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# Identification of Positive and Negative Regulatory Elements Governing Cell-Type-Specific Expression of the Neural Cell Adhesion Molecule Gene

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**The neural cell adhesion molecule (NCAM) is one of the most prevalent cell adhesion molecules in vertebrates. Its expression is subject to complex cell-type- and developmental-stage-dependent regulation. To study this regulation at the level of transcription, we analyzed the promoter region of the mouse NCAM gene. The NCAM promoter did not contain a typical TATA box. Transcription started at several sites that were used indiscriminately by different cell types, implying that the different NCAM isoforms are expressed from a single promoter. Sequences responsible for both promotion and inhibition of transcription resided within 840 base pairs upstream of the main transcriptional start site. The sequence from positions −645 to −37 relative to the translation initiation site directed high levels of expression in NCAM-expressing N2A cells. The same fragment was six times less active but still significantly active in L cells, but this activity was repressed by inclusion of an additional upstream segment. We mapped eight domains of interactions with nuclear proteins within the 840-base-pair region. The segment with maximum promoter activity contained two adjacent footprints, the occupation of which appeared to be mutually exclusive. One of them corresponded to an Sp1-factor-binding consensus site, the other one bound a factor with nuclear factor I activity. The single protected domain in the fragment harboring a repressor activity consisted of a GGA repeat resembling negative regulatory elements in other promoters. Three adjacent binding sites occupied an A+T-rich segment and contained ATTA motifs also found in the recognition elements of homeodomain proteins. These results show that negative and positive elements interact to regulate the tissue-specific patterns of expression of the NCAM gene and indicate that a factor related to nuclear factor I is involved in its transcriptional control.**

Cell-cell adhesion molecules (CAMs), the ligands or receptors that are involved in cell-cell contact formation, play important roles in controlling cell assembly and movement and in the maintenance of tissue architecture. On the basis of the timing and distribution of their expression, CAMs have been divided into two major classes: primary or general CAMs, which are expressed early in development and by a variety of cell types, and secondary or restricted CAMs, which are found on a limited number of differentiated tissues. General CAMs are believed to be critically involved in specifying cell patterning and movement in the embryo and thus the three-dimensional body plan of higher organisms (15, 16, 35, 43, 48).

The neural cell adhesion molecule (NCAM) is one of the most prevalent CAMs in vertebrates and belongs with the cadherins (47) to the general CAMs. It is encoded by a single gene, from which several mRNA species and proteins arise by alternative splicing and use of different poly(A) addition signals (3, 10, 44). NCAM expression is precisely regulated in terms of tissue distribution and developmental control. In the embryo, NCAM is expressed transiently in many structures on derivatives of different germ layers. This transient expression has sharply defined borders and occurs often in areas of embryonic induction (2, 9, 29, 30, 49). NCAM expression becomes more limited during further development. In the perinatal period, NCAM is found on neurones and glial cells of the central and peripheral nervous system, in skeletal and cardiac muscle, and in the kidney (8, 9, 17). In the nervous system, NCAM is expressed at the earliest stage of neural tube formation and persists throughout adult life. In the developing muscle, changes in NCAM protein expression are accompanied by similar changes in the steady-state levels of its mRNA, and denervation of adult skeletal muscle results in massive induction of NCAM gene transcripts (8). It is thus likely that NCAM expression is controlled in part at the level of transcription.

The mechanisms and in particular the nuclear factors that regulate the expression not only of NCAM but of any CAM are unknown at present. Detailed characterization of many eucaryotic genes has led to the concept that they are regulated at the level of transcriptional initiation by the combined action of multiple proteins that interact with short DNA sequences, usually located in the 5′-flanking regions of the corresponding genes. To begin to characterize the *cis*- and *trans*-acting elements involved in the transcriptional control of NCAM, we thus analyzed the upstream region of the mouse NCAM gene. We show that an 840-base-pair (bp) upstream sequence confers cell-type-specific expression onto a reporter gene. We also demonstrate that positive and negative regulatory elements are required to achieve cell-specific expression, and we have identified eight sites for nuclear factor binding. A more complete characterization of the factors that bind these sites should lead to the identification of transcriptional regulators that are important for morphogenesis.

