Silencer Elements Modulate the Expression of the Gene for the Neuron-Glia Cell Adhesion Molecule, Ng-CAM*

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The combined factors that regulate the expression of cell adhesion molecules (CAMs) during development of the nervous system are largely unknown. To identify such factors for Ng-CAM, the neuron-glia CAM, constructs containing portions of the 5′ end of the Ng-CAM gene were examined for activity after transfection into N2A neuroblastoma and NIH3T3 cells. Positive regulatory elements active in both cell types included an Ng-CAM proximal promoter with SP1 and cAMP response element motifs extending 447 base pairs upstream of a CAM proximal promoter with SP1 and cAMP response element motifs. Negative regulatory elements included five neuron-restrictive silencer elements (NRSEs) and a binding site for Pax gene products in a 305-base pair segment of the first intron. Constructs containing the promoter together with the entire first intron were active in N2A cells but were silenced in NIH3T3 cells. This silencer activity was mapped to the NRSEs. In contrast, the Pax motif inhibited activity of Ng-CAM constructs in both cell types. The DNA elements defined in these transfection experiments were examined for their ability to bind nuclear factors. The region within the first exon formed a DNA-protein complex after exposure to nuclear extracts prepared from both NIH3T3 and N2A cells. The NRSE region formed a more prominent complex with proteins prepared from NIH3T3 cells than it did with extracts from N2A cells. A member of the Pax protein family, Pax-3 bound to the Pax motif. Mutations introduced within the Pax motif in its ATTA sequence eliminated this binding whereas mutations in its GTTCC sequence did not, suggesting that paired homeodomain interactions are important for the recognition of Pax-3 by this DNA target sequence. The combined data suggest that negative regulation by NRSEs and Pax proteins may play a key role in the place-dependent expression patterns of Ng-CAM during development.

Cell adhesion molecules (CAMs) are essential for guiding tissue formation (1) and play key roles in the development of the nervous system. CAMs important in the nervous system include N-CAM-related molecules such as Ng-CAM (2, 3), Nr-CAM (4, 5), L1 (NILE) (6, 7), neurofascin/ABGP (8, 9), TAG-1/axonin-1/FJ3 (10–12), and contactin (13). Ng-CAM, L1, Nr-CAM, neurofascin/ABGP from vertebrates and neuroglian from Drosophila (14) comprise a subfamily of neural CAMs containing six immunoglobulin domains and five fibronectin type III repeats. Molecules of this Ng-CAM subfamily are expressed prominently in axonal pathways in both the central nervous system and peripheral nervous system and are involved in neurite fascilitation and outgrowth. Each of these neural CAMs has a characteristic spatial and temporal expression pattern during neural morphogenesis but the factors that restrict expression of Ng-CAM and other neural CAM genes to particular populations of neural cells are not well understood.

To define the sequences of DNA responsible for place-dependent expression of CAMs, we have focused on signals from homeobox and Pax gene products (15–18). An attractive hypothesis is that Ng-CAM and other neural CAMs are targets of homeodomain and Pax proteins (19). During neural development, a number of transcriptional regulators encoded by the homeobox and Pax gene families appear in defined expression patterns along the anterior-posterior and dorsal-ventral axes of the embryo that correlate with the patterns of a variety of CAMs. Moreover, mutations in Pax genes are known to alter the programs of neural differentiation and migration, processes that are influenced by the activity of neural CAMs. For example, mutations in Pax-6 and Pax-3 genes lead to developmental defects of the nervous system as shown, respectively, in small eye and splotch mutant mice (20). Some of these defects appear to be caused by aberrant neuronal migration (21, 22), a process normally modulated by neural CAMs.

