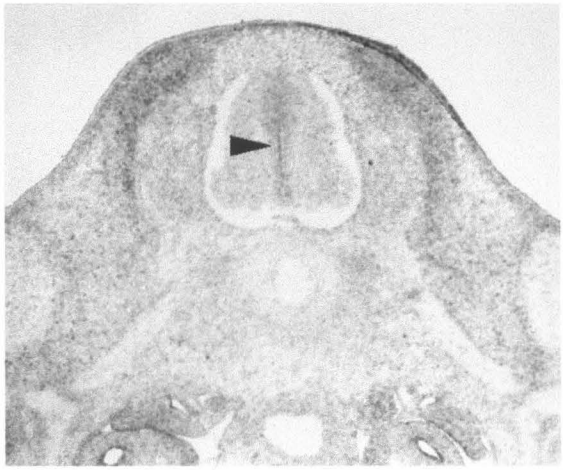


Fig. 2. Derepression of βIII-tubulin in chick embryonic fibroblasts infected with dnNRSF (A-C) but not control, nLuc (D-F) retrovirus. The same fields are shown in (A, D) as in (B, E), respectively, photographed with phase optics. (B, E) Infected cells stained with the anti-c-myc antibody, 9E10, show nuclear expression of the exogenous protein. The level of nLuc expression appears higher because luciferase contains five myc-epitope tags, whereas dnNRSF contains one. (C) dnNRSF-infected cells stained with the TuJ1 antibody against the βIII-tubulin protein show derepression, whereas nLuc-infected cells do not (F).

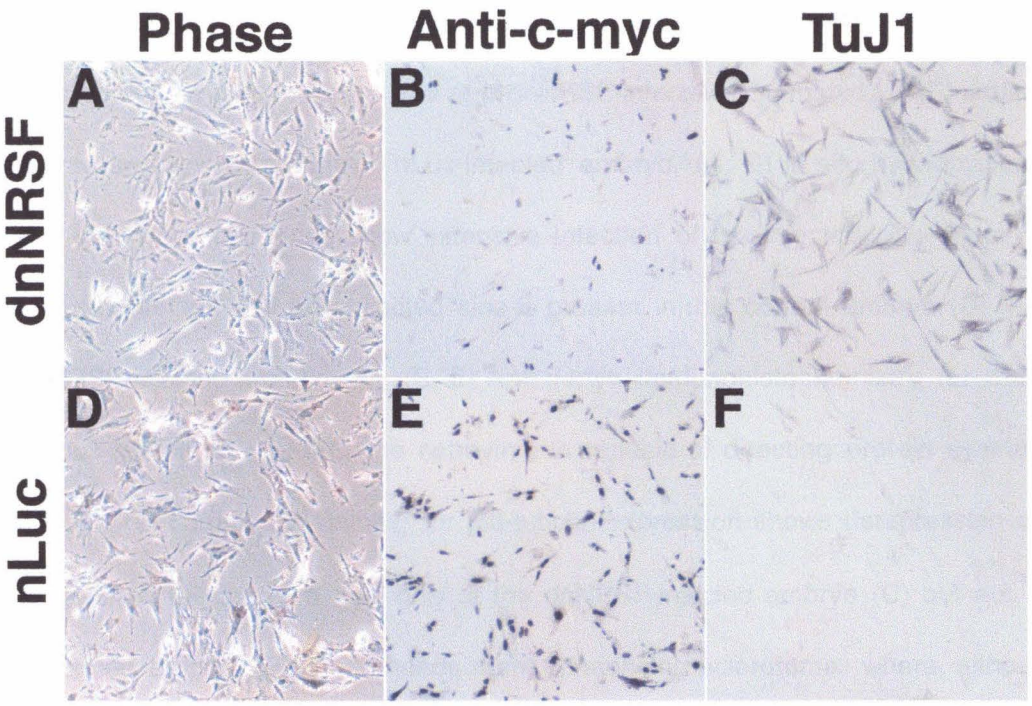

Phase
Anti-c-myc
TuJ1
dnNRSF
nLuc
A
B
C
D
E
F

Fig. 3. In vivo derepression of βIII-tubulin in dnNRSF-infected somite derivatives. Retroviral injections were made on E2 (St. 14-15) into the 5 somites, on one side of the embryo only, at the level of the presumptive wing bud. (A-C) Adjacent, transverse sections at the level of the wing bud of a dnNRSF-infected E5 embryo. (D-F) Adjacent, transverse sections of a control, nLuc-infected embryo. (A, D) In situ hybridization for the two different transgenes show extensive infection of the injected side. More secondary infection of the uninjected side is present in this control embryo (D) than in the dnNRSF-injected embryo (A). (B, E) Anti-c-myc immunohistochemistry for the exogenous protein shows that the retrovirus is capable of directing protein synthesis in vivo. (C, F) TuJ1 antibody staining for βIII-tubulin expression shows derepression of this target gene on the infected side only of the dnNRSF-injected embryo (C) but not the nLuc-injected embryo (F). Arrowheads mark condensing sclerotome, where, although the retrovirus is expressed, βIII-tubulin is not derepressed (A, C).

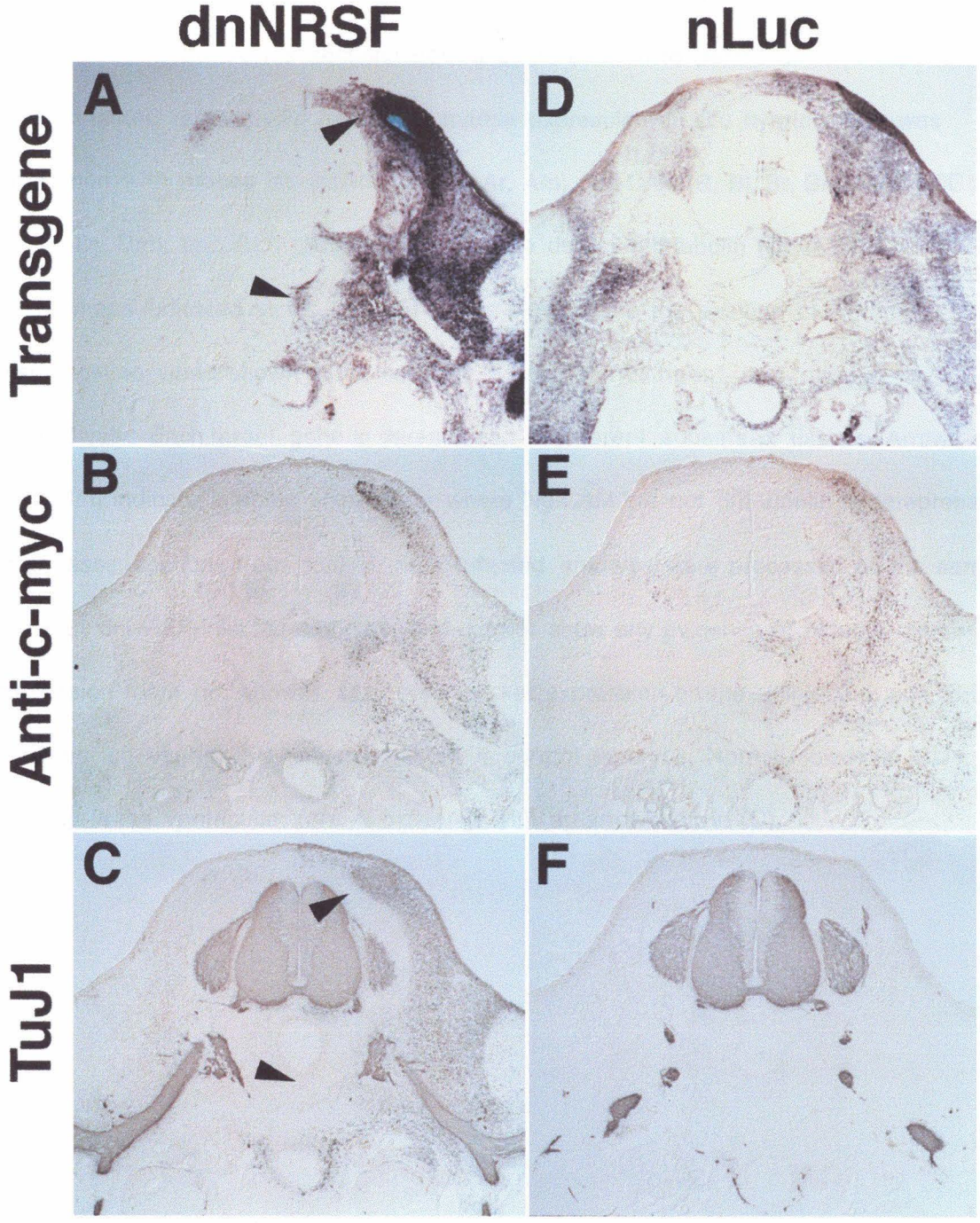

dnNRSF
nLuc
Transgene
Anti-c-myc
TuJ1
A
B
C
D
E
F

Fig. 4. Derepression of multiple neuronal target genes in non-neural tissues of embryos injected with dnNRSF virus into somites. Transgene and target gene expression are shown on serial transverse sections from a single embryo to permit direct comparison of virally infected regions with regions of marker expression. In situ hybridization was performed with probes for dnNRSF (A, Al, Ar, Am), Ng-CAM (B, Bl, Br, Bm), and SCG10 (D, Dl, Dr, Dm); and TuJ1 antibody was used to detect βIII-tubulin (C, Cl, Cr, Cm). The boxed areas indicated as "l," "r," and "m" in (A) indicate the position of higher magnification views shown in the columns labeled "left," "right," and "middle," respectively. Each target gene is derepressed in different subsets of tissues. Arrows in (Bm, Cm) indicate infected sclerotome where Ng-CAM but not βIII-tubulin is derepressed. In all cases, sections from control, nLuc-infected embryos were processed on the same slides as dnNRSF-infected embryos, and did not show any evidence of ectopic marker expression (data not shown). Scattered marker expression on the uninjected side (B-D, compare left vs. right) was also detected in control embryos. Normal, low-level TuJ1 staining in the ventricular zone is indicated with an arrowhead in (C).

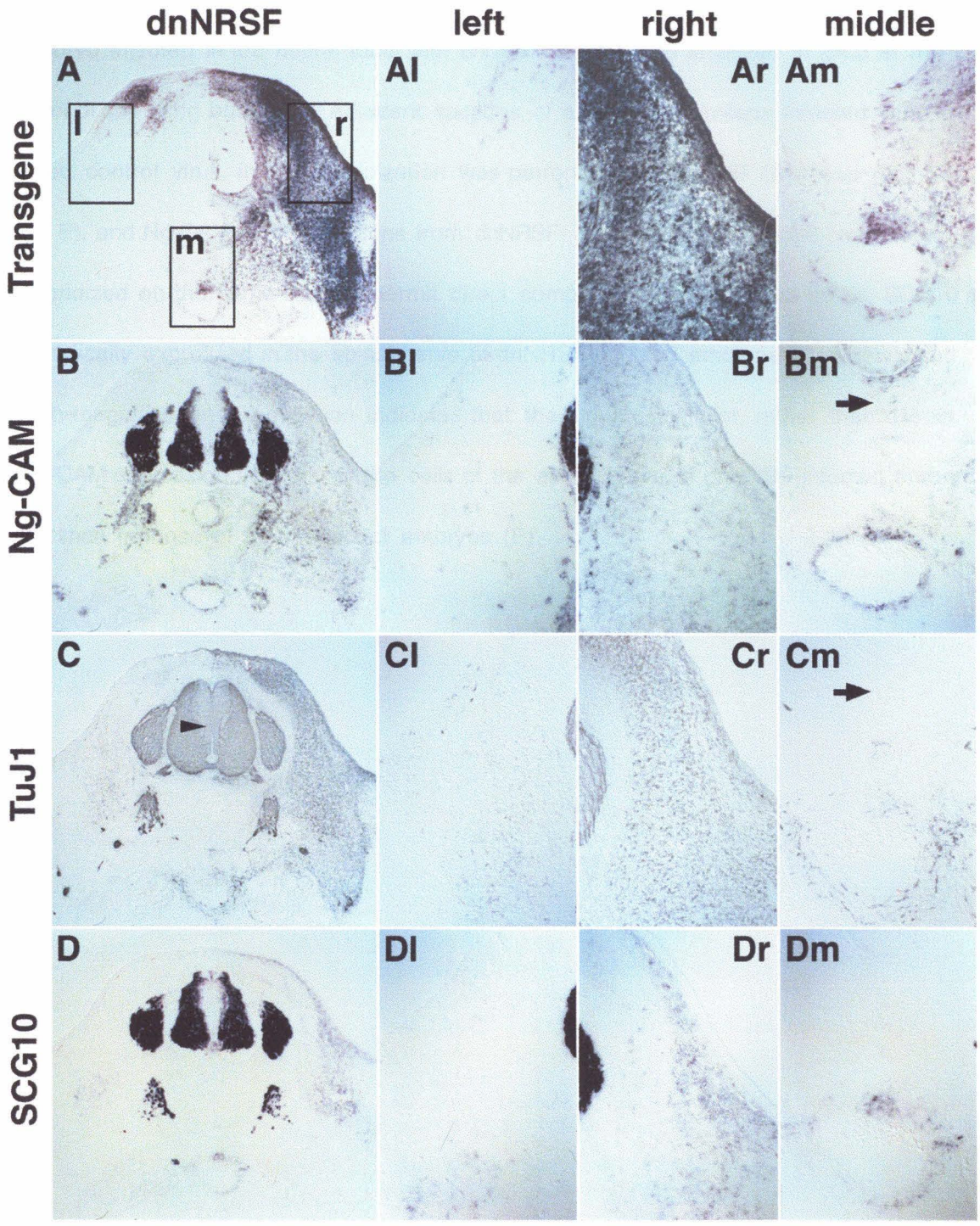

dnNRSF
left
right
middle
Transgene
Ng-CAM
TuJ1
SCG10
A
Al
Ar
Am
l
r
m
B
Bl
Br
Bm
C
Cl
Cr
Cm
D
Dl
Dr
Dm

Fig. 5. Derepression of neuronal genes in peripheral glial progenitors by infection of trunk neural crest with dnNRSF retrovirus. (A-C) Adjacent transverse sections of an embryo injected in the neural tube with dnNRSF at E1.5 and analyzed at E4.5 at the level of the wing bud. (D-F) Adjacent sections of an embryo similarly infected with the nLuc, control virus. In situ hybridization was performed for dnNRSF (A), nLuc (D), SCG10 (B, E), and Ng-CAM (C, F). Sections from dnNRSF- and nLuc-infected embryos were hybridized on the same slide to permit direct comparison of expression levels. SCG10 is ectopically expressed in the spinal nerve of dnNRSF-infected embryos (B, arrow), and high-magnification examination indicates that the signal is cellular, rather than axonal. Ng-CAM expression is higher in the cells of the spinal nerve of dnNRSF-infected embryos (C) than in those of nLuc-infected embryos (F).

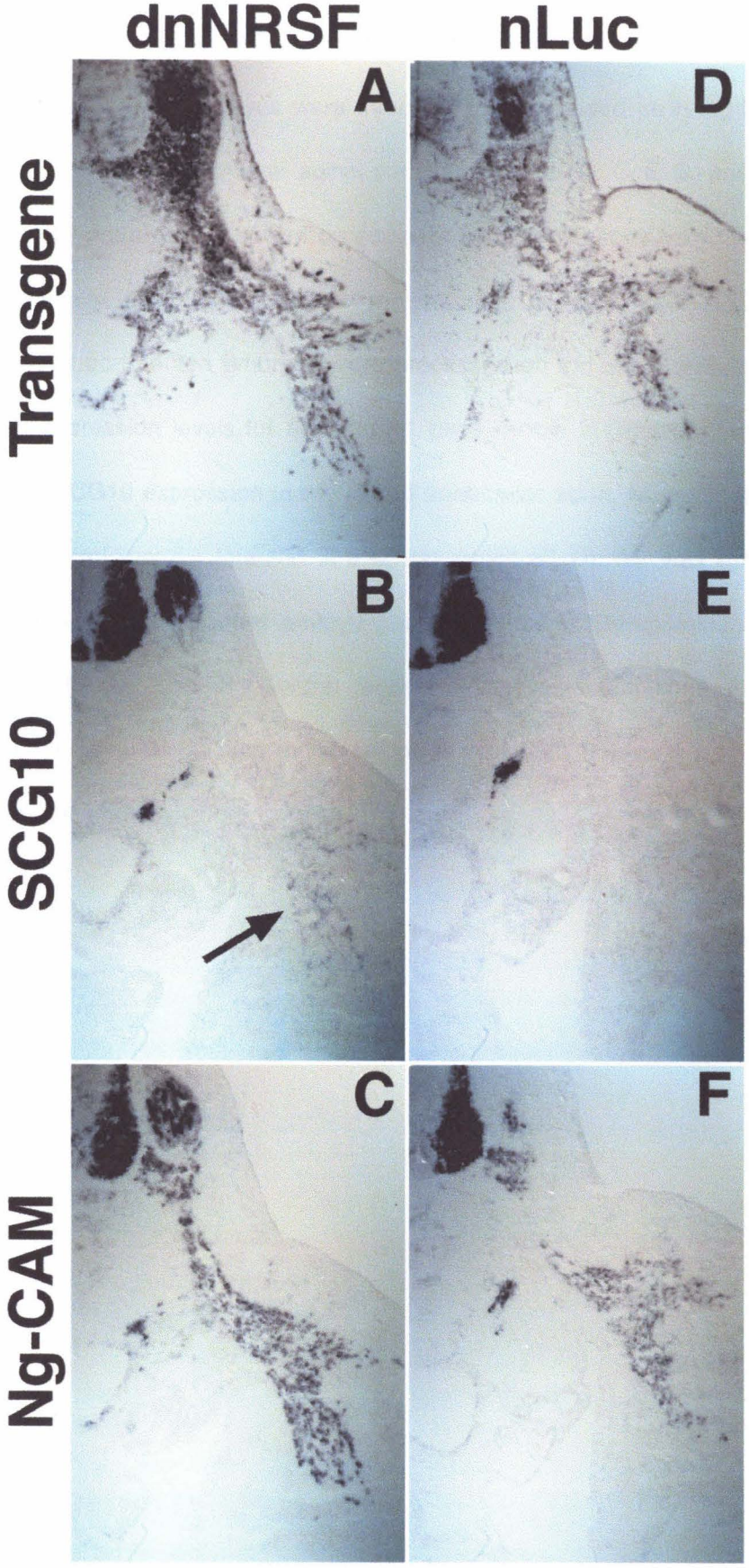

dnNRSF
nLuc
Transgene
SCG10
Ng-CAM
A
B
C
D
E
F

Fig. 6. Premature expression of neuronal target genes in CNS neural progenitors of dnNRSF-infected embryos. Embryos were infected and processed as in Fig 5. Shown are transverse sections of thoracic-level spinal cord. Transgene (A, C, E, G) and target gene (B, D, F, H) in situ patterns are shown on adjacent sections, respectively, for direct comparison of virally infected regions with regions of target-gene derepression. Sections of dnNRSF- and nLuc-infected embryos were processed on the same slide to allow direct comparison of expression levels for each target gene. Arrow in (B) indicates derepression of SCG10 expression in the dorsal ventricular zone. No such signal is detected in a control embryo infected in a similar region (C, D), nor was ventricular-zone staining seen in any nLuc- infected embryo (data not shown). Derepression of Ng-CAM (F, arrow) tends to occur in more ventral regions of the ventricular zone. In nLuc-infected embryos Ng-CAM staining in this region is very faint (H), as it is in uninjected controls (data not shown).