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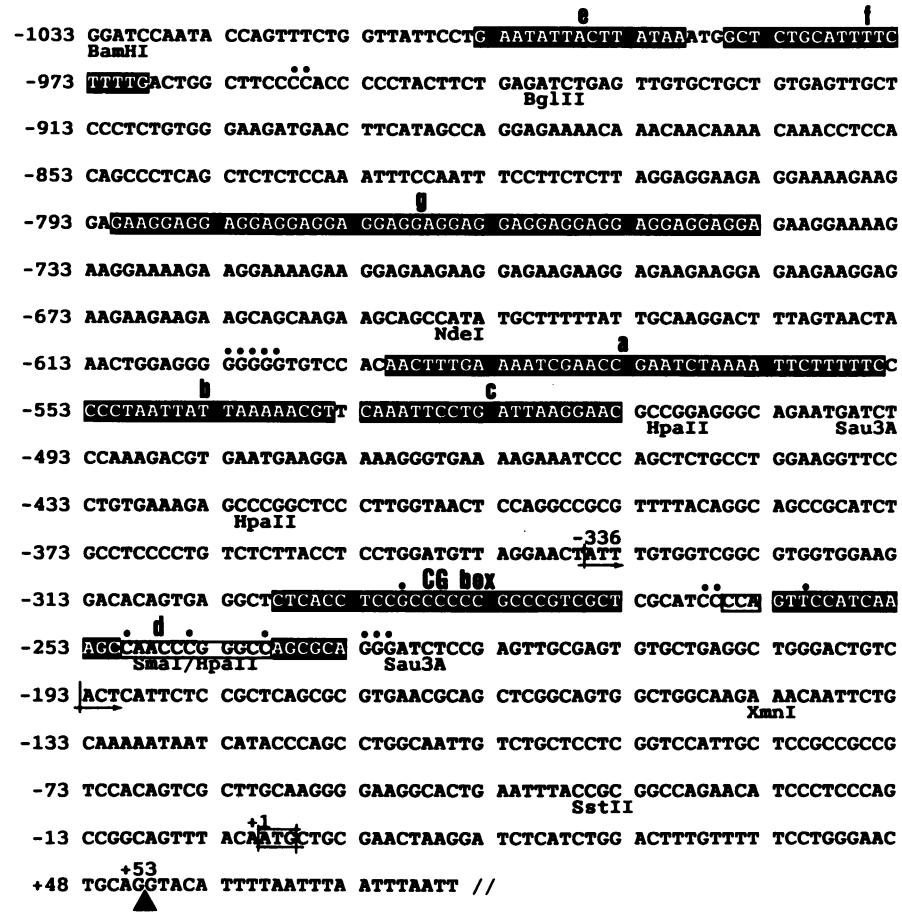



FIG. 1. Analysis of the 5′ end of the mouse NCAM gene and its flanking region. The 1,108-bp sequence contains the flanking sequence, the first exon, and the beginning of intron 1. All numbering is relative to the translational initiation site (boxed). The 5′ start site and the major 3′ start site of transcription are indicated by the arrows. The triangle marks the end of exon 1. The main restriction site landmarks are given. The segments that are protected from DNase I digestion by N2A cell nuclear proteins are indicated by the black boxes; the shaded boxes 5′ to and within domain d denote regions where DNase I protection could not be assessed due to failure of the enzyme to cleave in the absence of protein. DNase-hypersensitive sites are indicated by dots above the corresponding nucleotides.

## MATERIALS AND METHODS

**Cloning, sequencing, and determination of transcription initiation sites.** Clone lambda 21.1, which contained the first exon, part of the first intron, and 7 kbp of flanking sequence of the mouse NCAM gene, was isolated from a C57BL/6 library in EMBL3 (51) (a kind gift of Una Chen) by using a 5′ *Pst*I fragment of the NCAM cDNA M9 (4) as a probe. A *Bam*HI-*Sma*I fragment and the adjacent *Sma*I-*Hin*dIII fragment of the 21.1 insert that hybridized with the M9 probe were subcloned into M13 vectors and sequenced on both strands by using T7 DNA polymerase (Sequenase; U.S. Biochemical Corp.).