To examine the factors regulating Ng-CAM expression, we have isolated genomic clones containing the 5′ end of the Ng-CAM gene, characterized its proximal promoter, and located two regulatory regions within a 305-base pair segment of the first intron. One region of the first intron was found to contain five neuron-restrictive silencer elements (NRSEs) which extinguished expression of the Ng-CAM gene in a fibroblast cell line, but not a neuroepithelial cell line. Another region contained GTTCC and ATTA sequences characteristic of binding sites for Pax proteins. This Pax motif was found to bind Pax-3 in gel mobility shift experiments; such binding was disrupted when specific mutations were introduced in the ATTA sequence within the Pax motif. In transfection experiments with both NIH3T3 and N2A cells, this Pax motif was found to be a negative regulator of Ng-CAM gene expression independent of the silencing imposed by NRSEs. Our studies suggest that the NRSEs and Pax motif may play critical roles in the place-dependent expression of Ng-CAM in the nervous system.

MATERIALS AND METHODS

Analysis of Ng-CAM Genomic DNA—A chicken genomic DNA library in the cosmid vector pWE15 (Clontech) was screened using a 400-base pair 32P-labeled EcoRI-BstXI fragment derived from the 5′ end of the Ng-CAM cDNA sequence (3). A single cosmid clone (Cos-Ng) was isolated. Sequencing of the 2536-base pair segment of Ng-CAM genomic DNA was performed by the dyeoxy chain termination method using
Sequenase (U. S. Biochemical Corp.) (23) and the data were compiled using the GCG package (University of Wisconsin). The NRSEs and the Pax motif were not revealed in conventional data base searches, but were found after analysis of Ng-CAM constructs in transfection experiments. Ng-CAM NRSEs were aligned with the consensus NRSE sequence (24) using the fasta algorithm (25). Oligonucleotides were made using a 392 DNA synthesizer (Applied Biosystems, Forrestfield, CA).

Determination of the Transcription Initiation Site—The transcription start site was determined through a combination of primer extension and RNase protection analyses. Poly(A) + RNA was isolated from 12 day embryonic chick brains using the Fast Track mRNA isolation kit (Invitrogen, San Diego, CA). For primer extension, 4 µg of poly(A) + RNA was hybridized to a 32P-labeled oligonucleotide derived from the antisense strand of the first exon of the Ng-CAM gene (Fig. 2, from position +185 to +166). Primer extension was performed using Superscript reverse transcriptase (Life Technologies Inc.) at 42 °C for 1 h.

RNase protection analysis was performed using the RPA II kit (Ambion). The template for the RNA probe was made by polymerase chain reaction from the genomic DNA. The downstream primer used for the amplification was Ng-7T3, an oligonucleotide identical in sequence to oNg-7, but also containing the T3 RNA polymerase promoter at the 5′ end. The upstream primer used in the polymerase chain reaction was an oligonucleotide designated oNg-6, derived from the sense strand of the Ng-CAM genomic sequence (Fig. 3, position −172 to −156). A labeled Ng-A probe was synthesized from the Ng-CAM genomic DNA and labeled with T[32P]UTP. Template DNA was then digested with RNase-free DNase. Two fmol of probe in elution buffer was mixed with 1 µg of poly(A) + RNA from 12 day embryonic chick brains and hybridized at 45 °C for 16 h. RNase protection analysis was performed using different dilutions of RNase to determine the optimal conditions for cleavage. The products of both primer extension and RNase protection analysis were resolved on an 8% polyacrylamide sequencing gel.

Construction of Reproter Plasmids—Reporter gene plasmids were constructed in the promoterless chloramphenicol acetyltransferase (CAT) gene vector pCAT-basic (Promega, Madison, WI) and pBlue-script-II (Stratagene, La Jolla, CA) and are shown schematically in Fig. 3. Ng4, Ng15, and Ng447/182 plasmids were constructed by polymerase chain reaction amplification of DNA segments spanning the regions between −447 and +1644 and +1487 and +182, respectively, and inserted into the pCAT basic vector (Promega). The Ng4, Ng15, and Ng447 plasmids containing 4 kilobases, 1.5 kilobases, and 200 base pairs of the Ng-CAM gene 5′-flanking sequence, respectively, were made by insertion of appropriate restriction fragments into the pCAT-basic vector. The Ng447–1 construct was made by insertion of a 2536-base pair BamHI-PvuI fragment into pBluecript and the CAT gene was inserted into the KpnI site immediately downstream. The constructs SgrBsp, EE, EA, ES, and AS constructs containing internal deletions of the Proximal Promoter and Regulatory Sequences in the First Intron—A cosmid clone (designated Cos-Ng) was isolated after screening with a 32P-labeled 400-base pair EcoRI-BstXI fragment derived from the 5′ end of the Ng-CAM cDNA sequence (3). Cos-Ng contained an insert approximately 20 kilobases in length. The borders for the first seven exons and introns were located by restriction mapping and Southern blotting and their sequences were determined using oligonucleotide primers derived from the Ng-CAM cDNA sequence (Fig. 3).