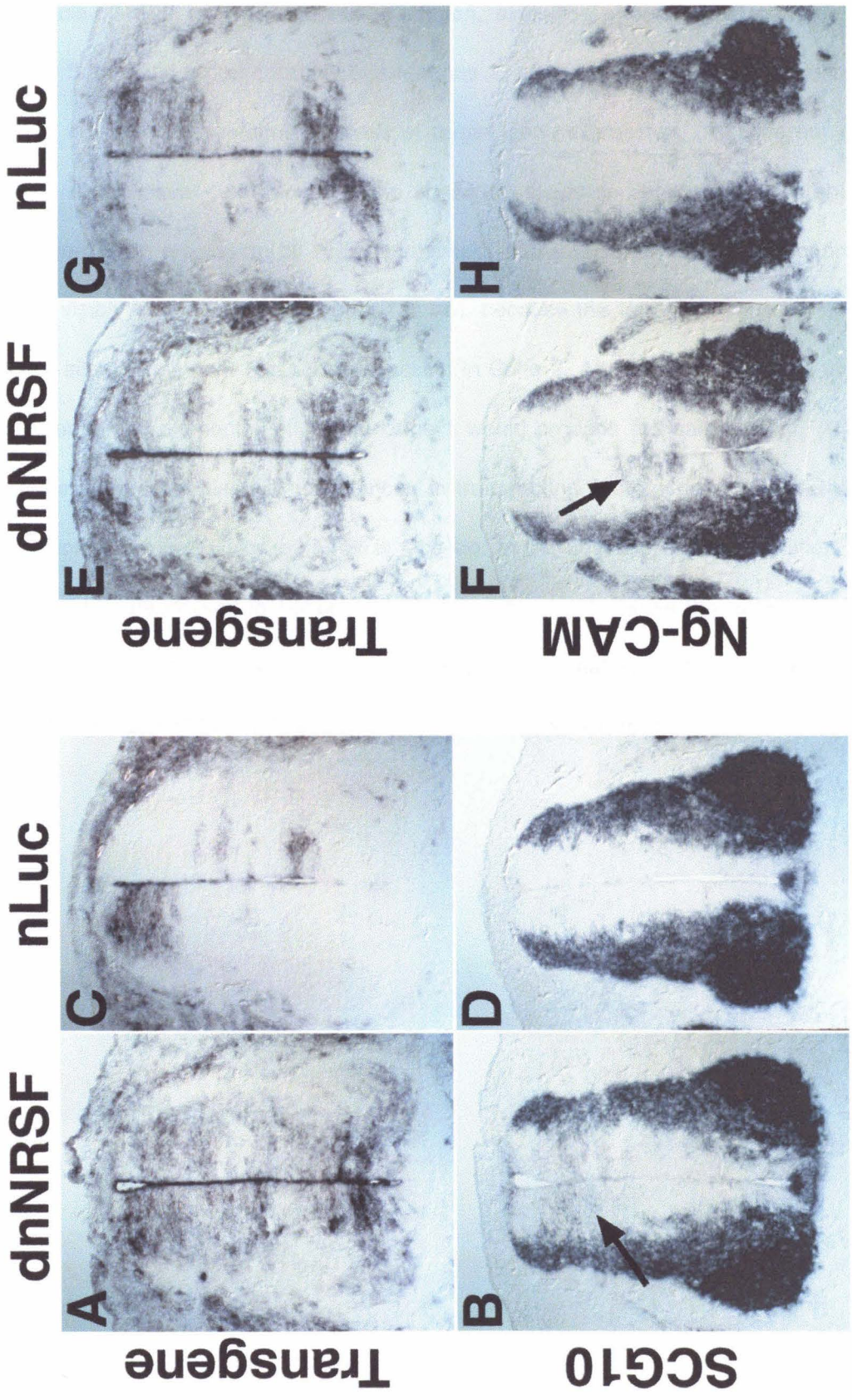
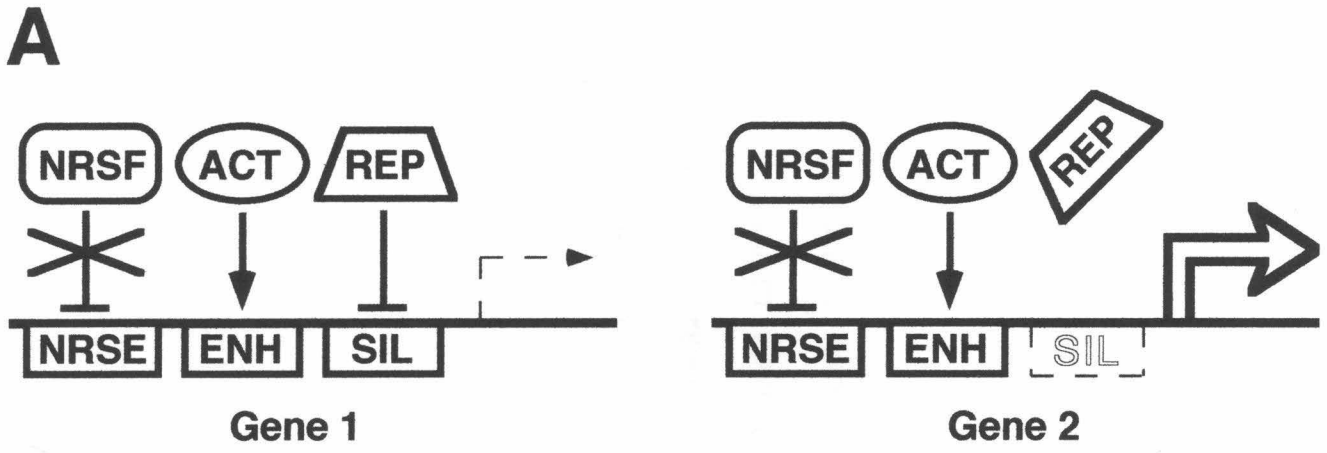
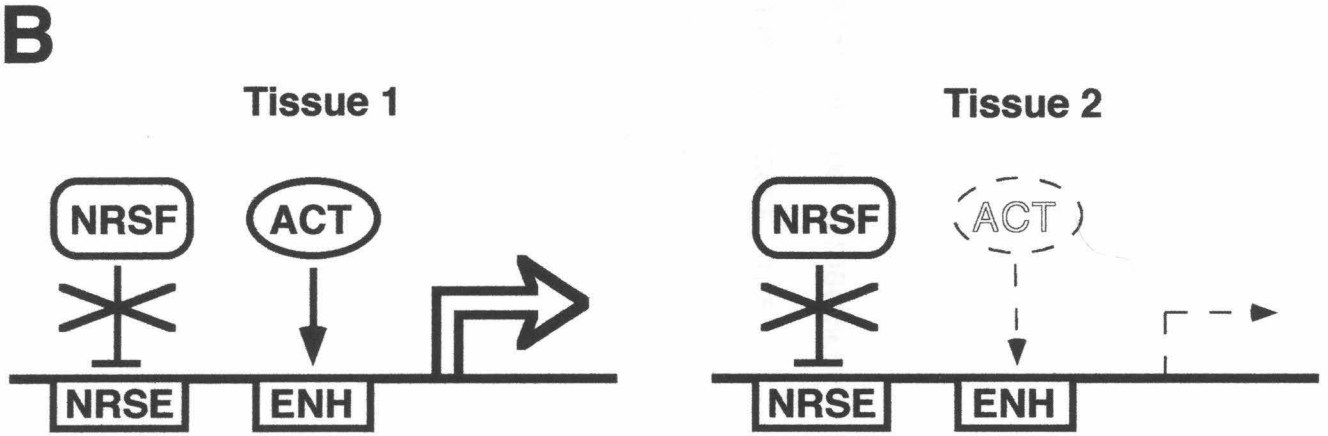

dnNRSF
nLuc
Transgene
SCG10
A
B
C
D
dnNRSF
nLuc
Transgene
Ng-CAM
E
F
G
H

Fig. 7. Role of NRSF in transcriptional regulation, excluding effects on chromatin structure. Schematic representation of situations in which relief from NRSF repression is and is not sufficient for detectable levels of target gene expression. (A) Different genes within the same tissue - differences in the cis-acting elements determine detectable derepression when repression by NRSF is inhibited. In the absence of NRSF function, Gene 2 is visibly derepressed, but Gene 1 is not, because the latter contains an additional silencer element (SIL) that is lacking in Gene 2. Alternatively, the lack of an essential enhancer element (ENH) from Gene 1 would produce the same effect. (B) Same gene in different tissues - differences in trans-acting factors determine detectable derepression when repression by NRSF is relieved. In the absence of NRSF function, the gene is visibly derepressed in Tissue 1 but not in Tissue 2, because the latter lacks an essential activator (ACT) that is present in Tissue 1. Alternatively, the presence of an additional repressor (REP) in Tissue 2 would have the same effect.


A
NRSF
ACT
REP
NRSE
ENH
SIL
Gene 1
NRSF
ACT
REP
NRSE
ENH
SIL
Gene 2


B
Tissue 1
NRSF
ACT
NRSE
ENH
Tissue 2
NRSF
ACT
NRSE
ENH

Chapter 4

GENERATION AND CHARACTERIZATION OF ANTIBODIES AGAINST DIFFERENT REGIONS OF MOUSE NRSF AND CHARACTERIZATION OF THE EXPRESSION PATTERN OF NRSF PROTEIN IN DIFFERENTIATING P19 CELLS

SUMMARY

Antibodies against the N- and C-terminal regions of mouse NRSF were generated and characterized by dot-blot, Western, gel-shift, immunocytochemical, and immunohistochemical analyses. One monoclonal antibody, 12C11, is functional in dot-blot and Western analyses and recognizes both mouse and human NRSF in gel-supershift experiments. This antibody recognizes endogenous levels of NRSF expression in cultured cells and over-expressed levels in sections of retrovirus-infected chicken embryos. This and another monoclonal antibody generated against the N-terminus, 13B10, were used to characterize the expression pattern of NRSF protein in P19 cells, a model system of neural induction and neuronal and glial differentiation. NRSF is expressed at a high level in undifferentiated P19 cells and is down regulated upon neural induction, at a time point when neuronal and glial markers have not yet appeared. After neuronal and glial differentiation NRSF is expressed at different levels in different cell types. Cells that express neither neuronal nor glial markers show high levels of NRSF, while neurons express undetectable levels. Glia, on the other hand, do express NRSF, but at low levels.

INTRODUCTION

The expression pattern of a transcription factor determines in what tissues and cell types it has the potential of having an important function. Initial studies characterized NRSF expression in different cell lines and in various embryonic tissues using Northern

analysis, RNAse protection, and in situ hybridization (Chong et al., 1995; Schoenherr and Anderson, 1995; Scholl et al., 1996). Northern analysis showed that the major transcript of NRSF was approximately 7.6 kilobases (kb) and was expressed at relatively high levels in HeLa cells, in the rat L6 skeletal muscle cell line, and in newborn rat dorsal root ganglia (DRGs), which contain both neurons and differentiating glia. In contrast, the neuronal cell line, PC12, had very low-level NRSF expression (Chong et al., 1995). RNAse protection analysis by another group showed that NRSF expression was relatively high in two different fibroblast cell lines, Rat1 and C3H10T 1/2; that expression levels ranged from relatively high to low but detectable among four different glial cell lines, RN22, JS1, C6 rat glioma, and NCM1; and that NRSF expression was undetectable in two neuronal cell lines, MAH and PC12 (Schoenherr and Anderson, 1995). The difference between the two groups' assessment of NRSF expression in PC12 cells may have resulted from differences in sensitivity between the two techniques or slight differences between PC12 cell lines grown in different laboratories. Together these population analyses suggested that overall NRSF was expressed at high levels in non-neural cell types, moderate levels in glial cell types, and very low levels in neuronal cells.

In situ hybridization analyses of mouse embryonic tissues demonstrated the NRSF expression pattern in vivo (Chong et al., 1995; Schoenherr and Anderson, 1995). The high level of NRSF expression detected in non-neural tissues agreed with the high-level expression of NRSF seen in non-neural cell lines. In situ analyses also indicated a previously unknown aspect of the neural expression pattern: NRSF expression was moderate in the neural epithelium, an area consisting of neural progenitors, but very low in the marginal zone, where neurons (but not glia) have differentiated at the time of analysis (Chong et al., 1995; Schoenherr and Anderson, 1995). In ganglia of the

peripheral nervous system neural progenitors and differentiating neurons are spatially intermingled (at the time of analysis, glia have yet begun to differentiate), complicating the interpretation of in situ signals detected therein. Nonetheless, one group reported significant signal in the cranial ganglia, which increased from embryonic day 11.5 (E11.5) to E13.5 (Chong et al., 1995); while another group detected decreasing signal in DRGs and sympathetic ganglia from E12.5 to E13.5 (Schoenherr and Anderson, 1995). A potential explanation for this pattern could be that NRSF is expressed at moderate levels in undifferentiated neural progenitors present in the ganglia, and as they differentiate into neurons, the levels decline (as seen in the trunk-level ganglia), but as glia then begin to differentiate, the levels increase (as seen in the more anterior, more mature, cranial ganglia).

A careful and detailed analysis of the NRSF mRNA and its expression pattern in adult rat brain and various primary cell preparations and cell lines added new layers of complexity to the issue (Palm et al., 1998). Analysis of NRSF expression in adult rat brain was carried out using radioactive in situ hybridization and sections were counterstained in order to visualize cell profiles. This allowed a precise analysis of level of NRSF expression per cell in various brain regions and identification of cells as neuronal or glial. As expected, the highest levels of NRSF expression were detected in the non-neural tissues of the brain: the choroid plexus, ependymal layer, and pia. Unexpectedly, however, neurons in almost all regions of the adult brain were found to express low levels of NRSF. There were a few regions, though, where the neurons did not express detectable levels, including the caudate putamen, globus pallidus, and accumbens. In contrast to most neurons, glia of the adult brain were not found to express NRSF. Only a single population of glia, within the olfactory nerve layer of the olfactory bulb, expressed

detectable levels of NRSF. These results are generally in opposition to the data described above (Chong et al., 1995; Schoenherr and Anderson, 1995), where glial cell lines expressed moderate amounts of NRSF and neuronal cell lines expressed little if any. This indicates, therefore, that NRSF expression level may not be predictable a priori, simply based on the identity of a cell as a neuron or glial cell.

Another important finding of this detailed report involved alternative splicing of the rat NRSF gene, referred to as rREST (Palm et al., 1998). In almost all tissues and cell lines examined, about 10% of the transcripts were found to be a truncated, alternatively spliced isoform, rREST1, which encodes a version of NRSF truncated after the fourth zinc finger. More interestingly, however, the group also reported another alternative splicing event, which was specific to neurons. This isoform, rREST4, includes a short extra exon that results in a frame shift that truncates the protein after the fifth zinc finger. (The transcript itself, however, is unaffected but for the few extra base pairs.) RNAse protection analysis of neural tissues and cultured neurons indicated that rREST4 usually accounts for 1% of NRSF transcripts in neurons. In the neuronal cell line, Neuro 2A, however, rREST4 was the only NRSF transcript detectable. A final interesting observation of this paper is that, while almost all glia in the adult rat brain showed undetectable levels of NRSF expression, both the C6 rat glioma cell line and cultured hippocampal astrocytes were found to have relatively high levels of NRSF expression. Perhaps glia in their adult, quiescent state in general do not express NRSF, but when they are proliferating, as in the case of acute culture, or a transformed cell line, or during differentiation, NRSF is up regulated.

The expression analyses described so far have been at the level of the NRSF transcript. Two antibodies have been generated against NRSF and used in a limited way

to characterize NRSF protein expression. A mouse polyclonal ascites was made against the zinc-finger domain of human NRSF and used to supershift the NRSF-NRSE complex from a HeLa cell nuclear extract (Schoenherr and Anderson, 1995). A rabbit polyclonal antiserum was made against full-length human NRSF and used in both gel-shift and Western analysis (Chong et al., 1995). This antiserum recognized an NRSE-binding complex from extracts of HeLa cells, L6 rat skeletal muscle cells, and primary cultures of rat skeletal muscles. Additionally, the antiserum used in Western analysis identified a 116 kilo Dalton (kDa) protein strongly expressed in L6 cells and very weakly expressed in PC12 cells. Upon affinity purification of this antibody, the migration of NRSF was amended to approximately 200 kDa (Tapia-Ramirez et al., 1997).

In an effort to generate an anti-NRSF antibody that was functional in a range of immunochemical analyses, the monoclonal antibody 1D3 was made against a segment of mouse NRSF including the N-terminal region and zinc-finger domain (C.J. Schoenherr and D.J. Anderson, unpublished). Unfortunately, this antibody was functional in Western analysis but not in gel-shift analysis, immunocytochemistry, or immunohistochemistry (C.J. Schoenherr and D.J. Anderson, unpublished). This report describes the generation of monoclonal antibodies that allow the analysis of NRSF expression at the cellular level. These antibodies were characterized by dot-blot, Western, and gel-shift analyses and their functionality tested in staining of cultured cells and tissue sections. In the process of generating the monoclonal antibodies, a polyclonal serum was made that was used to address the possibility of aberrant alternative splicing in mouse embryos with a targeted deletion of NRSF (Chen et al., 1998).

Analysis of NRSF expression at the population and cellular levels was conducted on the P19-cell model of neural induction and neuro- and gliogenesis (Bain et al., 1994;

Pevny et al., 1998; Rudnicki and McBurney, 1987). A number of interesting observations were made. First, undifferentiated P19 cells, which have the capacity of differentiating into either muscle or neurons and glia, express high levels of NRSF. Second, NRSF expression is down regulated in these cells by the end of the neural-inducing treatment, days before markers of differentiation appear. Third, while P19 cell-derived neurons do not express detectable levels of NRSF, P19 cell-derived glia express NRSF at low levels. This is in agreement with previous analyses of neuronal and glial cell lines (Chong et al., 1995; Palm et al., 1998; Schoenherr and Anderson, 1995).

MATERIALS AND METHODS

Plasmid constructions

Two inducible bacterial expression constructs were made in pGSTag to produce fusion proteins to glutathione S-transferase (GST) containing different regions of mouse NRSF. GST-5' codes for all but the first amino acid (aa) of NRSF up to but not including the first zinc finger, approximately 150 aa. It was made by first using PCR to generate a fragment containing the 5' end of the NRSF coding sequence with an exogenous Hind III site at the 3' end of the fragment. Primers used were T7 and Z1R-HIII: 5' G C A G A T C A A G C T T G G C C T T A G A A C T C C T G 3'. This PCR fragment was then digested with Msc I (the first two base-pairs (bp) of which constitute the TG of the ATG initiator codon of NRSF) and HIII. Finally, this fragment was ligated into pGSTag from Xba I (blunted with Klenow) to HIII, and the plasmid was sequenced across the Xba I/Klenow – Msc I junction to confirm that the reading frame was maintained from the GST through to the NRSF coding sequences. GST-3' codes for an approximately 250 aa, C-terminal

fragment of NRSF. It was constructed by ligating a Hind III fragment of NRSF into the Hind III site of pGSTag. This fragment begins 750 bp before the stop codon of NRSF and contains 70 bp of 3' untranslated region (UTR).

Preparation of GST-NRSF fusion proteins

Proteins were induced in and purified from DH5α bacteria according to the handbook, "GST Gene Fusion System" (Pharmacia). Briefly, competent DH5α were transformed with GST-5', GST-3', or uninserted vector. Large-scale cultures were innoculated and treated with IPTG to induce expression from the plasmid. Cultures were sonicated and GST-fusion protein was partially purified from the lysate using glutathione sepharose to bind the protein and glutathione to elute it. Finally, proteins were further purified by gel electrophoresis and electroelution. NRSF was not cleaved from GST before using it to immunize mice.

Immunization, serum titering, and spleen fusion

Susan Ou of the Monoclonal Antibody Facility innoculated mice with GST-5' and GST-3' proteins, took bleeds, and performed spleen fusions. Three mice were injected for each protein, once each week for two weeks with 10 μg protein per injection per mouse. One week later, bleeds were tested for serum antibody titer using dot blots of partially purified GST-fusion protein on nitrocellulose. Antibody binding was detected with an HRP-conjugated, goat anti-mouse IgG secondary antibody developed with the substrate, 4-chloro-1-naphthol (4CN). Weekly injections were continued, with the lower-titer, GST-5' animals receiving an increased dose of 20 μg per injection. When titers reached a level

where a dilution of 1:10,000 gave a signal with 300 ng of blotted protein, the mouse was considered ready for spleen fusion. One mouse was used per fusion protein, and a final bleed was collected just before removal of the spleen.