Primer extension analysis to determine the 5′ end of NCAM mRNA was done exactly as described previously (4). S1 nuclease protection assays were performed by the method of Ruppert et al. (42). Uniformly labeled antisense strands were prepared by using two different M13 templates, one starting at the *Xmn*I site (Fig. 1) of the NCAM gene (probe A) and the other starting at a *Pvu*II site 154 nucleotides (nt) within the chloramphenicol acetyl transferase (CAT) gene in BS2 (see below) (probe B).

**Plasmid construction and CAT assays.** Suitable restriction fragments of lambda 21.1 were subcloned in either one of the CAT vectors pBLCAT3 (32) and pconaCAT (28), kindly provided by G. Schütz and A. Israël, respectively. All fragments had an identical 3′ end, the *Sst*II site at position −37. The location of the fragments relative to the translation initiation codon in the NCAM gene and the resulting constructs are listed in Fig. 3. For construction of PPSS and PPSHS, respectively, a *Pst*I-*Pst*I fragment and a *Hin*dIII-*Pst*I fragment from lambda 21.1 (the *Pst*I site is within the first intron) were ligated to *Pst*I- and *Hin*dIII-*Pst*I-cut pBLCAT3. The 3′ *Sst*II-*Pst*I segment of the NCAM gene was then removed by cutting with *Sst*II and *Sal*I, followed by religation. NS2 was prepared from PPSS by cutting with *Nde*I and religation, thereby removing a 228-bp segment between the *Nde*I site in the NCAM gene and the single site in pBLCAT3. BS2 was constructed by cloning the *Bam*HI-*Sst*II fragment of the NCAM gene into *Bam*HI-*Hin*dIII-cut pconaCAT; SS2 was constructed by removing the *Bam*HI-*Sma*I segment from BS2. To obtain a CAT construct in which the segment containing footprints a through c was deleted, *Bal*31 exonuclease (New England BioLabs) was employed. Plasmid NS2 linearized with *Nde*I was treated with 1 U of *Bal* 31 under the conditions specified by the manufacturer, followed by the addition of *Nde*I linkers and religation. The boundaries of the deletion were determined by sequencing denatured plasmid templates. The *Nde*I-*Sst*II

fragment from one clone, in which 183 bp downstream of the *Nde*I site had been deleted, was cloned into the *Nde*I-*Sst*II sites of pBLCAT3 to make sure that the same vector sequences bordered the promoter region. The promoterless pBLCAT3 plasmid and plasmid PPAS, which contained the *Pst*I-*Sst*II fragment of lambda 21.1 in reverse orientation with respect to the CAT gene, were used as negative controls; RSVCAT (20) and pSRαCAT (46), respectively, were used as position controls. RSVCAT and pCH110, which harbors the bacterial β-galactosidase gene under control of the simian virus 40 early promoter, were obtained from A. Israël; pSRαCAT was obtained from N. Arai.

The mouse C1300 neuroblastoma-derived cell line N2A, which expresses high levels of NCAM protein and mRNA (18), and a subclone of Ltk$^-$ cells (33), which had been verified by RNase protection experiments to lack NCAM transcripts, were cultured in Dulbecco modified Eagle medium containing 10% fetal calf serum. Cultures of C2 myoblasts were grown as described by Moore et al. (36). All cells were transfected by the calcium phosphate coprecipitation method of Gorman (21), except that no dimethylsulfoxide or glycerol shock was applied. For each 10-cm dish, 5 to 10 μg of the test plasmid was coprecipitated with 10 μg of pCH110 DNA and the quantity of vector DNA required to obtain a total of 20 μg of DNA. For in vivo competition experiments, test CAT constructs (1 μg) were cotransfected with a 25-fold molar excess of either the −1033/−645, −645/−498, or −498/−245 fragment cloned in pGEM7Zf(+) (Promega Biotec Co.).