To determine the site of transcription initiation within the Ng-CAM gene, we performed primer extension and RNase protection analyses of poly(A) + RNA isolated from chick brain tissue at embryonic day 12. Extension of an antisense oligonucleotide primer (oNg-7) RNA was immobilized on nitrocellulose membranes and hybridized with the labeled probe. The membrane was washed and exposed to an X-ray film. The transcription start site was determined to be at position +1388, which is located immediately downstream of the first exon (Fig. 1).
SiIners and a Pax Motif Regulate the Ng-CAM Promoter

Fig. 1. Structure of the 5′ end of the Ng-CAM gene. Top, diagram of the 20-kilobase insert from the chicken cosmid clone Cos Ng containing the first seven exons of the Ng-CAM gene. The portions of exons 1 and 2 encoding 5′-untranslated sequences are indicated with an open box. Exons encoding translated mRNA sequence are indicated with black boxes. Bottom, nucleotide sequence of the borders for the first seven exons and introns of the Ng-CAM gene. The coding sequences of the exons are shown in upper case letters, while intronic sequences are indicated with lower case letters. The ATG codon is located in exon 2 and amino acids encoded by nucleotides 3′ of this position are shown over the appropriate triplet codons. In some cases borders were inferred solely from comparison of the Ng-CAM genomic sequence to the published Ng-CAM cDNA sequence (3).

A construct designated Ng447/182, containing the entire first exon was approximately 2-fold more active than the Ng447 construct in both NIH3T3 and N2A cells. These data suggested that the segment between +61 and +182 is a positive regulatory region of the Ng-CAM gene. A construct designated Ng447+1, containing the Ng-CAM proximal promoter together with the entire first exon, first intron, and a portion of the second exon was active in N2A cells but was completely silent in NIH3T3 cells. This finding suggested that sequences within the first intron of the Ng-CAM gene were capable of silencing the gene in non-neuronal cells.

NRSEs Within the First Intron Silence Ng-CAM Expression in NIH3T3 Cells, but Not in N2A Cells—To pinpoint the sequences that silenced Ng-CAM gene expression in NIH3T3 cells, we examined various constructs having deletions of segments of the first intron of the Ng-CAM gene for loss of silencing in transfection experiments. Constructs with deletions of the NRSEs were tested in both NIH3T3 and N2A cells (Fig.3). The Ng-CAM NRSEs show a high degree of similarity with the NRSE consensus sequence, but they are all in the orientation of the reverse complement from that found in the other genes (Fig. 4). Two constructs, designated EE and EA, which eliminated NRSEs 1–4 and NRSEs 1–5, respectively, showed an increase in activity (i.e. loss of silencer activity) as compared to the Ng447-1 construct. The same deletion constructs showed only a slight increase in activity in N2A cells.