Screening of hybridoma supernatants and subcloning of hybridomas

Between 750 and 800 initial supernatants were generated by Susan Ou from each of the spleen fusions. The GST-3' innoculation resulted in supernatants first, which were screened by immunocytochemistry on cells transfected with an NRSF expression construct. Ling Wang assisted in this screen. The supernatants derived later from the GST-5' innoculation were screened by immunocytochemistry on untransfected C3H10T 1/2 cells, the cell line from which mouse NRSF was cloned (C.J. Schoenherr and D.J. Anderson, unpublished). Antibody binding was detected using an HRP-conjugated goat anti-mouse IgG secondary antibody and developed with diaminobenzidine intensified with nickelous sulfate. A supernatant was scored as positive if it gave nuclear staining. Positive polyclones were subcloned by Susan Ou, and two of the resulting antibodies were isotyped, also by Susan Ou. Large-scale batches of hybridoma supernatant were prepared by Ling Wang.

Western analysis

Western analysis was conducted according to standard procedures. Cells were collected by trypsinization and counted before being lysed in SDS loading buffer and boiled. Lysate from equal numbers of cells were loaded in each lane of a gel. After blotting onto nitrocellulose, strips were cut and incubated with different hybridoma supernatants

either neat or diluted 1:10. Antibody binding was either detected with an alkaline phosphatase-conjugated secondary antibody and developed with BCIP and NBT or detected with an HRP-conjugated secondary antibody and visualized using ECL Western blotting detection reagents (Amersham). Chicken embryonic fibroblasts (CEFs) were generated and infected as described in Chapter 3. The virus used to express a truncated form of mouse NRSF in CEFs is B-SMA, which is the RCASBP(B) retroviral vector (Morgan and Fekete, 1996) containing a SLAX12NCO-derived insert encoding an N-terminally myc-epitope tagged version of NRSF that is truncated at the Afl II site. Since this site is 520 bp downstream of the zinc-finger domain, this truncated form of NRSF definitely contains the epitopes for both 12C11 and 13B10, which were made against the N-terminus, before the zinc fingers.

Gel-shift analysis

Gel shifts and supershifts were performed using the NRSE probe S36 as described in (Schoenherr and Anderson, 1995). HeLa cell nuclear extract was prepared by N. Mori (Mori et al., 1992). In vitro-synthesized mouse NRSF was made by cloning the coding region of mouse NRSF (C.J. Schoenherr and D.J. Anderson, unpublished) into the pCITE-1 vector (Novagen). RNA transcribed from this plasmid was then in-vitro translated in rabbit reticulocyte lysate (Promega). Mouse embryonic fibroblasts were made by Z.-F. Chen (Chen et al., 1998) from wild-type littermates of NRSF-/- embryos. Nuclear extracts of these fibroblasts were made according to a modification (Mori et al., 1992) of (Schreiber et al., 1989), except that no protease inhibitors were included except PMSF.

were either conjugated to HRP or Alexa 568 (Molecular Probes). HRP was developed as described above. Except in the case of GFAP staining, cells were fixed for 10 minutes (min) at room temperature (RT) in 3.7% formaldehyde in PBS. Primary antibody incubation was over night at 4° in 1% goat serum (GS) and 0.1% NP-40 (except for TuJ1, which was 1 hour at RT). Secondary antibody incubation was 1 hour at RT. For GFAP, cultures were fixed at 4°C for 20 min, primary antibody incubation was 1 hour at RT in 5% GS and 0.5% Triton-X 100, and secondary antibody incubation was 45 min at RT. Antibody staining done to compare differences in expression levels between differentiated and undifferentiated cells and between antibodies was performed side-by-side and developed for the same amount of time.

RESULTS

Protein generation and mouse innoculation

In order to improve the chances of obtaining a useful antibody, and to allow for the possibility of analyzing alternative splicing events, two GST-fusion proteins were made: one containing the N-terminus of NRSF, and the other containing the C-terminus. GST-5' encodes approximately the first 150 aa of mouse NRSF (C.J. Schoenherr and D.J. Anderson, unpublished), from the second codon to just before the zinc-finger domain. GST-3' encodes approximately the last 250 aa of mouse NRSF. Protein was generated in bacteria and purified first by batch binding to and subsequent elution from affinity resin and then by gel electrophoresis and electroelution. The GST moiety was not cleaved from the fusion protein before injection into mice.

Susan Ou of the Monoclonal Antibody Facility carried out the following procedures except for titering of mouse serum. Three mice per protein were injected with 10 μg of protein each week for two weeks. The following week bleeds were taken and serum was tested for antibody titer using dot-blot analysis. The mice responded to the GST-3' protein more quickly than GST-5', so the dosage of GST-5' innoculation was doubled. Weekly injections were continued for a number of weeks, serum was titered again, and spleen fusion of the most strongly responding GST-3' mouse was done first. Spleen fusion of the corresponding GST-5' mouse was done after the preliminary results of the first fusion were known. A final bleed was taken from each mouse before the spleen was removed, to be used as a mouse polyclonal antiserum if necessary (see below).

Screening of supernatants and subcloning of hybridoma cell lines

Susan Ou provided approximately 800 supernatants from the GST-3' spleen fusion. These supernatants were screened for antibodies against NRSF (with assistance by Ling Wang) by immunocytochemistry on cells transfected with an NRSF expression construct. Those that gave nuclear staining in a small proportion of the cells were considered to be putative positives. Five positive polyclones were subcloned by Susan Ou, and two survived subcloning: 6C1 and 7G1. A negative control test of staining of untransfected cells showed no staining for 7G1 but scattered staining for 6C1. Thus, 6C1 recognized an antigen that tended to be present in a subset of cultured cells, perhaps related to a cell-cycling event. It was confirmed that 6C1 recognized a protein independent of NRSF by dot-blot analysis (data not shown). On the other hand, 7G1 was found to be specific

for NRSF by dot-blot analysis (Fig. 1A), but the complete lack of staining on untransfected cells, even after the hybridoma was subcloned brought into question its future utility. Indeed, 7G1 has only ever been shown to be functional in dot-blot analysis and in detecting over-expressed levels of NRSF in tissue culture (see below).

Susan Ou provided approximately 750 supernatants from the GST-5' spleen fusion. This time supernatants were screened on untransfected C3H10T 1/2 cells, the cell line from which mouse NRSF was cloned (C.J. Schoenherr and D.J. Anderson, unpublished). A nuclear staining pattern was taken to indicate a potentially positive supernatant. Although some hybridomas might be missed this way, any that did come through the screen would definitely be functional in immunocytochemistry. There was also the possibility that antibodies against an unrelated nuclear antigen might be generated, but these would hopefully be in the minority and would be easily detected (see below).

Three positive polyclones were identified in the initial screen, and all of them survived subcloning: 15A1, 12C11, and 13B10. These three are independent isolates, and two subclones of each of the latter were isolated: 12C11-1 and –2, and 13B10-1 and –2. Dot-blot analysis confirmed that 12C11 and 13B10 specifically recognize the N-terminus of NRSF (Fig. 1A). 15A1, however, was shown to recognize a nuclear antigen that must share an epitope with GST (Fig. 1A), because 15A1 bound to all three proteins: GST-5', GST-3', and GST itself. The hybridoma supernatant, 1D3, generated by Chris Schoenherr, was also tested in this dot-blot analysis and shown to recognize the region included in GST-5' (Fig. 1A), even though this monoclonal antibody was raised against a segment of mouse NRSF that included the zinc-finger domain. Isotyping of

12C11 and 13B10 by Susan Ou indicated that both of these monoclonal antibodies are of class IgG_1 (data not shown).

Western analyses

Initially, Western analysis of the NRSF-specific antibodies on extracts from 10 T1/2 cells was done in order to confirm NRSF specificity by virtue of the size of the protein recognized and to determine functionality in this assay. The 1D3 antibody was used as a positive control, because it is known to be functional in Westerns (C.J. Schoenherr, unpublished). Unfortunately, too little protein was loaded for even 1D3 to recognize endogenous NRSF in these cells, which express the gene at relatively low levels. However, 12C11 was able to recognize a faint band of the appropriate mobility in this experiment (data not shown), indicating that 12C11 is more effective in Western analysis than 1D3.

An alternative experiment was then conducted to test the new antibodies by Western analysis. A retroviral construct, B-SMA, was made (originally for other purposes) that encoded a C-terminally truncated form of mouse NRSF with a myc-epitope tag at its N-terminus. This retrovirus was used to infect chick embryonic fibroblasts (CEFs) such that they would express a portion of mouse NRSF containing the epitopes for 12C11 and 13B10 (but, unfortunately not for 7G1). Two Westerns were done side-by-side, one of uninfected CEFs and another of B-SMA – infected CEFs. Strips from each blot were incubated with various antibodies and developed using alkaline phosphatase reagents (Fig. 1B).

Numerous points can be made from this experiment. First, the strips of the blot made from uninfected CEFs show background bands that, since they are the same across all the different antibodies, correspond to weak binding of the secondary antibody to CEF proteins. Second, the prominent band in the 9E10 strip of the blot from infected CEFs indicates the position of the truncated, myc-tagged NRSF protein (Fig. 1B, strip 7). Unfortunately, it migrates just above 87 kDa, at the same position of one of the background bands in the blot of uninfected cells. This background band can also be seen in the blot of infected cells in the 15A1 and 7G1 strips (Fig. 1B, strips 1 and 5), because 15A1 is known to bind a non-NRSF protein, and the epitope for 7G1 is outside the domain of the B-SMA construct. The four other antibodies tested, however, did show a more prominent band in the correct position. Of these, the two 12C11 subclones gave the strongest signal (Fig. 1B, strips 2 and 3). The weakest signal was that of 1D3 (Fig. 1B, strip 6), and 13B10 was slightly weaker than 12C11 (Fig. 1B, strip 4). In conclusion, the two newly generated monoclonal antibodies that recognize the N-terminus of NRSF were shown to be functional in Western analysis, but the antibody against the C-terminus remains untested.

Gel-shift analyses

The functionality of the antibodies was also characterized in gel-shift analysis using both mouse and human NRSF (Fig. 2). Gel shifts were done using a probe corresponding to two copies of the binding site for NRSF found in the rat SCG10 gene (Mori et al., 1992). A nuclear extract (made by N. Mori) of HeLa cells gives a prominent gel shift band corresponding to human NRSF (Fig. 2A, arrow). Antibody supershifting of

this band was only seen for 12C11 (Fig. 2A), indicating either that 13B10 and 7G1 are non-functional in gel-shift analysis, or that they do not recognize human NRSF. This question has not been resolved for 13B10; however, it has been addressed in the case of 7G1 (Fig. 2B). The gel-shift band representing mouse NRSF (Fig. 2B, arrowhead), both from in-vitro synthesized protein (Fig. 2B, "I") and from mouse embryonic fibroblasts made by Z.-F. Chen (Fig. 2B, "F"), migrates slightly more slowly than the band from human NRSF (Fig. 2B, arrow; compare lanes 1, 2, and 3). The reason for this is unknown. In the case of in-vitro synthesized protein, two prominent gel-shift bands were seen (Fig. 2B, lane 2). These two bands correspond to the full-length protein and either a break-down product of NRSF or a place where the ribosome commonly falls off in the process of in-vitro translation, because both bands were supershifted by the 12C11 antibody (Fig. 2B, lane 8). Neither of these bands was supershifted by the 7G1 antibody (Fig. 2B, lane 9) indicating that 7G1 is non-functional in gel-shift analysis.

A reagent for supershifting versions of NRSF that lack the N-terminus was desired in order to help detect potential aberrant splice forms that may be generated in a mouse carrying a targeted deletion of the 5' end of the NRSF gene (Chen et al., 1998). Although the 7G1 antibody was not useful in this regard, the final bleed of the mouse that was used to generate this hybridoma was used as a mouse polyclonal antiserum against the N-terminus of NRSF and tested for its ability to specifically supershift human and mouse NRSF (Fig. 2C). This polyclonal antiserum (Fig. 2C, "3' F.B.) was shown to be able to supershift both human NRSF (Fig. 2C, lane 3) and mouse NRSF (Fig. 2C, lane 6). This supershift was specific for the C-terminal region of NRSF, because the truncated form of NRSF found in preparations of in-vitro synthesized protein (Fig.

2B, lane 2) was not supershifted by the polyclonal antiserum, while the full-length protein was supershifted (data not shown).

Immunohistochemistry

Although both 12C11 and 13B10 were identified based on their functionality in immunocytochemistry, they have never been found to be able to detect endogenous levels of NRSF in immunohistochemistry, i.e., in tissue sections. They are, however, capable of detecting exogenous expression resulting from infection with a retrovirus encoding a C-terminally truncated form of NRSF, B-SA (Fig. 3). This virus is similar to the retrovirus described above, B-SMA, except that the myc-epitope tag has been deleted. When chicken embryos were injected with B-SA in the neural tube at St. 15 (Hamburger and Hamilton, 1992), widespread infection resulted after 2 days. Adjacent sections at the level of the wing bud were stained side-by-side with 12C11 (Fig. 3A) and 13B10 (Fig. 3B). These adjacent sections would be expected to be equally well infected, but clearly 12C11 is more effective at detecting NRSF expression than is 13B10. This is in agreement with all other assays in which the two antibodies have been directly compared. (The antibody concentrations of the two supernatants has never been quantified, however, so formally 13B10 could simply be of a lower titer than 12C11.)

Characterization of NRSF expression in P19 cells, a tissue-culture model of neuro- and gliogenesis

P19 cells are an embryonal carcinoma cell line that have the capacity of differentiating into either neurons and glia or muscle depending on the inducing treatment (Bain et al.,

1994; Rudnicki and McBurney, 1987). When undifferentiated P19 cells are aggregated in the presence of retinoic acid for 4 days, they become neural (Pevny et al., 1998) and then begin to differentiate into neurons by 2 days later (d6) and glia by 4 days later (d8) (Rudnicki and McBurney, 1987, and data not shown). This cell line was, therefore, an ideal system for studying the levels of NRSF expression at varying stages of neuronal and glial differentiation.

The 12C11 and 13B10 antibodies were first used in Western analysis in order to characterize the bulk properties of the cell line with respect to NRSF expression over the course of neural induction and differentiation (Fig. 4). Undifferentiated P19 cells (d0) express high levels of NRSF (Fig. 4A, arrowhead). After 4 days of neural-induction treatment (d4), a time at which no neuronal markers are yet expressed, NRSF expression has already decreased markedly (Fig. 4A). The lower molecular-weight bands seen with the ECL detection system used here are for the most part unrelated to NRSF, as they are seen in the control strip that was not exposed to primary antibody (Fig. 4B). A more detailed and extended time course of NRSF expression in differentiating P19 cells is shown in Figure 4C. Here it is apparent that NRSF expression does not decrease until after two days of neural induction (Fig. 4C, compare d2 to d4). In addition, NRSF levels do not further decrease while neurons and glia begin to differentiate (Fig. 4C, d6 and d8, respectively).

Because the newly generated anti-NRSF antibodies were functional in immunocytochemistry as well as in Western analysis, NRSF expression was examined at the cellular level in undifferentiated and neuronally differentiated P19 cells (Fig. 5). Cells at stages d0 and d6 were processed side-by-side with 12C11 and NeuN. The specific antigen recognized by NeuN is not known, but it is known to stain the nuclei of neurons.

This was particularly useful in comparing positive and negative signals in neuronal nuclei, as these rounded-up cells can appear to be stained when they are not. As indicated by the Western analysis, undifferentiated P19 cells express NRSF at relatively high levels (Fig. 5B, arrow). Although P19 cells are a clonal cell line, however, NRSF expression was not uniform in all cells in this state (Fig. 5B), an observation that could not have been made by assessing the bulk population as in Western analysis. Immunocytochemistry of undifferentiated P19 cells with 13B10 gave a similar pattern of staining, indicating that it was, indeed, endogenous NRSF that was being visualized (data not shown). In contrast to 12C11 staining, NeuN showed no signal in undifferentiated P19 cells (Fig. 5D, arrowhead). By two days after neural induction was complete, however, the majority of the cells showed high levels of NeuN staining (Fig. 5H, arrow). This staining is quite different to that seen in cells of this stage using the 12C11 antibody (Fig. 5F, arrowhead). NRSF levels at d6 are significantly lower than those seen in undifferentiated P19 cells (Fig. 5B), as expected from the Western data. The exact levels in the different cells present at this stage are admittedly difficult to discern, however, because the cells tend to aggregate at this time point.

Although NRSF levels were seen to decrease upon neural induction and neuronal differentiation, the Western analysis indicated that NRSF expression was not completely abolished by these transformations. Questions of NRSF expression in glia and at low levels in differentiated neurons have been a long-standing issue (see Discussion). Therefore, P19 cells were analyzed at a time when both neurons and glia had differentiated, and in a way that cells could interpretably be double-stained with neuronal and glial markers. To do this, cells were differentiated and aggregates were dissociated and replated as usual, but 6 days after this, the cells were trypsinized and

replated on dishes coated with poly D-lysine at a moderate density (Fig. 6A, D). The coating allowed both neurons and glia to attach directly to the tissue culture dish as opposed to other cells. Cells were fixed and stained the next day, the equivalent of d11.

At this stage numerous cells were found to express relatively high levels of NRSF (Fig. 6B, E, arrows). These highly expressing cells, however, were never found either to stain with TuJ1 (Fig. 6C, arrow), an early neuronal marker that indicates Class III β-tubulin expression, or to express glial fibrillary acidic protein (GFAP) (Fig. 6F, arrow), a marker of glial cells. The identity of these cells that express NRSF at levels similar to undifferentiated P19 cells, therefore, is unknown. Cells positive for TuJ1 were never found to co-express detectable levels of NRSF (Fig. 6B, C, arrowhead). In contrast, while most GFAP-positive cells expressed little if any NRSF (Fig. 6E, F, arrowhead), a few GFAP-positive cells did appear to express moderate levels of NRSF (Fig. 6F, open arrowhead). This indicates that expression of NRSF that remains in P19 cells after neural induction is present mostly in cells that may remain undifferentiated, but potentially in some glia as well.

DISCUSSION

This report describes the generation and characterization of multiple monoclonal antibodies generated against different regions of mouse NRSF. Each antibody has been characterized in a range of immunochemical analyses. One antibody, 7G1, raised against the C-terminal region of NRSF, is functional in dot-blots and immunocytochemistry of cells made to overexpress NRSF. It is unable, however, to recognize either mouse or human NRSF in gel shifts, and it cannot recognize endogenous levels of NRSF expression

in cultured cells. It remains untested in Western analysis. The serum from the final bleed of the mouse used to make 7G1 can be used as a mouse polyclonal antiserum specific to the N-terminal region of NRSF in gel-supershift experiments with both mouse and human protein. Two other monoclonal antibodies were raised against the N-terminal region of mouse NRSF, preceding the zinc-finger domain. These antibodies, 12C11 and 13B10, are functional in dot-blots, Westerns, immunocytochemistry of endogenous NRSF, and immunohistochemistry of embryos overexpressing the protein. 12C11 recognizes both mouse and human NRSF in gel-supershift experiments, and 13B10 does not supershift a human NRSF-containing complex, but remains untested for its ability to recognize mouse NRSF in gel-supershift experiments. In general the signal from 13B10 is slightly weaker than that of 12C11, but both are stronger than that of 1D3, a previously generated monoclonal anti-mouse NRSF antibody with limited immunochemical functionality (C.J. Schoenherr and D.J. Anderson, unpublished).

This study aimed at generating an antibody that could be used to assess the expression of NRSF at the cellular level. Previous reports indicated that neuronal cell lines express little if any NRSF (Chong et al., 1995; Schoenherr and Anderson, 1995), while different glial cell lines and acutely cultured glia express low to high levels (Palm et al., 1998; Schoenherr and Anderson, 1995). This was in contrast to an in situ hybridization analysis of adult brain that indicated that most neurons in situ express low but significant levels of NRSF, while almost all glia in situ express undetectable levels of NRSF (Palm et al., 1998). The antibodies generated against NRSF were ineffective at detecting endogenous levels of NRSF in sectioned tissue (data not shown), but were able to detect endogenous NRSF in cultured cells. Rather than simply characterize the expression levels of different cell types of static identity, however, analysis of NRSF

expression was performed in a tissue-culture model system of neural induction and neuronal and glial differentiation, P19 cells.