To examine whether the NRSEs were sufficient for silencing of the Ng-CAM proximal promoter, constructs were prepared in which the five NRSEs were placed upstream of the Ng447 region of the Ng-CAM gene linked to a CAT reporter. Two constructs were prepared in which the NRSEs were present either in the orientation normally found in the gene (SAS) or in the opposite orientation (SS). The SAS and SS constructs were both silent in NIH3T3 cells, showing CAT activities that were comparable to the Ng447-1 and pCAT-Basic constructs (Fig. 3). However, in N2A cells, the SAS and SS constructs were highly active and showed no reduction of Ng-CAM proximal promoter activity. These studies suggest that, while the NRSEs are sufficient to silence Ng-CAM promoter activity in NIH3T3 cells, they do not show silencing in N2A cells.
The Pax Motif Negatively Regulates Ng-CAM Expression in Both NIH3T3 and N2A Cells—To test whether the Pax motif had an effect on Ng-CAM expression, three constructs were prepared and examined for activity (Fig. 3). A construct designated AS, having a 145-basepair deletion which removed the Pax motif, showed increased activity (i.e., a release from silencing) as compared to the Ng447 construct. The AS deletion showed a release from silencing in both NIH3T3 and N2A cells (Fig. 3, compare AS to Ng447). The same effects were observed in a construct in which the GTTCC and ATTA sequences within the Ng-CAM Pax motif were deleted (see the construct designated Pax, Fig. 3). A construct designated ES, in which both the NRSEs and the Pax motif were removed was more active than all other deletion constructs in NIH3T3 cells. The effects of the NRSEs and the Pax region were additive, i.e., the level of activity from the ES construct was approximately equal to the sum of activities from the EA and AS constructs. The combined data suggest that, in some contexts, the NRSEs and the Pax motif may act together to regulate expression of the Ng-CAM gene.

Binding of Nuclear Proteins to Ng-CAM Regulatory Elements—To examine whether nuclear protein factors could bind to the various regulatory regions of the Ng-CAM gene, we conducted a series of gel mobility shift experiments using two segments of the Ng-CAM gene shown in these studies to be important for regulation in cellular co-transfection experiments. 

Fig. 2. DNA sequence of a 2536-base pair segment of the Ng-CAM gene starting at a position 447 base pairs upstream of the transcription start site and ending within the second exon, 2089 base pairs downstream from the RNA start site. The start of RNA transcription is indicated by a rightward pointing arrow. Potential regulatory motifs within the proximal promoter and the first intron of the gene are underlined. These sequences include the CRE, SP1, NRSE, and Pax motifs.
FIG. 3. Activity of Ng-CAM constructs in NIH3T3 and N2A cells. Top, diagram of the 5' end of the Ng-CAM gene showing the position of various restriction sites used to prepare deletion constructs. Sequences included in exons are indicated with boxes that are either solid black or cross-hatched. The cross-hatched region corresponds to the +82/+182 region of the first exon. The five NRSEs are represented by boxes numbered 1–5. The Pax motif is located immediately downstream from the NRSEs and is indicated by an open box. N2A or NIH3T3 cells were transfected with the promoterless CAT gene reporter vector (Bas) or 14 other constructs containing various segments from the 5' end of the Ng-CAM gene. Cell extracts were normalized to an internal reference standard of β-galactosidase activity and assayed for CAT activity as described under "Materials and Methods." CAT activity for all constructs was quantitated using a PhosphorImager from four separate experiments performed in duplicate in which the activity levels varied no more than 5%.

Fig. 4. Comparison of the five NRSEs (Ng-NRSE1–5) located between +1388 and +1566 in the first intron of the Ng-CAM gene with a consensus NRSE sequence derived from NRSEs found in several neural-specific genes (24). The Ng-CAM NRSEs are in the orientation of the reverse complement from those found in other genes.