The P19 cell line is an embryonal carcinoma that has the ability, under different inducing conditions, to differentiate into either muscle or neurons and glia (Bain et al., 1994; Rudnicki and McBurney, 1987). After 4 days of aggregation in the presence of retinoic acid, the cells acquire properties of neural tissue, including expression of the HMG-box transcription factor, SOX1 (Pevny et al., 1998). In 2 more days (d6), some cells begin to express markers of neuronal differentiation. These markers include putative direct targets of NRSF repression, including Class III β-tubulin, middle molecular weight neurofilament, and synapsin I (Thiel et al., 1994, and data not shown). In 2 more days (d8), many cells express neuronal markers, and some cells begin to express glial markers. Many glia have appeared by d10 and even more differentiate by d12 (data not shown).

Analysis of NRSF expression of P19 cells at various stages of neural determination and neuronal and glial differentiation resulted in a number of interesting observations. First, NRSF down regulation was apparent by d4, the time at which the cells are competent to later differentiate into neurons, but before neuronal or glial markers have begun to be expressed. This down regulation, therefore, may correspond more to the difference in NRSF expression level detected between non-neural tissue and neural progenitors of the neural tube (Chong et al., 1995), than to the difference in NRSF expression between neural progenitors and differentiated neurons (Chong et al., 1995; Schoenherr and Anderson, 1995).

Unexpectedly, no further down regulation was detected by the population-level analysis of Western blotting from d4 through d8. This may be explained by results of the

cellular-level analysis of differentiated cells. At a time point when many neurons and glia have differentiated, a significant fraction of cells expressed levels of NRSF equivalent to the undifferentiated state. These cells double-stained with neither an antibody against Class III β-tubulin, TuJ1, nor with an anti-GFAP antibody. These high-expressing cells may have contributed to the lack of further down regulation of NRSF in the overall population of P19 cells after d4. In addition to these cells, however, the detection of low-level NRSF expression in GFAP-positive cells indicates that glia may have contributed to some extent to the Western result as well. These data underscore the problems with analyzing mixed populations of cells, as demonstrated by the conflicting in situ data between different peripheral ganglia, where NRSF expression increased in cranial ganglia during development while it decreased in trunk-level ganglia (Chong et al., 1995; Schoenherr and Anderson, 1995). In addition, these data reflected the expression pattern found in neuronal and glial cell lines, as well as in acute cultures of hippocampal astrocytes, rather than that found in the intact brain. Further investigation is required to address the question of differences in NRSF expression associated with the transformed state characteristic of a cell line or with culturing of glia and the resulting changes in cell-cycling status.

A few other studies have analyzed the expression of NRSF in tissue-culture models of neurogenesis. In two of them, the expression level of NRSF transcript was compared among a family of neuroblastoma cell lines and contrasted with the levels of the neuronal NRSF target gene, synapsin I, in those lines (Lietz et al., 1998; Nishimura et al., 1996). Both groups found that the level of NRSF expression in each line was generally inversely correlated with the level of synapsin I expression. One of the groups took this analysis further by differentiating, with staurosporine plus forskolin, the

neuroblastoma cell line with the highest NRSF expression level (Nishimura et al., 1996). The expression of both NRSF and synapsin I is then analyzed at different time points over the course of neuronal differentiation. They found that NRSF expression decreased to undetectable levels by the end of the analysis, and as NRSF expression decreased synapsin I expression increased. This is similar to what was found in differentiating P19 cells in the current study. However, semiquantitative PCR was used to assess NRSF transcript levels in the neuroblastoma cell line, whereas immunocytochemistry was used to assess NRSF protein levels in the P19 cells. The current study is the first to analyze NRSF protein at the cellular level during neuronal and glial differentiation.

Another group assessed NRSF expression in the PNS-derived stem cell line, RT4-AC and its supposedly neuronal and glial derivatives (Donahue and Reinhart, 1998). RT4-AC apparently converts spontaneously and reproducibly to a glial derivative, RT4-D or either of two neuronal derivatives, RT4-B and RT4-E. The "stem cell" line expresses markers of both differentiated glia and neurons, including GFAP and SCG10 (a direct target of NRSF repression). The glial derivative has lost its neuronal characteristics including SCG10 expression and the ability to generate an action potential. The neuronal derivative, RT4-B, has lost its glial characteristics including GFAP and P_0. The other "neuronal" derivative, however, RT4-E, has not only lost the glia characters, but has also lost SCG10 expression. It retains the ability to generate an action potential, but the Na^+ channel expressed in the cells is the skeletal muscle M2 gene. The authors characterize the expression levels of NRSF in the "stem cell" line and these various glial and "neuronal" derivatives using Northern analysis. As might be expected, the two SCG10-expressing cell lines show the lowest levels of NRSF transcript. The glial derivative shows slightly more NRSF, and the "neuronal" derivative that does not express SCG10 shows

the highest level. While supportive of the role of NRSF in neural cell types to repress neuron-specific genes, the characteristics of differentiating P19 cells resemble in vivo events more closely than does the RT4 model system.

In addition to the work described here, three other laboratories have made use of the 12C11 antibody prior to publication (Chen et al., 1998). One group, David Askew and colleagues, used the antibody to show that a factor that binds to a negative regulatory element in the His-1 gene is not NRSF, even though the element bears some similarity to the NRSE (Xu et al., 1999). Another group, Noel Buckley and colleagues, used the antibody to demonstrate binding of an in-vitro synthesized preparation of a neuron-specific isoform of NRSF to the NRSE (Roopra et al., submitted). A third group, Louis Hersh and colleagues, used 12C11 to help demonstrate the mechanism whereby the REST4 isoform of NRSF regulates the cholinergic gene locus in PC12 cells via protein kinase A (Shimojo et al., 1999). Numerous groups have requested the antibody since publication as well, including Raymond Dingledine and colleagues, who used it in co-immunoprecipitation experiments to show interaction of endogenous NRSF with Sin3A and HDAC1 (Huang et al., 1999). Future studies may make use of the antibodies described here to investigate the regulation of NRSF gene expression itself or to confirm a down-regulation of protein expression in antisense and/or RNAi experiments.

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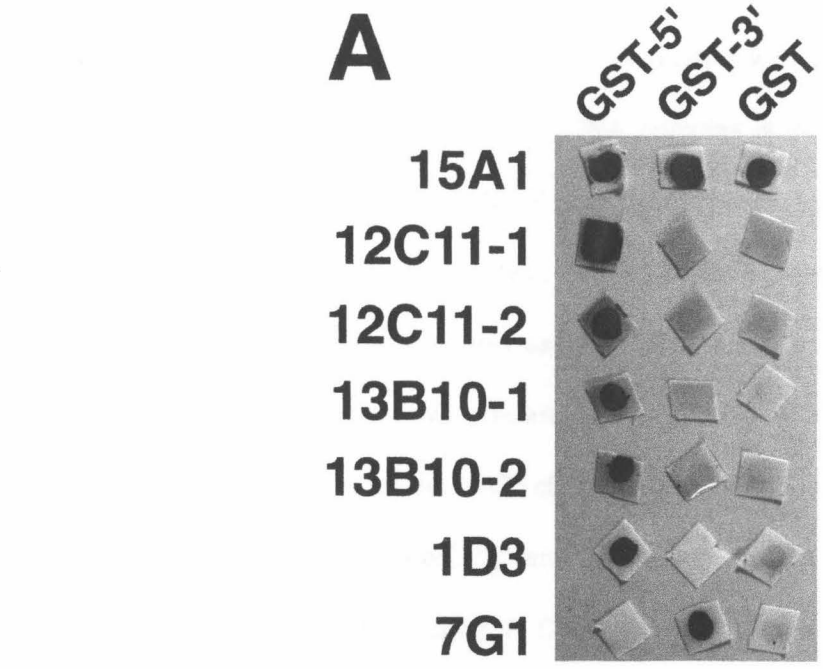
FIGURE LEGENDS

Fig. 1. Dot-blot and Western analysis of anti-NRSF and control monoclonal antibodies. (A) Partially purified GST or GST-NRSF fusion proteins were spotted onto nitrocellulose and exposed to various hybridoma supernatants. Positive binding is indicated by a dark spot. GST-5' and GST-3' are GST fusions to the N-terminus and C-terminus, respectively, of mouse NRSF. All supernatants except 1D3 (C.J. Schoenherr and D.J. Anderson, unpublished) were generated in the present study. Subclones of independent hybridomas are indicated by "-1" and "-2." All but 15A1 were found to bind to a specific region of NRSF. This analysis narrowed down the region of the 1D3 epitope to the N-terminal region before the zinc-finger domain. (B) Chicken embryonic fibroblasts (CEFs) either were left uninfected or were infected with the B-SMA retrovirus, which encodes a C-terminally truncated form of mouse NRSF with a myc-epitope tag at its N-terminus. Extracts of these cells were run on a gel and blotted. The blots were cut into strips and incubated in various hybridoma supernatants. Uninfected cells show a set of weak non NRSF-specific bands. The 9E10, anti-c-myc antibody indicated the position of the exogenous NRSF protein. 15A1 served as a negative control, and lack of staining with 7G1 is because the region against which 7G1 was made was not included in the truncated NRSF protein. The 12C11 subclones show the strongest binding, with 13B10 slightly weaker, and 1D3 giving the weakest signal.


A
GST-5'
GST-3'
GST
15A1
12C11-1
12C11-2
13B10-1
13B10-2
1D3
7G1

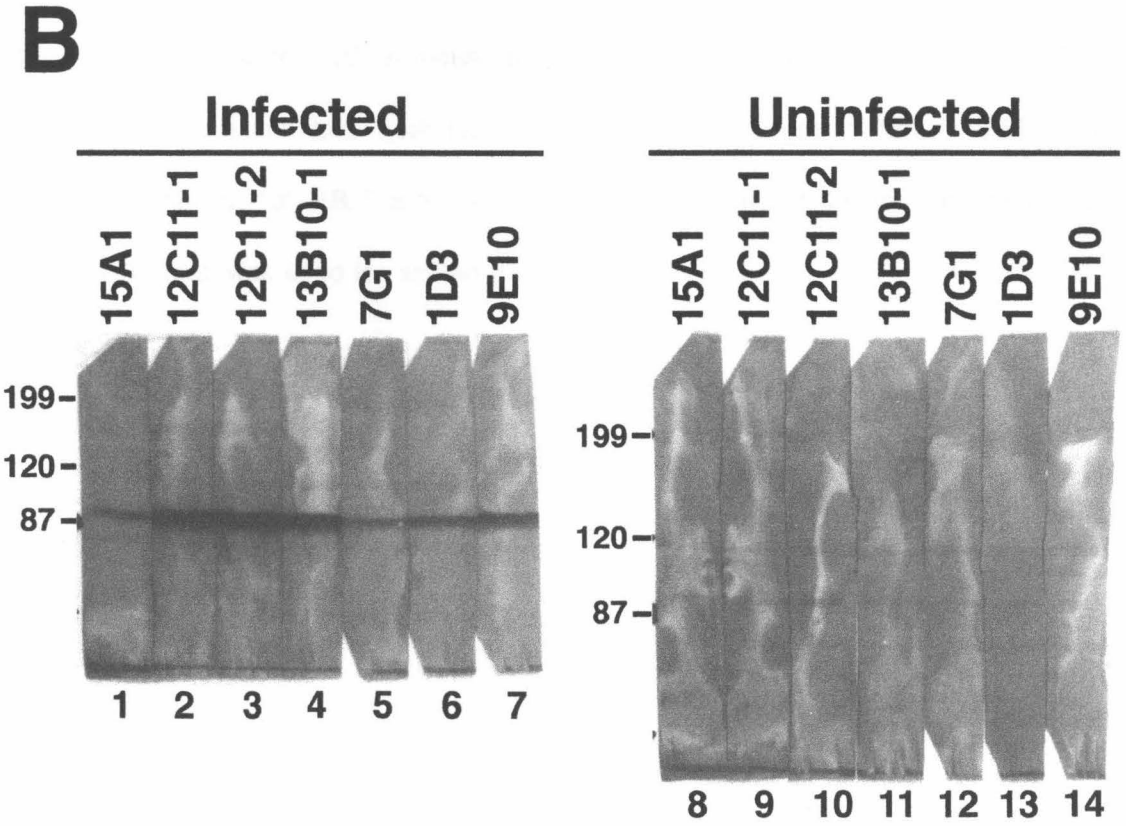

B
Infected
Uninfected
15A1
12C11-1
12C11-2
13B10-1
7G1
1D3
9E10
199
120
87
1 2 3 4 5 6 7
8 9 10 11 12 13 14

Fig. 2. Gel-shift analysis of anti-NRSF antibodies on human and mouse NRSF. (A) HeLa cell nuclear extract ("H," made by N. Mori) was incubated with probe containing two copies of the NRSF binding site. The protein-DNA complex is indicated by an arrow. The supershift with 12C11 indicates that NRSF is present in this complex, and that 12C11 is functional in this assay with human protein. The other antibodies either do not recognize human NRSF or are not functional in this assay. 15A1 served as a negative control. (B) In addition to HeLa cell extract, two preparations of mouse NRSF were tested in supershift experiments. The mouse NRSF cDNA (C.J. Schoenherr and D.J. Anderson, unpublished) was in-vitro translated, "I," and an extract was made from mouse embryonic fibroblasts ("F," made by Z.-F. Chen) derived from wild-type littermates of NRSF-/- embryos. Note that the complex containing mouse NRSF (arrowhead) migrates slightly faster than the human complex (arrow). 7G1 is non-functional in this assay. 13B10 remains untested. (C) A mouse polyclonal antibody generated against the N-terminus of NRSF supershifts both human (arrow) and mouse (arrowhead) NRSF. This polyclonal antibody, "3' F.B.," is the serum of the final bleed of the mouse, immunized with GST-3', that was used for spleen fusion to generate 7G1.

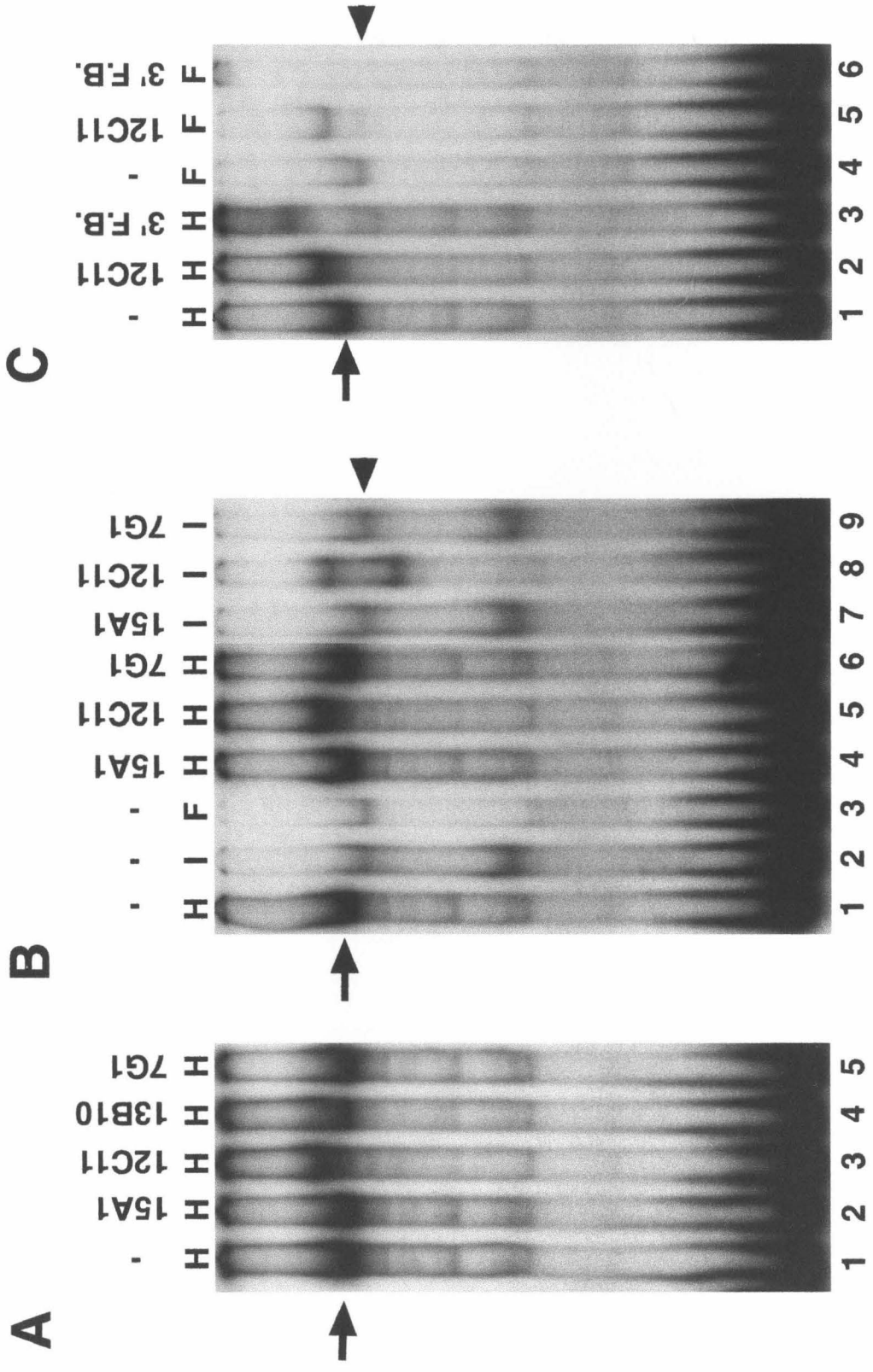

A
H - 15A1 12C11 13B10 7G1
1 2 3 4 5
B
H - - F - 15A1 12C11 7G1 I 15A1 I 12C11 I 7G1
1 2 3 4 5 6 7 8 9
C
H - 12C11 H 3' F.B. F - 12C11 F 3' F.B.
1 2 3 4 5 6

Fig. 3. Immunohistochemistry of sections of B-SA – infected chicken embryo with 12C11 and 13B10. Chicken embryos were injected in the neural tube at St. 15 [Hamburger, 1992 #55] with retrovirus expressing a C-terminally truncated form of mouse NRSF. Two days later embryos were fixed and sectioned at the level of the wing bud. Adjacent sections were stained side-by-side with 12C11 (A) and 13B10 (B). Although both sections should be equally well infected, signal with the 12C11 antibody is clearly stronger.

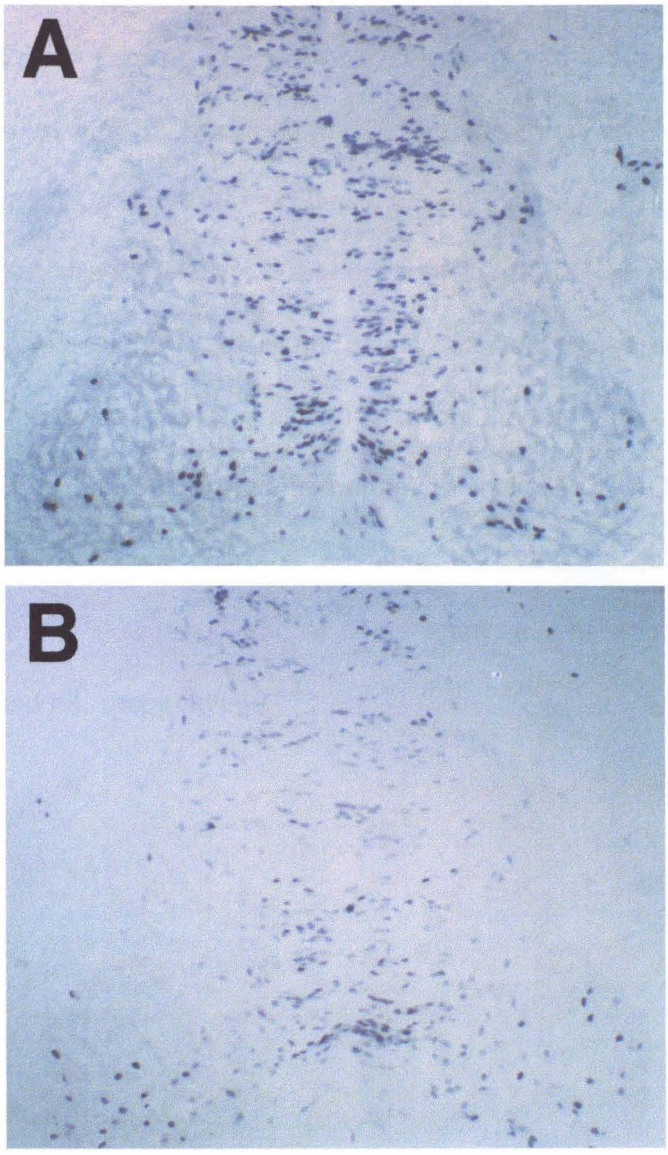

A
B

Fig. 4. Bulk characterization of NRSF expression in P19 cells at different stages of differentiation. Upon neural-induction treatment, P19 cells become neural by d4, begin to differentiate into neurons by d6, and begin to differentiate into glia by d8 [Rudnicki, 1987 #65, and data not shown]. Western analysis was performed on equal numbers of cells from sister dishes of P19 cells at different days after initiation of treatment. (A) Both 12C11 and 13B10 indicate that NRSF expression (arrowhead) is relatively high in undifferentiated P19 cells (d0) and is downregulated by d4, before markers of differentiation are expressed. (B) Additional bands detected by the secondary antibody and ECL detection reagents are not specific to the primary antibody. (C) A detailed time course of P19 cell neural induction and differentiation indicates that NRSF is not down-regulated until after the second day of neural-induction treatment (d2), and that NRSF expression is not further down-regulated at times when neurons and glia begin to differentiate (d6 and d8, respectively).

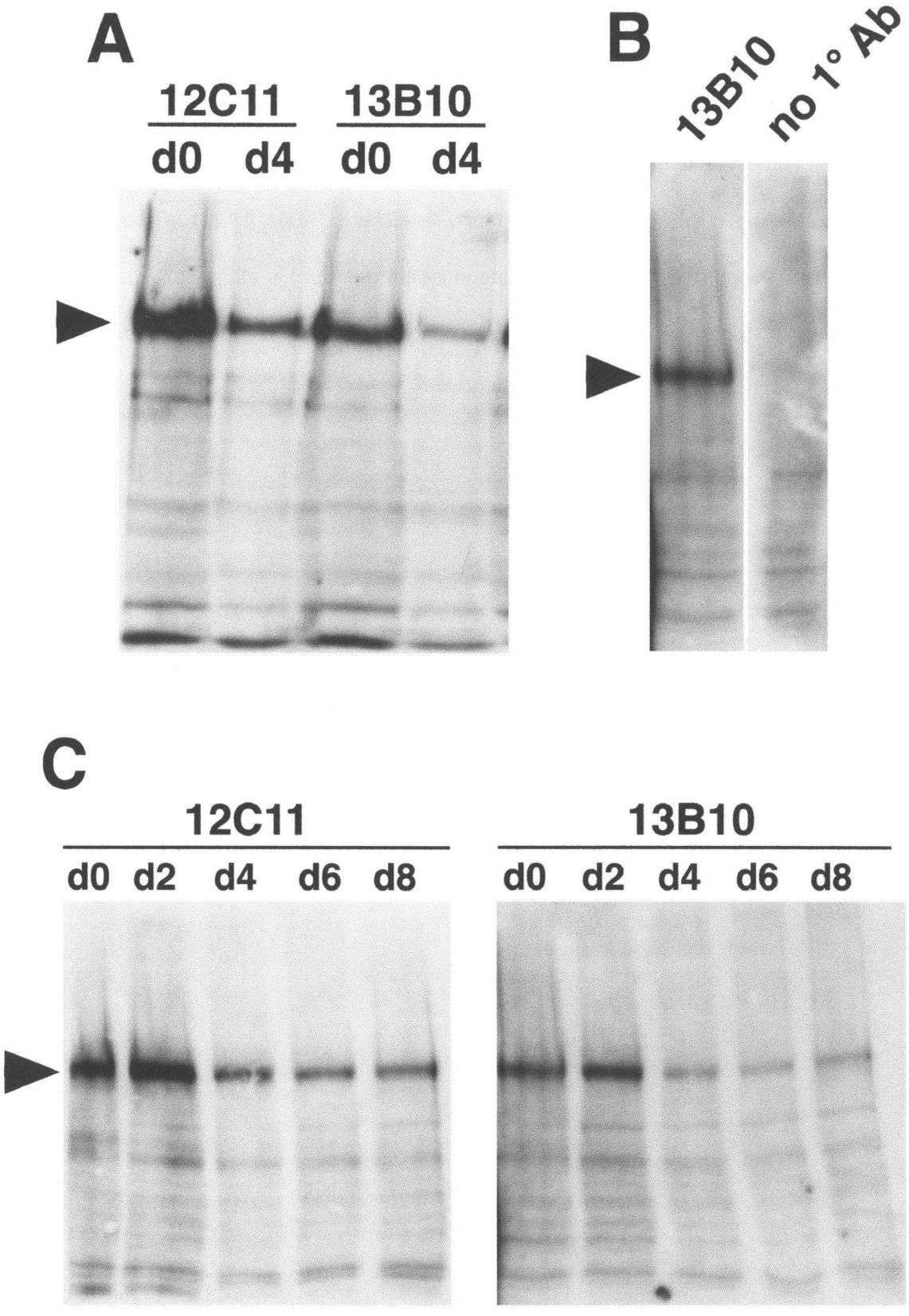

A
12C11
13B10
d0
d4
d0
d4
B
13B10
no 1° Ab
C
12C11
13B10
d0
d2
d4
d6
d8
d0
d2
d4
d6
d8

Fig. 5. Immunocytochemistry of undifferentiated and differentiated P19 cells. Expression of NRSF and the nuclear, neuronal antigen NeuN was examined in P19 cells at d0 (A-D) and d6 (E-H). All staining was done side-by-side and developed for the same amount of time. Subsets of undifferentiated cells express high levels of NRSF (B, arrow) as detected by 12C11 staining, while all undifferentiated cells are negative for NeuN (D, arrowhead). Supernatant from the independent hybridoma, 13B10, gave similar, though weaker, staining (data not shown). Upon neuronal differentiation, NeuN is upregulated (H, arrow), and NRSF is downregulated (F, arrowhead).

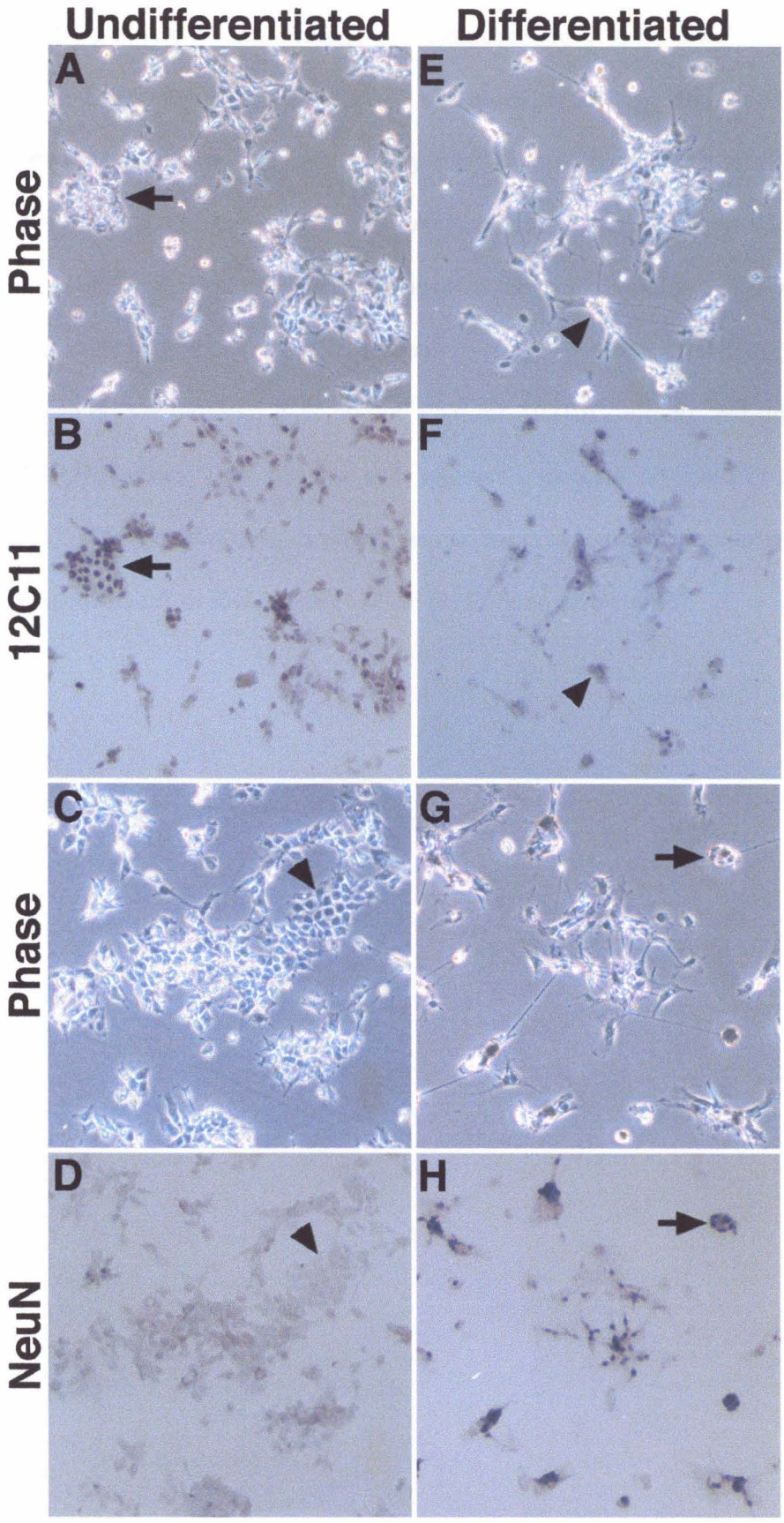
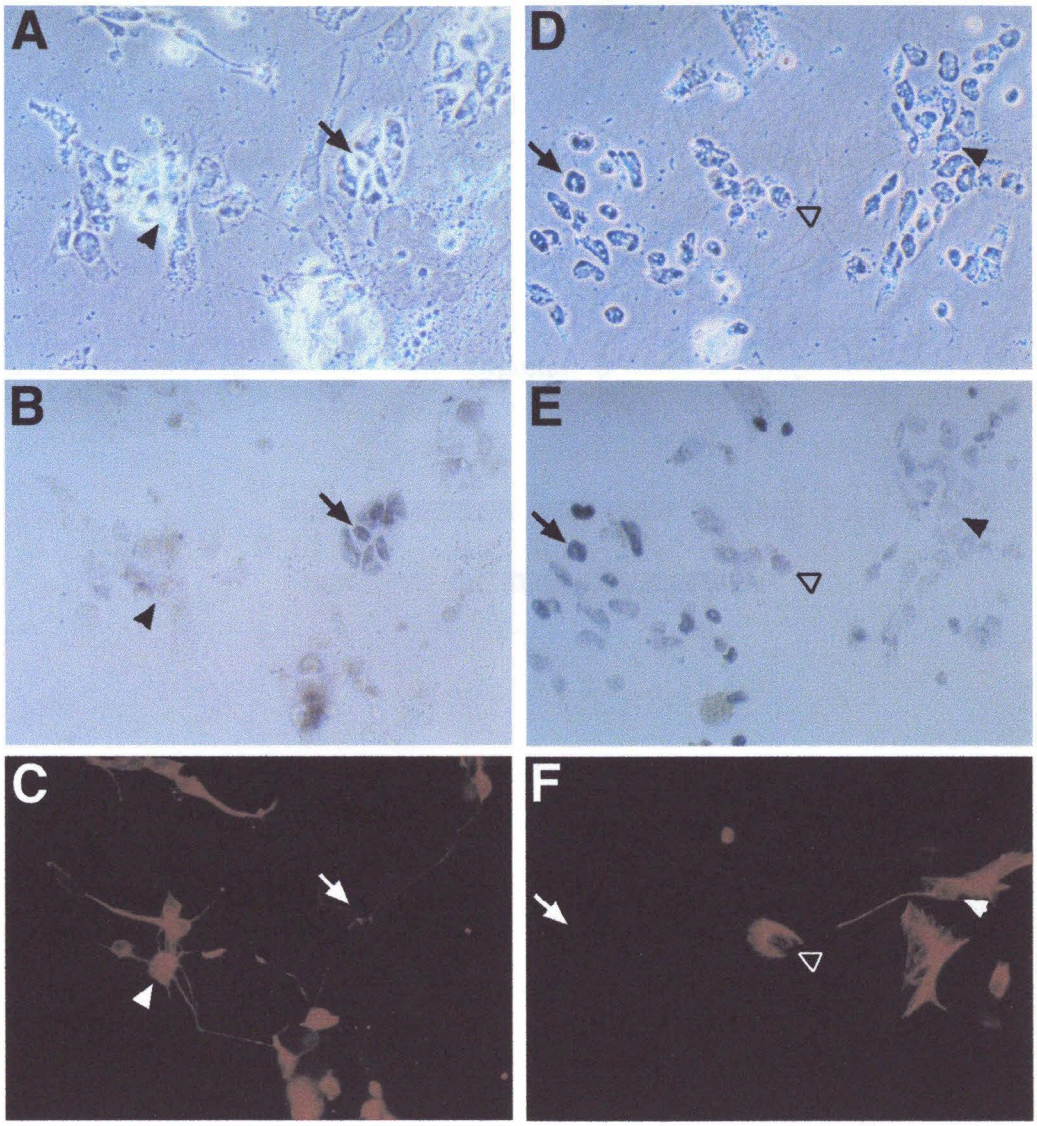

Undifferentiated
Differentiated
Phase
12C11
Phase
NeuN
A
B
C
D
E
F
G
H

Fig. 6. Double staining of differentiated, re-disassociated P19 cells with 12C11 and either TuJ1 or anti-GFAP. P19 cells at stage d10 were trypsinized and replated at moderate density on dishes coated with poly D-lysine. Cells were fixed and processed the next day. (A, B, C) Phase, bright-field, and fluorescent images of the same field. (D, E, F) Phase, bright-field, and fluorescent images of the same field of a different dish. A proportion of cells at this time point expresses relatively high levels of NRSF (B, E, arrows). These high-expressing cells do not stain for TuJ1 (C, arrow), nor do they express GFAP (F, arrow). TuJ1-positive cells express undetectable levels of NRSF (B, C, arrowheads). GFAP-positive cells, however, either express almost undetectable levels of NRSF (E, F, arrowhead) or low levels of NRSF (E, F, open arrowhead).


A
D
B
E
C
F

Chapter 5

OVEREXPRESSION OF NRSF IN VIVO DISRUPTS NEURAL DEVELOPMENT

SUMMARY

The neuron-specific silencer factor (NRSF) is normally expressed at moderately high levels in the ventricular zone of the neural tube, where neural progenitors are located. NRSF expression is downregulated in the marginal zone, where neurons differentiate. In addition, NRSF is known to be a transcriptional repressor with many neuronal putative direct target genes. We therefore asked whether overexpression of NRSF in the developing spinal cord of chicken embryos would result in abnormalities in aspects of neural development such as neurogenesis, neuron-specific gene regulation, and axon targeting. NRSF overexpressing cells were found in the marginal zone, and apparently equal numbers of neurons were generated on both the NRSF-overexpressing and control side of the neural tube, indicating that NRSF overexpression does not prevent neurogenesis. Two targets of NRSF, however, were found to be repressed by exogenous NRSF, including N-tubulin and Ng-CAM. NRSF overexpressing cells in the marginal zone were morphologically neuronal. Some axons, however, appeared to make pathfinding errors. This is potentially because of changes in the expression of cell adhesion molecules, such as Ng-CAM. These data indicate that the downregulation of NRSF during neuronal differentiation is required for proper neural development.

INTRODUCTION

The generation and maturation of neurons is a key aspect of neural development. Neural progenitors of the central nervous system proliferate in the ventricular zone of the neural tube and produce daughter cells that no longer divide. In the developing spinal cord these post-mitotic cells then migrate laterally into the marginal zone and begin to exhibit neuronal properties. A number of genes have been identified that play a role in regulating the generation and differentiation of neurons in vertebrates, including members of the Notch signaling apparatus, neuronal determination and differentiation genes, and anti-neuronal HLH genes (Beatus and Lendahl, 1998; Kageyama and Nakanishi, 1997; Lee, 1997).

NRSF is a zinc-finger transcription factor isolated according to its ability to repress the transcription of neuron-specific gene promoters in non-neuronal cell types (Chong et al., 1995; Schoenherr and Anderson, 1995). It is expressed widely outside of the nervous system, and during neural development it is expressed at moderately high levels in the ventricular zone of the neural tube but at very low levels in the marginal zone (Chen et al., 1998; Chong et al., 1995; Schoenherr and Anderson, 1995). In the adult brain NRSF has been found to be expressed at low levels in most neurons (Palm et al., 1998). Comparatively much higher levels were seen, however, in the non-neural tissues in the brain such as the choroid plexus and meninges. Among neural cell types in vivo, therefore, NRSF expression appears to be highest in undifferentiated neural progenitors.

NRSF has been implicated in the transcriptional repression of a large number of primarily neuron-specific genes: SCG10 (Chen et al., 1998; Mori et al., 1992), the type II sodium channel (Kraner et al., 1992), synapsin I (Li et al., 1993; Schoch et al., 1996),

brain-derived neurotrophic factor (Timmusk et al., 1999), choline acetyltransferase (Lonnerberg et al., 1996), the neuron-glial cell adhesion molecule (Chen et al., 1998; Kallunki et al., 1995), L1 (Kallunki et al., 1997), the m4 subtype of muscarinic acetylcholine receptor (Mieda et al., 1997; Wood et al., 1996), the β2-subunit of the neuronal nicotinic acetylcholine receptor (Bessis et al., 1995), neuronal tubulin (Chen et al., 1998; Schoenherr et al., 1996), the AMPA receptor subunit, GluR2 (Myers et al., 1998), the *N*-methyl-D-aspartate receptor 1 (Bai et al., 1998), the γ2 subunit of the type A receptor for γ-aminobutyric acid (Mu and Burt, 1999), and others (Schoenherr et al., 1996).

The expression pattern of NRSF along with its long list of neuronal putative direct target genes suggested that it might have an important role in neural development – that it might be involved in coordinately regulating the expression of the neuronal phenotype during neurogenesis. In vivo inhibition of NRSF function in the developing spinal cord of chicken embryos resulted in upregulation of neuronal target genes in neural progenitors (Chen et al., 1998). Neuronal differentiation, however, was not found to be accelerated, and no ectopic neuronal differentiation within the ventricular zone was observed. These results indicate that, while NRSF expression in neural progenitors is not required to prevent premature neurogenesis, NRSF is required for the proper regulation of neuronal gene expression during neural development.

In the current study we address the question of whether downregulation of NRSF during neurogenesis is required for normal neuronal differentiation. We have electroporated NRSF expression constructs into one side of the neural tube of early chicken embryos at a stage when neuronal differentiation has not yet begun. This has allowed us to examine the effects of overexpressing NRSF in neural progenitors and of

maintaining NRSF expression in their daughters. We have found that, while maintenance of NRSF expression does not appear to inhibit the generation of morphologically differentiated neurons, neuronal target gene expression is significantly reduced and axon pathfinding is affected, likely due to changes in expression levels of relevant cell adhesion molecules. This indicates that proper regulation of NRSF expression during neuronal differentiation and maturation is required for normal neural development. (Please note: The data presented here are preliminary. This is apparent in the low number of samples analyzed and in the lack of essential negative controls in some cases. These aspects of the data are explicitly stated in the Results section. The Discussion, however, is based on the assumption that the results will be supported by further, controlled experiments, which are in progress.)