FIG. 5. The +82/+182 and NRSE regions of the Ng-CAM gene bind to nuclear factors prepared from NIH3T3 and N2A cells. Either the +82/+182 (panels A and B) or the NRSE (panels C and D) probe was mixed with nuclear extracts prepared from NIH3T3 cells (panels A and C) or from N2A cells (panels B and D). For binding reactions, probes were incubated without extract (all panels, lane 1) or with the appropriate nuclear extract (lanes 2–6). DNA-protein complexes formed between the +82/+182 and NRSE regions of the Ng-CAM gene with nuclear extracts are indicated with brackets labeled EX and NRSE, respectively. Competitor DNAs were added to some binding reactions (all panels, lanes 3–6). Competitors included either a 10- or 100-fold excess of the unlabeled DNA probe used in the specific binding reactions (all panels, lanes 3 and 4, respectively). To test whether the NRSE sequence could compete for binding of the +82/+182 probe to nuclear extracts, either a 10-fold or a 100-fold excess of cold NRSE DNA was added to the +82/+182 binding reactions (panels A and B, lanes 5 and 6, respectively). A similar test for cross-competition was performed by adding either a 10- or 100-fold excess of cold +82/+182 DNA to the NRSE binding reactions (panels C and D, lanes 5 and 6, respectively). Reactions were subjected to electrophoresis on a 4% polyacrylamide gel in 0.25 TBE buffer at 400 V for 2 h at 4 °C.
for binding of Pax proteins to DNA are the Ng-CAM Pax motif. GTTCC and ATTA motifs known to be important for binding to the Ng-CAM Pax motif to show binding of the GST/Pax-3 fusion protein to TPA labeled Ng-wt, Ng-HP, Ng-H, and Ng-P probes. The individual probe used for binding is indicated at the bottom of each panel. Binding reactions contained the indicated probe either: without added protein (lanes marked 1), with 2.5 μg of GST control protein purified from E. coli NM522 cells transformed with pGEX-2T (lanes marked 2), or with 0.5, 2.5, or 5 μg of GST/Pax-3 or GST/Pax-6 fusion (lanes marked 3-5, respectively). Binding reactions were subjected to electrophoresis on 6% polyacrylamide gels, dried, and autoradiographed for 4 h.

6). The Ng-wt probe contained the wild-type Pax motif (Fig. 3, the region between 1679 and 1708). The Ng-H variant contained 3-base pair substitutions that altered the ATTA motif. The Ng-P variant contained 11 base pair substitutions. Four substitutions destroyed the GTTCC motif which has been shown to be essential for the binding of the e5 DNA sequence to the paired domains of Pax-1 and Pax-3 (30, 32, 35). The seven other substitutions made in Ng-P were introduced after comparing the sequence of the Ng-CAM gene Pax motif to the consensus binding sequences for Pax proteins (31, 36, 37) and took account of critical base pair substitutions that may disrupt paired domain interactions with DNA. The third variant, Ng-HP (Fig. 6), contained the combination of mutations made in both Ng-H and Ng-P variants.

The Ng-wt probe showed binding to the GST/Pax-3 protein, but no binding to the GST control protein. Similar binding experiments using the Ng-HP and Ng-H variants showed little or no binding to Pax-3. The Ng-P variant showed no detectable decrease in binding to Pax-3 as compared to that of the Ng-wt. This result suggested that the GTTCC motif and other base pairs important for paired domain interactions were not necessary for Pax-3 binding. Rather, these experiments suggested that it was the ATTA motif that was important for Pax-3 binding to the Ng-CAM Pax motif.

**DISCUSSION**

In the chicken, Ng-CAM is first detected in the central nervous system at embryonic day 3 in cells of the ventral neural tube that are the precursors of motor neurons (38). At later stages, Ng-CAM appears on a number of neurons and is distributed mainly on axons rather than cell bodies. In the cerebellum, Ng-CAM appears in Purkinje cells, is expressed by a number of different fibers, and plays a role in the migration of granule cell neurons on radial glia. The synthesis of Ng-CAM also shows dynamic changes during both myelination and during nerve regeneration. At the onset of myelination, Ng-CAM expression decreases in the central nervous system but not in the peripheral nervous system. Ng-CAM expression is increased after peripheral nerve injury in the spinal cord and in the nerve at the lesion site, but is decreased in dorsal root ganglia (39). These dynamic patterns of Ng-CAM synthesis in the nervous system prompted us to identify factors that regulate Ng-CAM gene expression.