MATERIALS AND METHODS

Plasmid construction

In order to insert the coding region of mouse NRSF (C.J. Schoenherr and D.J. Anderson, unpublished) into both pCS2+ (Rupp et al., 1994; Turner and Weintraub, 1994) and RCASBP(B) (Morgan and Fekete, 1996), a portion of the original cDNA clone was inserted first into the subcloning vector, SKSP (S.T. Suhr, personal communication). This was done in several steps in order to include as little of the 5' and 3' untranslated regions (UTRs) as possible and to avoid using the polymerase chain reaction, such that sequencing of the entire 3.25-kilobase (kb) coding region of the subclone was not necessary. The final product, SP-NRSF, contains a 3.43-kb fragment of the NRSF cDNA including 140 base pairs (bp) of 5' UTR and 40 bp of 3' UTR.

pCS2+NRSF was constructed by inserting the BamH I – Cla I fragment from SK-NRSF into pCS2+ from BamH I to Cla I. pCS2+nβgal was a gift from Ralf Rupp and David Turner (Rupp et al., 1994; Turner and Weintraub, 1994). pCS2+N-IRτG was generated by inserting an EcoR I – Xba I fragment from Shuttle-IRES-tau-mGFP6 (Mombaerts et al., 1996; E.L. Dormand, unpublished; Schuldt et al., 1998) into pCS2+NRSF from EcoR I to Xba I. NRSF was inserted into RCASBP(B) in two steps. The Msc I – Cla I/Klenow-blunted fragment of SK-NRSF was first inserted into the subcloning vector, SLAX 12 NCO, which was cut with Hind III and Klenow blunted, then cut with Msc I. (Note, SLAX12NCO technically contains two Msc I sites, but the upstream site is Dcm methylated. This allows the second one to be used to insert a cDNA in frame, as required, with the ATG at the Nco I / second Msc I site.) The Cla I fragment of this subclone, SLAX-NRSF, was then inserted into RCASBP(B) at the Cla I site.

Electroporation

White Leghorn chicken eggs were incubated at 38°C until stages (St.) 12 to 15 of development (Hamburger and Hamilton, 1992), or about 16-25 somites, E2. Supercoiled plasmid for electroporation was used at a concentration of 1 or 2 μg/μL in TE with 0.025% Fast Green (Sigma). Embryos were injected with DNA solution into the lumen of the neural tube, and then subjected to electroporation using a BTX ElectroSquarePorator, BTX Genetrodes, and accompanying equipment (Genetronics, Inc.). The electrodes were spaced 6-8 mm apart and positioned such that the DNA was driven into the cells on only one side of the neural tube. Embryos were pulsed 5 times for 50 msec each at 25 V. Eggs were then re-incubated for 2 or 3 days before the

embryos were fixed for 2 hours at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS). Embryos were then rinsed 2 times in PBS, washed in PBS for 1 hour at 4°C, rinsed one last time in PBS, then sunk in 30% sucrose in PBS over night. Embryos were mounted in OCT (Tissue-Tek) and sectioned at 20 μm at the thoracic level.

Immunohistochemistry

Antibody staining of sectioned tissue was carried out by incubating sections over night at 4°C with the following primary antibodies at the dilutions indicated: 12C11, neat (Chen et al., 1998); α-β–galactosidase, 1:500 (5 Prime – 3 Prime); α-GFP, 1:1000 (Clontech); TuJ1, 1:1000 (BAbCo); 8D9, 1:10 (Developmental Studies Hybridoma Bank); cyn-1, 1:5 (Ericson et al., 1997); α-cngn2, 1:10,000 (S.E. Perez, unpublished); α-SCG10, 1:1000 (Stein et al., 1988); and NN18, 1:250 (Sigma). All of these antibodies are mouse monoclonal IgGs except α-β–galactosidase, α-GFP, α-cngn2, and α-SCG10, which are rabbit polyclonal antibodies. HRP-conjugated secondary antibody (Chemicon) was used at a 1:200 dilution for 1 hour at room temperature (RT) and developed with diaminobenzidine intensified with nickelous sulfate. Alexa dye-conjugated secondary antibodies (488 and 568, Molecular Probes) were used at a 1:250 dilution for 30-45 minutes at RT. HRP staining was photographed under bright-field optics, and fluorescent images were obtained by confocal microscopy.

RESULTS

Migration into the marginal zone of NRSF-overexpressing cells

An early step in neurogenesis is the lateral migration of daughters of neural progenitors from the ventricular zone of the neural tube into the marginal zone. NRSF is normally expressed at moderately high levels in the ventricular zone, where the neural progenitors reside. In contrast, NRSF expression is much lower in the marginal zone, where maturing neurons are located (Chong et al., 1995; Schoenherr and Anderson, 1995). In order to test if maintaining NRSF expression in the progeny of neural precursors prevented this migration, we electroporated an NRSF expression construct into the neural tube of developing chicken embryos. This was done such that the plasmid was driven into only one side of the neural tube at a stage (St. 12-15) when spinal cord neurons have not yet begun to differentiate (Oppenheim et al., 1988). The plasmid, pCS2+NRSF, contains the entire coding region of mouse NRSF along with 140 bp and 40 bp of 5' and 3' UTR, respectively. After electroporation eggs were re-incubated for 2 days, at which time expression of the exogenous protein can still be detected. Embryos were sectioned at the thoracic level and immunostained for NRSF using the monoclonal antibody 12C11 (Chen et al., 1998).

Cells overexpressing NRSF were found in the marginal zone of all embryos examined, n=4. These cells were detected at all dorsal-ventral levels of the developing spinal cord (Fig. 1), including regions containing commissural neurons (Fig. 1A, arrowheads) and motor neurons (Fig. 1B, arrows). This pattern of cells expressing exogenous NRSF was similar to that in embryos electroporated with an expression construct for nuclear-localized β-galactosidase, pCS2+nβgal (data not shown). Forced

expression of NRSF, therefore, does not prevent neural progenitors from giving rise to daughter cells that can migrate into the marginal zone.

Repression of endogenous target genes by overexpression of NRSF

Another important step in neurogenesis is the upregulation of neuronal gene expression. While NRSF expression decreases during neuronal differentiation, neuronal gene expression increases. NRSF is known to be a transcriptional repressor (Chong et al., 1995; Schoenherr and Anderson, 1995), and a large number of neuron-specific genes are thought to be direct targets of NRSF regulation (Schoenherr et al., 1996). We were interested in determining, therefore, if overexpression of NRSF was sufficient to perturb neuron-specific gene regulation during neural development.

Class III β-tubulin (or N-tubulin) and the neuron-glial cell adhesion molecule (Ng-CAM) are two NRSF target genes that have been previously shown to be sensitive to inhibition of NRSF function during embryogenesis (Chen et al., 1998). In the developing spinal cord, neural progenitors express low levels of N-tubulin, and newly born neurons quickly upregulate it to high levels. Ng-CAM expression is also upregulated during neuronal differentiation, but unlike N-tubulin, neural progenitors express it at undetectable levels, and upregulation is more gradual as neurons extend processes and mature.

We generated a different type of expression construct to assess potential changes in the regulation of these two genes upon overexpression of NRSF. NRSF was inserted into the chick retroviral backbone, RCASBP(B) (Morgan and Fekete, 1996). The NRSF coding region, at 3.25 kb, is high above the limit of 2.4 kb (Morgan and Fekete,

1996) for generation of replication-competent retrovirus from this construct, termed B-NRSF. When electroporated into chicken embryos, however, this construct allows for expression of the exogenous gene out to at least 3 days after electroporation, while expression from pCS2+NRSF is almost extinguished by this time point (data not shown). The enduring expression is likely because of integration of the retroviral DNA into the cell's genome.

We used the B-NRSF construct for this experiment for two reasons: (1) Neural progenitors are mitotically active. Plasmid is more easily diluted out, therefore, in this cell population. Using an integratable expression construct would ensure overexpression of NRSF within the ventricular zone as well as in the marginal zone. (2) Neuron-specific gene expression may take some time to reach mature levels. Using an integratable expression construct would allow the effects of NRSF overexpression to be observed at a later stage of development. At this time the difference in target gene expression between the NRSF overexpressing and control sides of the spinal cord may be more obvious.

B-NRSF was electroporated into St. 12-13 chicken embryos, and the eggs were re-incubated for 3 days. Widespread expression of NRSF was detected within both the ventricular and marginal zones (Fig. 2A, C, E, G). Adjacent sections immunostained for either N-tubulin or Ng-CAM showed repression of these genes by overexpression of NRSF, but in different cell populations, n=2. Overexpression of NRSF within the neural progenitors resulted in repression of the low-level N-tubulin expression normally seen in these cells (Fig. 2B, F, arrows). In contrast, overexpression of NRSF in the differentiating neurons of the marginal zone resulted in a decrease in the expression of Ng-CAM (Fig. 2D, H,). Interestingly, Ng-CAM is known to be expressed in the commissural neurons of

the developing spinal cord (Stoeckli and Landmesser, 1995). These neurons are located in the dorsal neural tube and send axonal projections ventromedially toward the floor plate. This axon tract is less apparent on the NRSF-overexpressing side of the neural tube (Fig. 2D, H, arrowheads). Electroporation of a negative control construct, B-nβgal, will be used to show that these effects are specific to NRSF.

No differences in the expression of other neuronal genes were detected at this time point (data not shown). Apparently equal numbers of cells expressing the neural determination gene, chick ngn2 (Perez et al., 1999), and the pan-neuronal cytoplasmic antigen, cyn-1 (Ericson et al., 1997), were detected on the NRSF-overexpressing and control sides of the neural tube. Also, two additional putative direct targets of NRSF, the axon growth-associated protein, SCG10 (Mori et al., 1992), and middle molecular weight neurofilament (Schoenherr et al., 1996), were not found to be obviously repressed by NRSF overexpression at this stage (data not shown).

Alterations in axon pathfinding in NRSF-overexpressing neurons

Relatively late steps in the process of neuronal differentiation are neurite extension, which gives neurons their characteristic morphology, and proper axon pathfinding, which allows neurons to receive and convey signals appropriately. Putative direct targets of NRSF include genes encoding proteins involved in these processes, such as neuron-specific cytoskeletal elements and neural cell adhesion molecules (Kallunki et al., 1997; Kallunki et al., 1995; Schoenherr et al., 1996). We wanted to test, therefore, if overexpression of NRSF in differentiating neurons would inhibit neurite outgrowth or alter axon pathfinding.

In order to visualize cellular morphology and axon targeting independent of the expression of neuron-specific genes, we made a third NRSF-expression construct. Downstream of NRSF in pCS2+, we inserted an internal ribosome entry site, followed by the coding sequence for a fusion protein of the microtubule-binding protein, tau, to the green fluorescent protein (GFP). This construct allows the cytoskeleton of the cell body and cellular processes of only the cells expressing exogenous NRSF to be viewed (Mombaerts et al., 1996).

This plasmid, pCS2+N-IRτG, was electroporated into St. 13-14 chicken embryos, and eggs were re-incubated for 2 days. Sections were first immunostained for NRSF. The abundant expression of NRSF in the ventricular zone as well as the marginal zone (Fig. 3A), even though this plasmid is not integratable, is likely due simply to more efficient electroporation with practice, although using embryos younger than St. 15 may have contributed. Double staining for NRSF and GFP indicated that the vast majority of cells expressing one protein also expressed the other (Fig. 3C). Some cells expressing NRSF at high levels showed low-level GFP expression, but GFP-expressing cells that had very low levels of NRSF were rare.

The tau-GFP fusion revealed that NRSF overexpression does not prevent cells from attaining a neuronal morphology. Cells expressing exogenous NRSF sent axons into the spinal cord from the dorsal root ganglion (Fig. 3C, top arrow), and projected axons out of the ventral root (Fig. 3C, bottom arrow). These are the normal projection patterns of dorsal root ganglion neurons and motor and preganglionic sympathetic neurons, respectively. A more detailed assessment of the axon targeting of these neurons was not made. Interestingly, the axons of NRSF-overexpressing commissural neurons showed some normal and some abnormal features. These axons grew towards

the floor plate, and many appeared to cross the ventral commissure. A number of these axons, however, did not then immediately turn anteriorly as expected. They instead remained at the same rostro-caudal level and projected dorsally either within the neural tube (Fig. 3C, arrowhead), or along the outer margin (data not shown). This kind of pathfinding defect was detected in all embryos analyzed (n=4). Electroporation of a negative control construct, pCS2+nβgal-IRτG, will be required to show that such axon-targeting errors do not normally occur at a similar frequency.

Ng-CAM has been shown to be involved in ipsilateral fasciculation of the axons of commissural neurons in the developing spinal cord (Stoeckli and Landmesser, 1995). We checked, therefore, if the repression of Ng-CAM expression by NRSF overexpression was detectable at this earlier time point. Adjacent sections did, indeed, show reduced levels of Ng-CAM expression on the NRSF-overexpressing side (Fig. 3B). Staining of the commissural axon tract also appeared reduced (Fig. 3B, arrow). Interestingly, when this section was double-labeled with GFP, the growing axon of an NRSF-overexpressing commissural neuron was found within the contralateral commissural axon tract (Fig. 3D, arrowhead; green axon). This indicates that mis-targeted commissural axons of NRSF-overexpressing neurons may continue to fasciculate with commissural axons after crossing the midline, instead of associating with the fibers of the ventral funiculus as they normally do (Yaginuma et al., 1991).

DISCUSSION

In this report we describe the effects of overexpressing NRSF in vivo in the developing nervous system of chicken embryos. We electroporated NRSF expression constructs into

one side of the neural tube at a stage when neurons of the spinal cord have not yet begun to differentiate. This allowed us to observe the results of overexpressing NRSF in neural progenitors and maintaining NRSF expression in their daughters.

Within the developing central nervous system NRSF is normally expressed at moderately high levels in the ventricular zone, where neural progenitors reside, and is expressed at much lower levels in the marginal zone, where newly born neurons differentiate (Chen et al., 1998; Chong et al., 1995; Schoenherr et al., 1996). NRSF is a negative transcriptional regulator (Chong et al., 1995; Schoenherr et al., 1996) with a large number of putative direct, neuronal target genes (see Introduction). Although inhibition of NRSF function during neural development in chicken did not result in premature neurogenesis, the expression of neuron-specific target genes was derepressed in neural progenitors (Chen et al., 1998). Targeted disruption of NRSF in mouse resulted in embryo deterioration and death too soon for an effect within the nervous system to be detected (Chen et al., 1998).

Given the expression pattern of NRSF, its potential direct target genes, and its requirement for proper gene expression during neurogenesis, we wanted to assess how enhanced levels of NRSF expression would affect neuronal development.

Maintenance of NRSF expression does not prevent neurogenesis

When neural precursors on one side of the neural tube were made to express exogenous NRSF, their progeny did migrate out into to marginal zone. Expression of the neural determination gene chicken ngn-2 was not disrupted, nor were the numbers of cyn-1$^+$ neurons on the NRSF-overexpressing side of the tube obviously different from those on

the control side. Most NRSF-overexpressing cells in the marginal zone had a neuronal morphology and expressed many neuronal genes, such as SCG10, N-tubulin, and middle molecular-weight neurofilament. Thus, maintenance of NRSF expression in daughters of neural progenitors does not prevent neurogenesis.

The only transcription factor currently known to inhibit neurogenesis is Hes1 (Ishibashi et al., 1994; Sasai et al., 1992; Tomita et al., 1996). (The evidence for another gene, Hes5, is limited (Akazawa et al., 1992; Ohtsuka et al., 1999).) Overexpression of Hes1 in neural progenitors of the forebrain prevents cells from migrating out of the ventricular zone (Ishibashi et al., 1994), and in both forebrain and retina overexpression prevents neuronal differentiation (Ishibashi et al., 1994; Tomita et al., 1996). Conversely, targeted mutation of Hes1 in mouse results in premature neurogenesis in both the forebrain and retina (Ishibashi et al., 1995; Tomita et al., 1996).

Hes1, like NRSF, is expressed in neural progenitors and is downregulated in differentiated neurons (Sasai et al., 1992). Also like NRSF, Hes1 is a transcriptional repressor (Sasai et al., 1992). The only known direct targets of this basic helix-loop-helix (bHLH) factor, however, are two transcription factors: itself (Takebayashi et al., 1994), and the neuronal differentiation gene, hASH1 (Chen et al., 1997). Hes1 is not only able to repress the transcription of positive regulators of neurogenesis, but, because of the structure of Hes1, it is also able to directly inhibit their function. In contrast to NRSF, therefore, Hes1 has the potential to regulate many early steps in the process of neurogenesis. The targets of NRSF are almost exclusively non-regulatory genes that are directly involved in the structure and function of mature neurons. Thus, it is perhaps not

surprising that maintaining NRSF expression does not prevent the process of neurogenesis itself.

NRSF overexpression represses endogenous neuronal target genes

Two neuronal direct target genes were found to be repressed upon overexpression of NRSF in the developing chicken spinal cord. N-tubulin, which is normally expressed at low levels in neural progenitors and is highly upregulated in differentiating neurons, was found to be repressed to almost undetectable levels in neural progenitors upon overexpression of NRSF. Any repression in the marginal zone was undetectable, probably due to the extremely high expression level of this gene in neurons. Ng-CAM, which is normally only detectable, within the central nervous system, in differentiated neurons, was repressed in the marginal zone upon NRSF overexpression. The reduction of Ng-CAM expression was detectable both in neuronal cell bodies and in their processes. As mentioned above, neither SCG10 nor middle molecular-weight neurofilament, both putative direct targets of NRSF, were obviously repressed by overexpression of NRSF.

Although these two genes may be detectably repressed at later stages of development not analyzed here, the varying responsiveness of NRSF target genes to perturbation of NRSF expression levels is consistent with previous findings. Several investigations have described the effects of deleting/mutating the binding site of NRSF (the NRSE) from the regulatory region of their target gene of interest. This has been done both in tissue culture and in transgenic mice. Comparisons of different NRSE-deleted/mutated reporter constructs in cell lines has indicated that the relative contribution of NRSF to the transcriptional regulation of a reporter gene depends on the

other regulatory elements present in the construct (Lonnerberg et al., 1996; Mieda et al., 1997). The regulatory regions of three different NRSF target genes have been analyzed in transgenic mice so far (Bessis et al., 1997; Kallunki et al., 1997; Kallunki et al., 1998; Timmusk et al., 1999). In each case, the NRSE deleted/mutated reporter gene was derepressed in a different set of tissues, indicating that NRSF was more crucial to proper gene expression in some tissues than in others, and that those tissues varied according to the specific target gene. The sensitivity of endogenous target genes to lack of regulation by NRSF was similarly varied when NRSF function was inhibited during chicken embryogenesis by introduction of a dominant-negative form of NRSF (Chen et al., 1998). Each target gene was derepressed in different sets of tissues. Thus, both inhibition and increase of NRSF function leads to the same conclusion, that the relative importance of NRSF in gene regulation is both gene- and cell type-dependent.

Overexpression of NRSF in neurons perturbs axon pathfinding

When NRSF was overexpressed in commissural neurons of the embryonic chicken spinal cord, not only was Ng-CAM expression repressed, but commissural axon pathfinding was affected. The axons of some NRSF-overexpressing commissural neurons did not turn anteriorly into the longitudinal axis after crossing the floor plate. Instead, these axons continued in the same rostro-caudal segment of the spinal cord and projected dorsally. Staining with Ng-CAM showed that some of these erroneously projecting axons were traveling along the axon tract of the contralateral commissural neurons.