Structure of the 5' End of the Ng-CAM Gene—We have isolated the 5' end of the chicken Ng-CAM gene (Fig. 7). The first seven exons encode Ng-CAM mRNA sequences which include the 5'-untranslated region, the initiator methionine, the signal peptide, the amino terminus, and the first two immunoglobulin domains of Ng-CAM (Fig. 1). Comparison of the partial Ng-CAM gene structure to the gene structures for other closely related neural CAMs such as L1 (40) and Nr-CAM reveals a close correspondence in how untranslated and coding sequences are partitioned into exons. The immunoglobulin domains of these molecules are all encoded by two exons, a feature that sets them apart from immunoglobulin genes, consistent with an evolutionary divergence (41). Chicken Ng-CAM, mouse L1, and chicken Nr-CAM, and chicken neurofascin proteins share approximately 40% identity in their amino acid sequences (3, 4, 6). It has been long debated whether Ng-CAM and L1 are functional equivalents in the chicken and mouse. Based on the comparison of the 5' gene structures of these neural CAMs, it is still not possible to establish whether the Ng-CAM and L1 genes are in the same evolutionary lineage. Further comparison of the cis control elements of these neural CAM genes may resolve the issue and may also reveal similarities and differences in regulatory regions affecting expression of these genes for neural CAMs.

The Proximal Promoter—The Ng-CAM gene was determined to have a single RNA start site, yet the Ng-CAM promoter was found not to contain a TATA box, a sequence typically contained in promoters for genes having a single RNA start site. However, the sequence GGGCCGCGGGG, which matches a consensus binding site for the SP1 transcription factor (28) was found immediately upstream of the RNA start site at -15. Thus, it is possible that SP1 and associated proteins may be part of a mechanism controlling transcription of the Ng-CAM gene (42).

A region of 447 base pairs of 5'-flanking upstream sequence was sufficient for basal Ng-CAM promoter activity in cells;

2 V. P. Mauro, G. M. Edelman, and F. S. J ones, unpublished data.
addition of up to 4 kilobases of Ng-CAM 5′-flanking sequence showed no further increases in this activity. Potential regulatory sequences located in the proximal promoter included an additional SP1 motif at -134 and a consensus CRE (TGACGTCA) at -255 (Fig. 7). In both NIH3T3 and N2A cells, a 3-fold decrease in expression was observed for constructs that had a deletion in the -200 to -447 region of the Ng-CAM proximal promoter. This region contained the CRE and thus, trans-factors of the CREB family (43, 44) may control Ng-CAM gene expression.

Inclusion of the region between +82 and +182 in the first exon of the Ng-CAM gene in constructs led to a 2-3 fold stimulation of Ng-CAM promoter activity in both NIH3T3 and N2A cells. It is likely that this sequence may impart either additional stability of Ng-CAM mRNAs or binds a transactivator important for transcription of the Ng-CAM gene. We found that the +82 to +182 region bound to nuclear proteins from both NIH3T3 and N2A cells.

Silencer Activity of NRSEs Located in the First Intron of the Ng-CAM Gene—We have located five NRSEs within the first intron of the Ng-CAM gene (Fig. 4). Single copies of the NRSE have been found in several neurally expressed genes including in the SCG10 gene (24), the gene for the rat type II sodium channel (45), and the gene encoding synapsin I (46). Five NRSEs appear in tandem within the first intron of the Ng-CAM gene and a tandem arrangement of NRSEs has been observed, for example, in the first intron of the rat brain derived neurotrophic factor gene (47), although so far no experiments have addressed whether this sequence has silencer activity.

In cellular transfection experiments, we found that the Ng-CAM NRSEs silenced the Ng-CAM proximal promoter in NIH3T3 cells. In contrast, NRSEs did not silence the promoter in N2A cells. Thus, the NRSEs silenced Ng-CAM gene expression in non-neuronal cells. The multiple copies of NRSEs in the Ng-CAM first intron suggest that they may bind proteins cooperatively. Recently, a neuron-restrictive silencer factor (NRSF, also called REST) has been identified which binds to the NRSE (48, 49). The protein contains eight zinc fingers which are related to those found in proteins of the GLI-Krüppel family.

Consistent with their activity patterns, we found that the Ng-CAM NRSEs formed a more prominent DNA-protein complex with nuclear proteins from NIH3T3 cells than they did with proteins from N2A cells. It is therefore likely that the protein enriched in NIH3T3 cells which binds to the NRSEs and silences Ng-CAM gene expression is NRSF/REST or a related protein. Interestingly, constructs with deletions in the NRSEs also show slightly elevated expression in N2A cells when compared to constructs containing the NRSEs (see Fig. 3). Thus, in N2A cells a minor amount of NRSF/REST may contribute to silencing, but the predominance of positive factors may greatly override this activity. In a preliminary search of the published L1 gene sequence (40) we have located an NRSE with the sequence TCTGCTGTCCGTGGTGCGA within the first intron at position 277-297. The possibility must therefore be considered that NRSEs may be used in the negative regulatory programs of other neural CAM genes in the Ng-CAM family.

Function of the Pax Motif and Binding to Pax-3—We have previously suggested that cell adhesion molecules are likely to be a major class of target genes regulated by homeobox and Pax transcription factors during embryonic development (15-19). In the present study, we were particularly interested in whether the regulatory sequences of the Ng-CAM gene, encoding a CAM that is restricted to the nervous system, are potential targets for Pax proteins. The cellular transfection experiments showed that constructs having deletions in either the region containing the Pax motif, or site-directed mutations within the GTTCC and ATTA sequences in the Pax motif, were more active than similar constructs containing the unmutated Pax motif. Furthermore, in NIH3T3 cells, the combination of NRSEs and the Pax motif was more effective in silencing than was the NRSE region alone. We found using gel mobility shift experiments that the Pax motif bound to Pax-3. This binding was disrupted by mutations in the ATTA sequence, but not by mutations in the GTTCC sequence and adjacent base pairs. These data demonstrate that Pax-3 binds directly to the Pax motif in the Ng-CAM gene and that this interaction is mediated primarily by the homeodomain and not by the paired domain of Pax-3. Previous studies showing specific Pax-3 binding to DNA have employed synthetic binding sequences derived from the even-skipped gene promoter from Drosophila (32, 35). The Pax motif in the Ng-CAM gene described in the present study represents the first naturally occurring vertebrate target sequence found for the Pax-3 gene product.

Recently, a sequence containing an ATTA motif has been located at -170 in the promoter of the gene for the neural CAM L1 (40). While this motif has some similarities with the Ng-CAM Pax motif, it also shows some important differences. The two Pax motifs are similar in that they both contain ATTA motifs and share a few identities in the base pairs flanking this sequence. However, in contrast to the Ng-CAM Pax motif, the L1(-170) motif closely resembles the consensus binding site for Pax-6 (31, 37). L1(-170) has been shown to bind to Pax-6, but does not bind to Pax-3. Unlike the binding of the Ng-CAM Pax motif to Pax-3 which is eliminated when mutations are introduced into the ATTA sequence, the binding of L1(-170) to Pax-6 was eliminated by mutations disrupting sequences which interact with the paired domain (50). The binding was unaffected by mutations in the ATTA motif that disrupt homeodomain interactions. It will be revealing to determine whether other genes encoding neural CAMs that are related to Ng-CAM and L1 at the amino acid level, such as Nr-CAM and neurofas, also contain Pax motifs. Genes encoding this family of neural CAMs may all contain Pax motifs with subtle variations in sequence composition that may determine binding preferences and selective CAM gene control by different Pax proteins.

It will be necessary to determine in vivo what roles the DNA control elements described here play in the developmental expression pattern of Ng-CAM in the nervous system. It is likely that particular combinations of Ng-CAM regulatory elements identified in this study (see Fig. 7) are utilized to control specific contexts of Ng-CAM expression during neural development and regeneration. For example, the NRSEs and Pax motif and their bound proteins may act combinatorially to restrict Ng-CAM expression to particular classes of neural cells during development. This possibility may be explored in chicken embryos by using retroviral vectors (51). Chicken Ng-CAM constructs can also be tested in transgenic mice, an approach that has been used successfully across species to analyze the brain-specific expression directed by regulatory sequences from the chicken gene encoding the a2 neuronal acetylcholine receptor (52). Such animal studies will be particularly useful in determining how the regulatory regions identified in the present study function to determine place-dependent expression of Ng-CAM.

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