Commissural neurons are known to express Ng-CAM from the time of neurite extension (Stoeckli and Landmesser, 1995). Inhibition of Ng-CAM function during

chicken spinal cord development by injection of a polyclonal antibody against Ng-CAM resulted in the defasciculation of commissural axons projecting toward the floor plate (Stoeckli and Landmesser, 1995). In contrast to treatment with antibodies to Nr-CAM and axonin-1, however, the axons still crossed the floor plate before projecting anteriorly. These results are, in part, consistent with the data presented here. The reduced Ng-CAM staining in the commissural axon tract of the NRSF-overexpressing side of the spinal cord may be due to reduced fasciculation of the commissural axons heading toward the floor plate in addition to the reduction in Ng-CAM expression in the commissural neurons. A disruption of fasciculation of the axons of NRSF-overexpressing commissural neurons will only be able to be demonstrated in comparison to the axonal outgrowth of cells expressing a control construct encoding tau-GFP. The axons so far observed, however, do appear to be relatively spread out. In obvious agreement with the results of the α-Ng-CAM treatment, the axons of the NRSF-overexpressing commissural neurons did cross the floor plate.

Why was a lack of anterior-turning not reported in the Ng-CAM antibody-inhibition experiment? This is possibly because antibody injection is a bilateral perturbation. In our experiment NRSF is only overexpressed on one side of the neural tube. Thus, the axon tract of the contralateral commissural neurons contains its normal repertoire of cell-adhesion molecules. Since axon pathfinding involves a delicate balance of positive and negative cues, the contralateral axon tract of normally Ng-CAM-expressing fibers (the NRSF-overexpression case) may be a more attractive substrate for Ng-CAM-deficient axons than other Ng-CAM-deficient axons (the antibody-injection case). This may explain the apparent difference in the two results. Interestingly, mis-targeted commissural axons not only projected up the contralateral tract, but

sometimes instead projected around the periphery of the developing spinal cord. In the chick, the first commissural neurites, known as the pioneer fibers, elongate down the lateral edge of the neural tube (Stoeckli and Landmesser, 1995). The wayward axons that grew around the edge of the contralateral spinal cord, therefore, may have been adhering to the axons of these early contralateral commissural neurons.

The fact that axon pathfinding is a finely tuned process may explain why this particular type of phenotype was detected upon overexpression of NRSF. The alterations in neuronal gene expression caused by changes in levels of NRSF activity, i.e., through inhibition of the NRSF protein or overexpression of the NRSF gene, are relatively subtle. NRSF may, therefore, be important in the regulation of processes where neuronal gene expression is carefully modulated, such as axon targeting and refinement of neuronal connections during development and perhaps the modification of synaptic strength associated with learning and memory (Abel et al., 1998). A role in the latter process may account for the continued low-level expression of NRSF in adult neurons (Palm et al., 1998). Consistent with this idea, the NRSE has been found to be important in preventing overactivation of the BDNF promoter in adult mice in response to kainic-acid induced seizures (Timmusk et al., 1999).

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FIGURE LEGENDS

Fig. 1. NRSF-overexpressing cells migrate into the marginal zone. A, B, and C each show sections from different chicken embryos electroporated with pCS2+NRSFsuch that only one side of the neural tube received plasmid. Sections were immunostained for mouse NRSF with the monoclonal antibody 12C11. The dashed line indicates the approximate border between the ventricular and marginal zones on the non-expressing side. Arrowheads in A point to potential commissural neurons. Arrows in B indicate potential motor neurons. Open arrowheads in C point to potential interneurons or preganglionic sympathetic neurons.

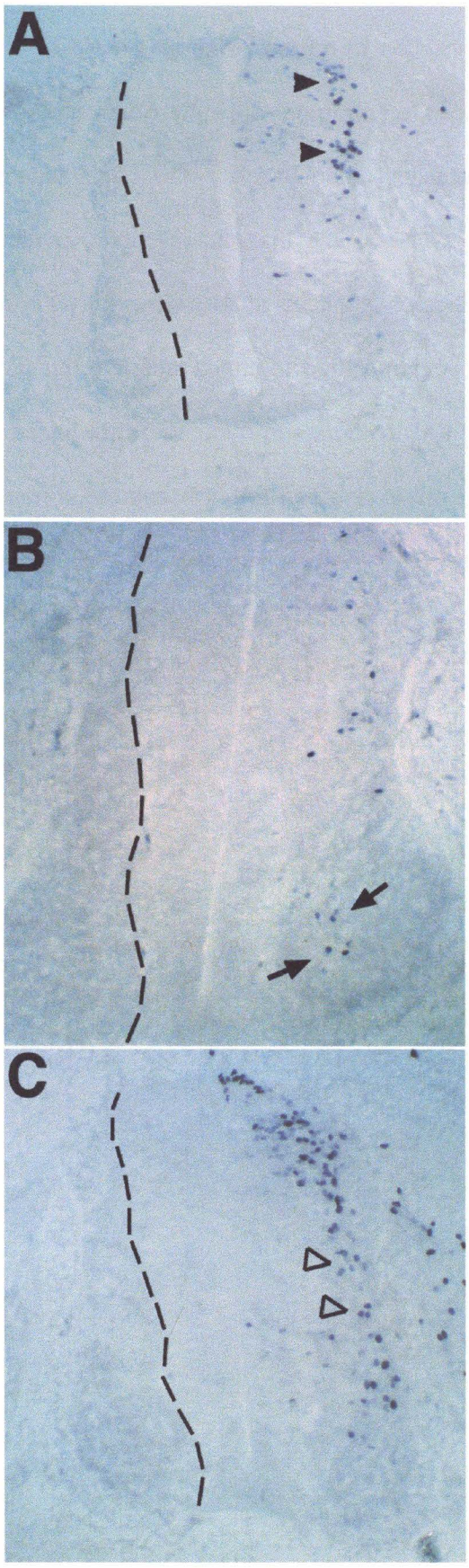

A
B
C

Fig. 2. Overexpression of NRSF results in repression of neuronal direct target genes. Chicken embryos were electroporated with the integratable expression construct B-NRSF. Sections were immunostained with the antibodies indicated. A-D represent sections from a single embryo, and E-H represent sections from another embryo. Pairs of panels, A+B, C+D, E+F, and G+H show adjacent sections. Exogenous NRSF is expressed throughout the ventricular and marginal zones of both embryos (A, E, C, and G). N-tubulin expression is repressed in the neural progenitors of the ventricular zone (B, F, arrows). Ng-CAM expression is repressed in the neurons of the marginal zone, including the axons of commissural neurons (D, H, arrowheads).

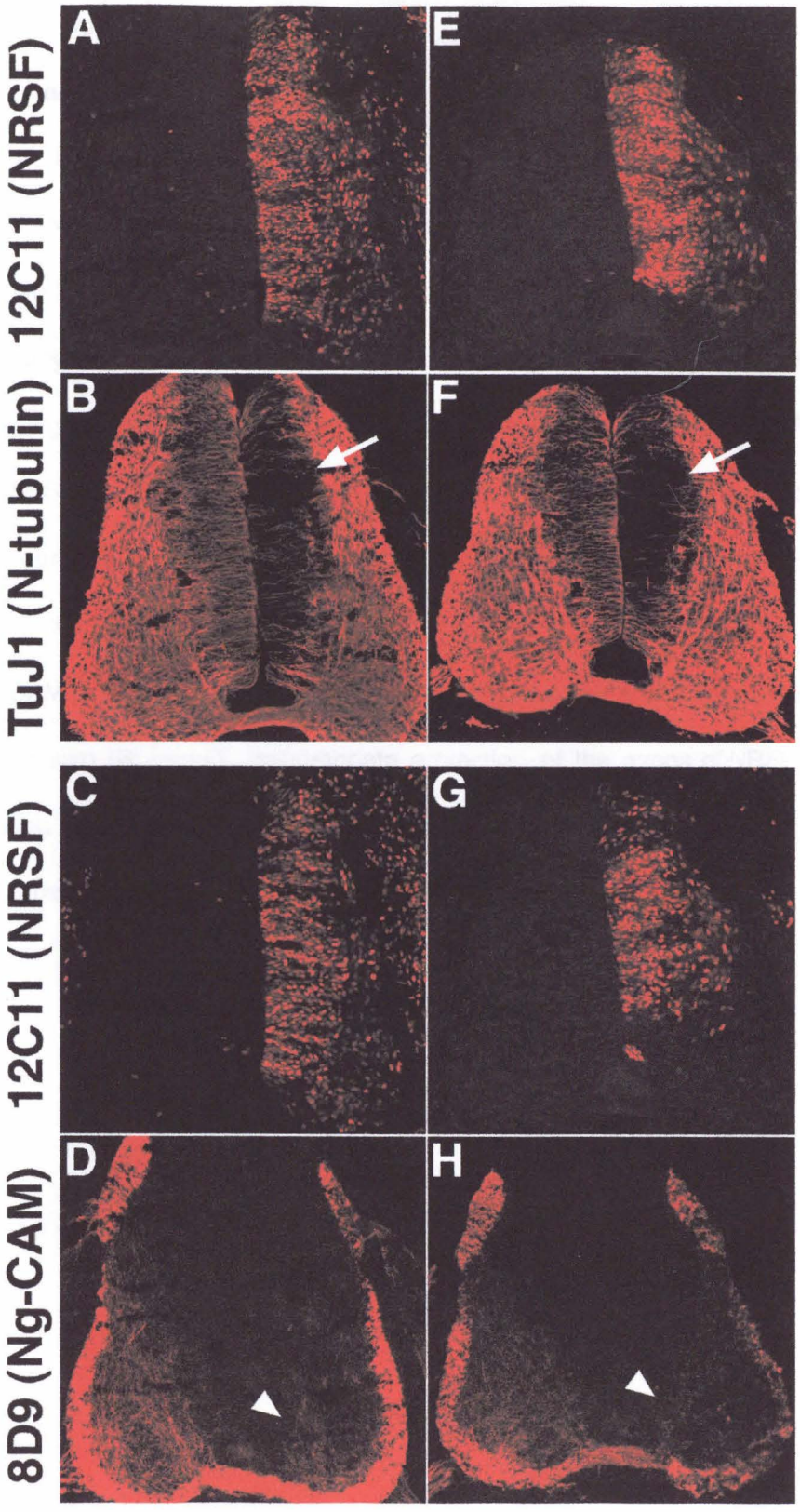
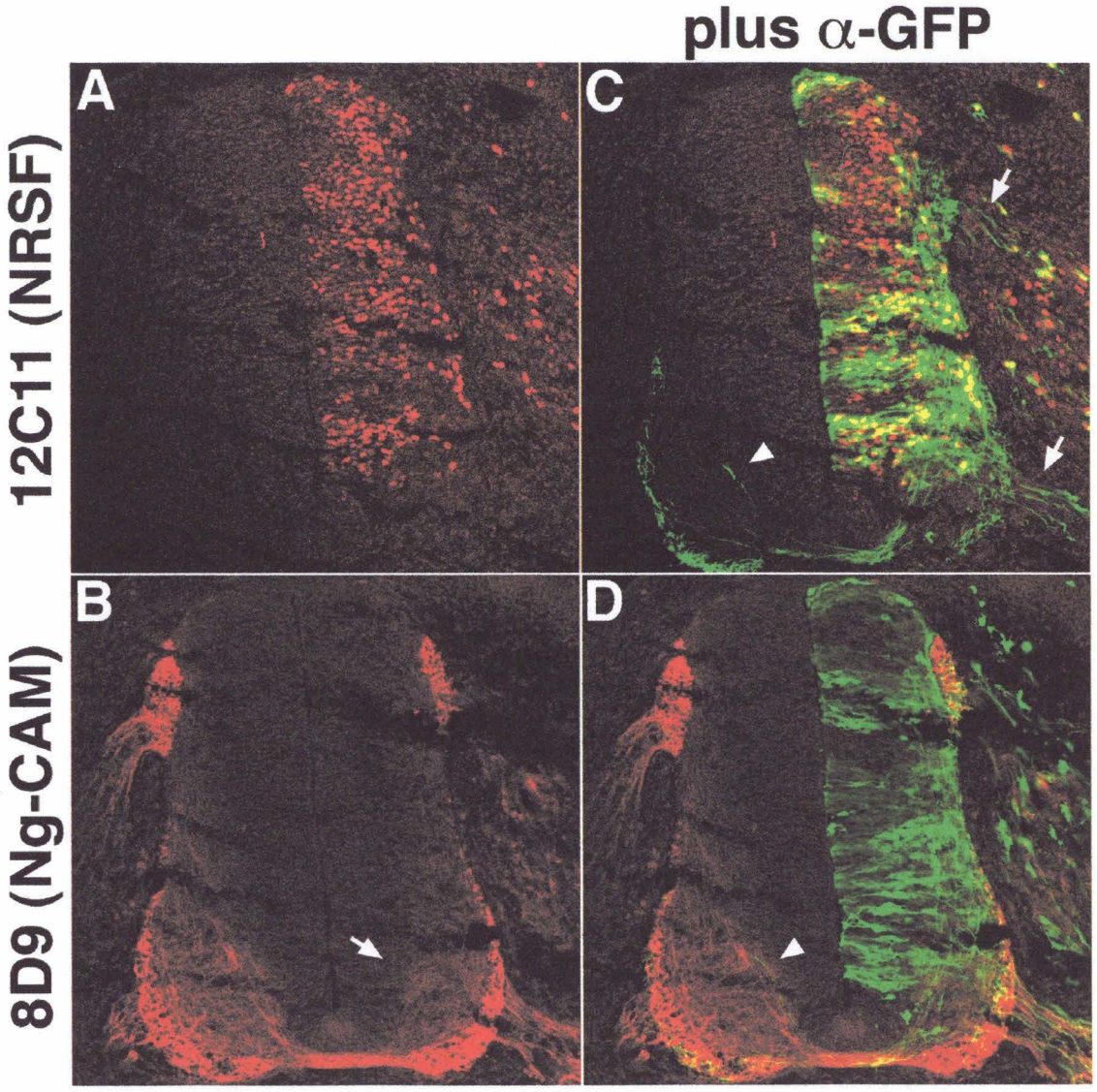

12C11 (NRSF)
TuJ1 (N-tubulin)
12C11 (NRSF)
8D9 (Ng-CAM)
A
B
C
D
E
F
G
H

Fig. 3. NRSF-overexpressing cells in the marginal zone have a neuronal morphology, but some project aberrantly. Chicken embryos were electroporated with an expression construct for NRSF including an internal ribosome entry site followed by a fusion of tau to GFP, pCS2+N-IRτG. A and C show a single section visualized with either a 568-nm laser alone (A) or with both a 568-nm and 488-nm laser (C). B and D show an adjacent section similarly visualized. Sections were stained with the indicated antibodies. Most cells expressing exogenous NRSF show GFP staining (C). Cells expressing GFP but only expressing NRSF at low levels are rare. NRSF-overexpressing neurons of the dorsal root ganglia appropriately send processes into the dorsal spinal cord (C, top arrow), and those of the spinal cord appropriately send processes out of the ventral root (C, bottom arrow). Ng-CAM is repressed in NRSF-overexpressing neurons and commissural axons of this young embryo (B, arrow). Inappropriate projection of the axons of NRSF-overexpressing commissural neurons is found within the contralateral commissural axon tract (C, D, arrowhead, green process).


plus α-GFP
12C11 (NRSF)
8D9 (Ng-CAM)
A
B
C
D

Appendix

SEARCH FOR NOVEL NRSE-BINDING PROTEINS USING THE YEAST ONE-HYBRID METHOD

SUMMARY

The only protein currently known to bind to the NRSE is NRSF. A number of observations, however, have suggested that another NRSE-binding protein may exist. One approach to identify proteins that bind to a specific sequence element is the yeast one-hybrid method. In this technique a library of cDNA fragments fused to the coding sequence of the activation domain of the yeast GAL4 protein is made. This library is then transformed into a yeast reporter strain that contains two different reporter genes. Each gene is under the transcriptional control of a different minimal promoter that has multiple copies of the regulatory element upstream of it. If a library clone contains a cDNA fragment that encodes a DNA-binding domain that can recognize the regulatory element, then reporter gene expression is activated. Here, the creation and characterization of a number of different reporter strains using both the wild-type and mutant NRSE is described. The production and screening of an activation domain-fusion library of adult mouse brain cDNA is also reported.

INTRODUCTION

NRSF is the only protein currently known to bind to the NRSE. Also, no genes have yet been identified that would be considered to be close structural family members of NRSF. Attempting to identify alternative NRSE-binding proteins is useful for a number of reasons.

The only known dominant-negative form of NRSF is one that does not interact directly with NRSF itself. This dominant-negative, which consists simply of the DNA-binding domain alone, presumably acts as a competitive inhibitor of the binding of NRSF to the NRSEs in its target genes (Chen et al., 1998; Chong et al., 1995; Tapia-Ramirez et al., 1997). Given this mode of action, this protein fragment has the potential to interfere with the binding of any NRSE-binding protein. Even though the fragment consists of NRSF sequences, therefore, it formally cannot be considered to be NRSF-specific. This is an important issue when a comparison is made between the characteristics of a chicken embryo that has been made to express this dominant-negative form of NRSF and the phenotype of mouse embryo that has a targeted deletion of a portion of the NRSF gene. A greater number of neuron-specific genes are seen to be derepressed in a more widespread fashion in the dominant-negative case than in the targeted deletion. This can be explained in a number of ways, one of them being the inhibition of additional NRSE-binding proteins. It is therefore important to search for additional NRSE binding proteins in case the dominant-negative form of NRSF is inhibiting more than just NRSF.

One particularly suspicious example of derepression of neuron-specific genes in response to dominant-negative NRSF expression is that in the peripheral nerve (Chen et al., 1998). In situ hybridization analysis at the stage when embryos were analyzed for the expression of normally neuron-specific genes showed that NRSF expression within the peripheral nerve was completely undetectable. Yet, this was a region where both Ng-CAM and SCG10 expression was observed to be derepressed by the dominant-negative protein. One possible explanation is that the protein inhibited NRSF function at an earlier stage, when these cells potentially did express NRSF. However, another explanation is

that the dominant-negative inhibited an alternative NRSE-binding protein present in peripheral nerve.

Another situation where the possibility of alternative NRSE-binding proteins is relevant is the normal lack of neuron-specific gene expression in CNS glia. Almost all cells outside of the nervous system, which do not, of course, express neuron-specific genes, express NRSF (Chong et al., 1995; Schoenherr and Anderson, 1995; Scholl et al., 1996). However, one of the largest populations of cells within the nervous system – the glia of the brain – do not express neuron-specific genes, but also do not express NRSF (Palm et al., 1998). The mechanism of repression of neuron-specific genes within glia is unknown. The NRSEs present in neuron-specific genes may be irrelevant to their lack of expression in glia. On the other hand, an alternative NRSE-binding protein expressed in glia could potentially be involved.

For these reasons, the yeast one-hybrid system was set up in order to potentially identify NRSE-binding proteins in addition to NRSF. Yeast reporter strains were generated that contain both wild-type and mutant forms of NRSF, for use in demonstrating specificity of any proteins identified. Two different reporter constructs were employed, containing different minimal promoters in order to reduce the number of false positives. Adult mouse brain was selected as the source of mRNA for the activation domain-fusion library that was constructed, because NRSF expression is known to be very low in neurons, and glia would represent a major component of the tissue.

MATERIALS AND METHODS

Except where noted, plasmids and yeast strains were as described in the MATCHMAKER One-Hybrid System User Manual (Clontech), and protocols were performed as described in the Yeast Protocols Handbook (Clontech).

Plasmid Constructions

Plasmids carrying four copies of either the wild-type or mutant NRSE from the rat SCG10 gene were generated using $S36^2$ and $Sm36^2$, made by N. Mori (Mori et al., 1992) and described again here for clarity. $S36^2$ contains two copies of the following sequence from the rat SCG10 gene regulatory region, cloned into pBSKS at the Hind III site: 5' a g c t G C A A A G C C A T **T T C A G C A C C A C G G A G A G T G C C** T C T G C 3' (where lower-case letters indicate exogenous sequence added to create a Hind III-compatible 5' overhang, and the bold letters represent the 21-bp NRSE. $Sm36^2$ contains two copies of the following sequence: 5' a g c t t G C A A A G C C A T **T T C A G C A C C A C T T A G A G T G C C** T C T G C a 3'. The differences between these two sequences are underlined: The "G G" has been changed to "T T," resulting in an approximately 100-fold decrease in binding affinity of NRSF. The exogenous sequences have been added to such that the Hind III site is regenerated when it is cloned into pBSKS. In this way, plasmids can be distinguished as carrying the wild-type or mutant NRSEs by restriction digest. The mutant is cut by Hind III, while the wild-type is not.

$S36^4$ and $Sm36^4$, pBSKS carrying four copies of either the wild-type or mutant NRSEs, respectively, were constructed as follows. One aliquot of plasmid was digested with Cla I (which cuts just after the NRSEs), blunted with Klenow, and then digested with

Sca I (which cuts within the Bluescript backbone). Another aliquot of the same plasmid was digested with Eco RV (which cuts just before the NRSEs) and with Sca I. The NRSE-containing fragment of the first aliquot was then ligated to the NRSE-containing fragment of the second aliquot to generate a plasmid containing four copies of the NRSE sequence. Between the two pairs of NRSEs, therefore, is a short piece of polylinker with the following sequence: 5' (A)AGCTT ATCG ATC AAGCT(T) 3', with the bases in parentheses present or not depending on whether the NRSEs are mutant or wild-type, respectively. (Note: fusion of the Klenow-blunted Cla I site to the Eco RV site regenerates the Cla I site.)

Yeast integration and reporter constructs containing four copies of either the wild-type or mutant NRSE were all generated using inserts excised from $S36^4$ or $Sm36^4$, respectively. The NRSEs were inserted into pLacZi from Eco RI to Xho I to make pLacZi-$S36^4$ and pLacZi-$Sm36^4$. The NRSEs were inserted into pHISi and pHISi-1 in the following way. $S36^4$ or $Sm36^4$ were first digested with Xho I and Klenow-blunted, then digested with Eco RI. The resulting fragments were then cloned into the vectors that had been digested first with Xba I and Klenow-blunted, then digested with Eco RI. This produced the following plasmids: pHISi- $S36^4$, pHISi- $Sm36^4$, pHISi-1- $S36^4$, and pHISi-1- $Sm36^4$.

The two positive-control, activation domain-fusion constructs, pACT2-ZnF and pGAD424-ZnF, were constructed as follows. The Eco RI fragment from RCASBP(B)-dnNRSF (Chen et al., 1998), which contains the Zinc-finger domain of mouse NRSF, was inserted into the Eco RI site of pACT2 to form pACT2-ZnF. The ZnF domain was then inserted into pGAD424 by cloning the Sma I – Bgl II fragment of pACT2-ZnF into pGAD424 from Sma I to Bgl II.

Generation and testing of yeast reporter strains

Yeast single-reporter strains were generated from strain YM4271, which has deletions at the *ura3*, *his3*, and *leu2* loci. LacZ reporter constructs were integrated into the *ura3* locus, and His reporter constructs were integrated into the *his3* locus using the small-scale LiAc yeast transformation procedure with linearized plasmid. All strains were struck out on –Leu plates in order to confirm Leu$^-$ auxotrophy, indicating that they were neither spontaneous revertants nor wild-type yeast contaminants.

The activation domain-fusion construct, pACT2-ZnF, was made in order to test the single-reporter strains. This high-expressing construct was first tested for toxicity by transforming the parental strain, YM4271, to determine whether growth was slowed. Single-reporter strains were then tested for good signal and low background using this positive-control construct and the uninserted vector, pACT2, respectively. Transformation of these constructs was performed according to the procedure mentioned above, but with supercoiled plasmid. β-galactosidase (β-gal) activity was measured by the colony-lift filter assay at 30°C, and growth on –His plates was tested with various concentrations of 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of the HIS3 gene product. Double-reporter strains were then generated from good single-reporter strains by transforming each with the other reporter construct using linearized plasmid. These final strains were tested for good signal and low background using both pACT2-ZnF and pGAD424-ZnF and the two uninserted vectors, respectively.

Production of activation domain-fusion library

Poly A^+ RNA was isolated from 2 female 129SVEV mouse brains using the FastTrack 2.0 kit (Invitrogen). An activation domain-fusion, plasmid library was produced from this mRNA using reagents and protocols as described in the Two-Hybrid cDNA Library Construction Kit User Manual (Clontech), with two exceptions: (1) Both cDNA synthesis reactions were performed with random-hexamer primers rather than using oligo(dT) primers for one of the reactions. (2) pACT2 was used as the library vector instead of pGAD10. After determining the complexity of the library, it was amplified and a large-scale preparation of the DNA was made. The library was also characterized with respect to percent insertion and range of insert length by isolating the plasmid from 18 random colonies and digesting with Eco RI.

Library screening

The adult mouse brain library described above was screened for inserts that encode NRSE-binding domains by using it to transform the yeast double-reporter strain, ZHw_2, according to the large-scale yeast transformation procedure described in the MATCHMAKER One-Hybrid System User Manual (Clontech). The transformation was plated on –His, -Leu plates containing 5 mM 3-AT. Colonies that appeared over the first six days after plating were restreaked over two plates – one to confirm the growth characteristics and the other to test for β-gal activity. Plasmid was rescued from clones showing relatively high levels of β-gal activity, and 10 were sequenced.

RESULTS

Yeast integration and reporter constructs

In order to identify potential NRSE binding proteins in addition to NRSF, the yeast one-hybrid method was employed (MATCHMAKER One-Hybrid System, Promega). As a first step in this procedure, a number of different reporter constructs that contained multiple copies of the NRSE were generated. All constructs were made in two versions in order to be able to confirm the specificity of the DNA-binding protein for the NRSE: One version contained four copies of the wild-type NRSE, and the other contained four copies of a mutant NRSE. This mutation is known to decrease the binding of NRSF by approximately 100 fold and was used in the isolation of NRSF itself (Schoenherr and Anderson, 1995). The reporter constructs were made in the following vectors: pLacZi, pHISi, and pHISi-1. The LacZ reporter vector contains the minimal promoter of the yeast iso-1-cytochrome C gene directing the transcription of the *lacZ* gene. The NRSEs were inserted just upstream of the promoter, such that a fusion protein between an NRSE-binding domain and the activation domain of the yeast GAL4 transcriptional activator would stimulate transcription from this promoter, resulting in β-gal production. The two HIS reporter constructs contain the minimal promoter of the *HIS3* locus directing the transcription of the *HIS3* gene. The difference between pHISi and pHISi-1 lies in the vector sequence flanking the minimal promoter, such that basal activity of the former is somewhat higher than that of the latter. The NRSEs were inserted just upstream of the promoters in these constructs such that an NRSE-binding GAL4 fusion protein would activate expression of the HIS3 gene, allowing growth on His^- plates. The two different HIS vectors are used because sometimes the background expression of pHISi is too high

after the regulatory elements have been inserted, and pHISi-1 must be used instead. This must be determined empirically for each sequence.

Creation and testing of yeast reporter strains

All 6 reporter plasmids were separately transformed into the *ura3*- and *his3*-deleted yeast strain, YM4271. The reporter plasmids do not contain an origin of replication, and therefore cannot autonomously replicate. The plasmids are designed to integrate into a specific locus (*ura3* for the LacZ construct and *his3* for the HIS constructs) when transformed as linearized DNA. In this way, reporter activity is only ever observed from one genomic location, and variability is reduced. Growth of the HIS transformants was slower than the LacZ transformants. This was expected because integration of the HIS plasmid was selected for by growth on –His plates; and in this case, expression of the rescuing gene, HIS3, was driven by leaky expression from the minimal promoter. Many more LacZ-transformed colonies resulted from the transformation than HIS-transformed colonies. This was expected because the LacZ plasmid has been reported integrate into the *ura3* locus more efficiently than the HIS plasmid integrates into the *his3* locus. A number of independent isolates were collected for each transformation (except for pHISi-1-Sm36[4], which yielded no transformants), and characterized further before one of each was selected for future use.

A positive control construct was made to test these single-reporter strains. An expression construct encoding a fusion of the zinc-finger domain of NRSF to the GAL4 activation domain was made in pACT2, a vector in which many yeast two-hybrid libraries are made. (The same activation domain-fusion libraries used in two-hybrid analysis are

used in the one-hybrid method.) This vector expresses the fusion proteins at high levels, and so the construct, pACT2-ZnF, was tested for toxicity in the parental yeast strain, YM4271, before being introduced into the single-reporter strains. When pACT2 and pACT2-ZnF were transformed side-by-side, the colonies grew at the same rate, indicating that expression of the positive control fusion protein was not toxic. These two plasmids were then transformed into each of the single-reporter strains in order to check whether their positive reporter signal was strong enough and their background reporter signal was low enough that the one-hybrid approach would be feasible in the case of the NRSE.

The LacZ strains, Zw_1-Zw_4 and Zm_1-Zm_4, containing wild-type or mutant NRSEs, respectively, were first simply tested for background β-gal activity without any activation domain-fusion construct. A filter-lift assay showed that the Zw strains gave a very faint blue color after about 2 hours (hr) at 30°C, which remained faint after over-night incubation. The Zm strains gave no color at all. This indicated that background LacZ reporter expression levels were likely to be acceptable. Zw_4 and Zm_4 were then transformed with pACT2-ZnF and pACT2 to assess the positive signal as well as the background of these single-reporter strains. Filter lifts were completely colorless from all combinations of reporter strain and activation domain-fusion plasmid except for Zw_4 and pACT2-ZnF. This combination began showing a faint signal by 20 minutes (min) and was obviously positive by 30 min.

The HIS strains, iw_1 and im_1 (made from the transformation of pHISi-S36[4] and pHISi-Sm36[4], respectively) and $-1w_1$ (made from the transformation of pHISi-1-S36[4]), were tested for their ability to grow on various concentrations of 3-AT. This was done first in the absence of expression of any activation domain-fusion protein and then after

transformation with pACT2-ZnF and pACT2. This titration was required in order to determine whether background growth on –His plates could be sufficiently suppressed in order for the strain to be effective in screening an activation domain-fusion library. In the absence of any pACT2 construct, 5 mM 3-AT could completely block the growth of $-1w_1$ on –His plates, but 15 mM was required for im_1, and 30 mM was required for iw_1. While background growth of $-1w_1$ was clearly most easily controlled, it was not chosen for creation of the double-reporter strain for two reasons. First, the mutant-NRSE partner of this strain ($-1m_1$) had not been obtained. Second, $-1w_1$ appeared to grow very slowly, and thus might be difficult to transform. Strains iw_1 and im_1 were then transformed with pACT2-ZnF and pACT2 and their growth tested on a range of 3-AT concentrations. Under these conditions, no growth was seen even at 5 mM 3-AT except for the case of iw_1 transformed with pACT2-ZnF. At 15 mM the growth of even this strain-plasmid combination was noticeably inhibited.

Double-reporter strains containing both the LacZ and HIS reporter constructs were generated by transforming both the LacZ strains with pHISi constructs and the HIS strains with pLacZi constructs. The transformation of im_1 with pLacZi-Sm36[4] became contaminated, so the strain that would have resulted, HZm, was never obtained. Four independent isolates of the transformation of iw_1 with pLacZi-S36[4], $HZw_1 - HZw_4$, were collected, however. In addition, 4 isolates of both wild-type- and mutant-NRSE versions of the strains generated by transformation in the opposite order were obtained as well: $ZHw_1 - ZHw_4$ and $ZHm_1 - ZHm_4$. The wild-type NRSE double-reporter strains were tested in parallel for growth at various concentrations of 3-AT in order to select one of each kind with the lowest background growth. Of the HZw strains, one was resistant to 5 mM 3-AT, but the other three only grew below this concentration. Among these three was

HZw_1, which was selected for potential future use. Of the ZHw strains, two were resistant to even 15 mM 3-AT to some degree, but the other two only grew below 5 mM. Of the latter two, ZHw_2 was selected for potential future use. Two ZHm strains were saved as well, ZHm_3, which grew as fast as ZHw_2, and ZHm_2, which grew more slowly.

Up until this point the library that was intended for screening using these double-reporter strains was a commercially available activation domain-fusion library of adult mouse brain cDNA (Clontech). This particular library was made in the vector, pACT2, and, unfortunately, the first-strand cDNA was oligo(dT)-primed. The position of the DNA-binding domain within the NRSF transcript is over 5 kilobases (kb) away from the poly(A) tail (Scholl et al., 1996). It was not expected, therefore, that NRSF would be detected by the yeast one-hybrid method using an activation domain-fusion library that had been made by oligo(dT) priming. If an alternate NRSE-binding protein was structurally related to NRSF, then that protein would also be unlikely to be detected with this library. It was decided, therefore, that a new, random-primed library would be made. The Two-Hybrid cDNA Library Construction Kit (Clontech) was used for this purpose, but the vector that was included was not pACT2. The vector, instead, was pGAD10, which expresses fusion proteins at a lower level, less likely to be toxic to the host cell. In order to decide which vector to use for the new library, a positive control construct was made in pGAD424, a vector identical to pGAD10, except for its multiple-cloning site. (This vector was less precious, as it was included in the MATCHMAKER One-Hybrid System kit (Clontech)). This construct, p424-ZnF, contained the zinc-finger domain of mouse NRSF fused in frame with the GAL4 activation domain.

Both constructs along with their corresponding uninserted vectors were transformed into the double-reporter strains, ZHw_2 and ZHm_3. Transformants were

tested for both β-gal activity and growth on –His plates containing a range of 3-AT concentrations. The filter-lift assay showed no color, after even 24 hr, for any of the strain-plasmid combinations except for the wild-type-NRSE strain with the two positive control constructs. The signal from each of these constructs, however, was markedly different. Blue color first became apparent with pACT2-ZnF at 20 min and was obvious at 30 min. In the case of p424-ZnF, however, even after 40 min no signal was apparent. After 24 hr the signal from p424-ZnF was faint, while that of pACT2-ZnF was deep blue. Growth of transformants of –His plates showed a similar, large difference between the two activation domain-fusion constructs. When transformants were struck out on –His plates containing either 0 or 5 mM 3-AT, expression of pACT2-ZnF allowed growth of all colonies in the presence of 3-AT, while only a subset of p424-ZnF-expressing colonies grew with 3-AT present. Interestingly, however, when transformations were plated directly onto 3-AT-containing plates, colonies were able to grow at 15 mM concentrations even when carrying either uninserted vector. This was probably due to yeast feeding off of their dead neighbors, and made it difficult to choose a concentration of 3-AT to use in the actual screen. In both assays pACT2-ZnF was found to have a much stronger signal than p424-ZnF with only a slightly higher background. It was therefore chosen for use in construction of the new, random-primed, adult mouse brain, cDNA library.

Production of activation domain-fusion library

Poly A^+ RNA from adult mouse brain was purified from two female 129SVEV mice using the Fast Track 2.0 kit (Invitrogen). A total of 8 μg of mRNA was used in two random

hexamer-primed cDNA reactions using a tracer amount of [α ^{32}P]-dCTP to follow the products. After adapter ligation and phosphorylation, the cDNA was size-selected to enrich for fragments over approximately 500 base pairs (bp). Home-made linearized, dephosphorylated pACT2 vector was generated and cDNAs were ligated into it in batches. Ligations were transformed into commercially available electrocompetent bacteria (Clontech) and 1/100 of the each transformation was plated in order to determine the complexity of the library. Unfortunately, the total complexity was only 73,400 independent clones. For a plasmid library this is less than 1/10 of what would be expected. The percentage of recombinant clones and range of insert length were assessed by isolating the plasmid from 18 randomly selected colonies and digesting with Eco RI to excise the insert. One hundred percent of the clones contained an insert, and insert length ranged from approximately 200 to 2800 bp. The library was amplified by plating the rest of the transformations and using the colonies to perform a large-scale preparation of the plasmids. Three hundred μg of this small library were obtained.

Library screening

Although it was unlikely that a novel NRSE-binding protein would be found in this low-complexity library, it was screened nonetheless. The double-reporter yeast strain, ZHw_2, was transformed with 20μg of the small library, and high efficiency was achieved, 1.9 x 10^4 cfu/μg. The transformation was plated over 15 15-cm plates containing 5 mM 3-AT. After 3 days the 35 colonies that had appeared were restreaked over two fresh 3-AT plates, one to confirm their ability to grow when isolated from other yeast and the other to test for β-gal activity. Five days after transformation 24 more colonies were

restreaked, and the next day an additional 42 colonies were picked. All but one of these colonies grew on 5 mM 3-AT when restreaked. Many of these clones gave blue signal in the β-gal assay; however, none turned blue as quickly as the pACT2-ZnF positive control had. Plasmid was isolated and sequenced from 10 of the clones that had the highest β-gal levels. Many of these were segments of ribosomal RNA, apparently a common type of background in one- and two-hybrid studies. One was a portion of a serotonin receptor cDNA, but in the opposite orientation. In the end, none of the 10 clones appeared to be true positives.

DISCUSSION

Described here is the generation of a set of yeast strains and a small library for use in the yeast one-hybrid method of identifying a factor that can bind to the NRSE. The single-reporter strains, Zw_4 and Zm_4, contain a wild-type and a mutant set of 4 NRSEs, respectively, upstream of a minimal promoter driving *LacZ* expression, integrated into the *ura3* locus. Zm_4 gave low background, and Zw_4 gave obvious signal at 30°C within 30 min when transformed with the activation domain-fusion expression construct, pACT2-ZnF, and low background when transformed with uninserted vector. The single-reporter strains, iw_1, im_1, and $-1w_1$, contain the appropriate versions of a set of NRSEs upstream of a minimal promoter (different from that of *LacZ*) driving *HIS3* expression, integrated into the *his3* locus. Expression from $-1w_1$ is lower than that from iw_1 and im_1. Growth of $-1w_1$ was completely inhibited by 5 mM 3-AT. Upon transformation of iw_1 and im_1 with uninserted control vector, 5 mM 3-AT was sufficient to prevent their growth as well.

The yeast one-hybrid screen was eventually performed using the double-reporter strain, ZHw_2, derived by transforming Zw_4 with pHISi-S36[4]. The complexity of the activation domain-fusion library was very low. This is probably why no true positives were obtained. However, why were so many apparently His^+ colonies isolated from the screen, when none of them showed β-gal activity comparable to the positive control? While this is difficult to answer, some suggestions can be made for improvement of the screen.

When ZHw_2 was transformed with pACT2 and pACT2-ZnF, and the transformation was directly plated on different concentrations of 3-AT, colonies did appear even with the negative-control vector at 15 mM 3-AT. The colonies from pACT2 were significantly smaller than those from pACT2-ZnF, but clearly, under these conditions, the background was not zero. When colonies were restreaked, however, no negative control colonies grew at 5 mM 3-AT. This indicates that any false positives should be able to be eliminated at the restreaking step. In the case of screening a library with the normal complexity of at least 1×10^6 independent clones, however, eliminating false positives by restreaking may be prohibitively labor intensive. It is, therefore, suggested that screening of a new library with the strain ZHw_2 be done in the presence of at least 15 mM 3-AT, and possibly more.

Making a new double-reporter strain based on the pHISi-1 plasmid may potentially reduce false-positive background. Either the-$1w_1$ strain could be transformed with pLacZi-S36[4], or Zw_4 could be transformed with pHISi-1-S36[4]. Each method has its advantages and disadvantages. The former method is likely to be easier (even though $-1w_1$ doesn't grow very well), because, as mentioned above, the LacZ vector integrates with higher efficiency than do the HIS vectors. Unfortunately, making the double-

reporter strain this way leaves the corresponding mutant-NRSE strain unmade. Transforming the Zw_4 and Zm_4 strains with pHISi-1-S36^4 and pHISi-1-Sm36^4, solves that problem. However, the reason why the –1m strain doesn't exist is because no transformants could be generated. The first method is probably best, because specificity of any putative positive resulting from the screen can be tested in the single-reporter strains, Zw_4 and Zm_4. This would probably be sufficient demonstration of binding-element specificity.

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