At 48 h after transfection, the cells were harvested and lysed, and CAT activity was measured (21). Care was taken that the rate of conversion of chloramphenicol was linear with time; the extracts were diluted accordingly. The [$^{14}$C]chloramphenicol concentration was 1.38 μCi/ml in most experiments. Enzyme activity was expressed as the percent conversion of chloramphenicol by 30 μl of extract in 1 h. These numbers were then corrected for variations in transfection efficiency by dividing by the β-galactosidase activity determined in the same extracts (28). Finally, the values were normalized with respect to the activity determined for pSRαCAT.

**Preparation of nuclear extracts and gel retardation experiments.** Nuclear extracts were prepared by the method of Piette et al. (40) with the following modifications: 0.5 M NaCl was used in the extraction step, and the $(NH_4)_2SO_4$ precipitation was replaced by a dialysis concentration step (dialysis for 1 h in 10 mM HEPES [*N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 8.0]–1 mM $MgCl_2$–150 mM KCl–25% glycerol–1 mM dithiothreitol, then for 1 h in the same buffer containing 75 mM KCl, and finally for 2 h in buffer with 30 mM KCl and 50% glycerol). Insoluble material was removed by centrifugation, and the supernatant (3 to 7 mg of protein per ml) was stored at −80°C.

Gel retardation assays were done by the method of Henninghausen and Lubon (23) with minor modifications. Suitable restriction fragments were 3′ end labeled with Klenow polymerase. Binding reactions (16 μl) contained $10^4$ cpm of probe fragment, 1 to 3 μg of poly(dI-dC) or poly(dA-dT) (Pharmacia Fine Chemicals), 10 mM HEPES (pH 8.0), 0.1 mM EDTA, 30 mM KCl, 1 mM $MgCl_2$, 0.1 mg of bovine serum albumin per ml, 2 mM of dithiothreitol, and 2 to 5 μg of nuclear extract protein. After incubation for 30 min at 4°C, protein-DNA complexes were separated from free DNA on 3.6% polyacrylamide gels made up in 12.4 mM Tris hydrochloride (pH 7.5)–6.6 mM sodium acetate–1 mM EDTA and run in the same buffer at 10 V/cm and 4 to 10°C.

Competition for factor binding was done using the following double-stranded synthetic oligonucleotides: (i) oligonucleotide b derived from the footprint b sequence (nucleotides [nt] −559 to −532), (ii) oligonucleotide b0 with the mutated sequence 5′ TTTTCCCCCTAACTATCAAAAACGTTCA 3′, (iii) a 26-mer oligonucleotide with the Hox1.3-protein-binding sequence (38), and (iv) an oligonucleotide 5′ TATTTTGGATTGAAGCCAATATG 3′, corresponding to the adenovirus type 2 nuclear factor I (NFI) binding site (26).

**DNase I footprinting.** Probes were 3′ end labeled as described above. The DNA was cut again with a suitable restriction enzyme; the fragment was labeled at one end and separated on an agarose gel. Footprinting assays were done as described by Hoeveler and Doerfler (24), except that the binding reaction was carried out in the buffer used for the gel shift experiments. Each reaction mix contained 5 to 35 μg of nuclear extract proteins, 5 to 10 μg of poly(dI-dC) or poly(dA-dT), and 1 to 2 ng of labeled probe ($2 \times 10^4$ to $4 \times 10^4$ cpm). When more than 10 μg of protein was used, a proteinase K digestion step (0.3 mg of enzyme per ml in 0.14% sodium dodecyl sulfate–35 mM EDTA at 42°C for 30 min) was added before phenol extraction. Maxam and Gilbert (34) sequence reactions were run alongside as size markers.

## RESULTS

**Analysis of the 5′ end of the NCAM gene.** We isolated a clone containing the 5′ end of the NCAM gene and sequenced a 1.2-kbp segment that extended for 1,033 nt upstream of the initiation codon (position +1) (Fig. 1). The sequence was identical to the published cDNA sequence (4) from position −161 to the consensus splice site at position +53. The transcription initiation sites were mapped by S1 nuclease protection experiments with two probes that extended up to the *Sma*I and *Bam*HI sites, respectively. Poly(A)$^+$ RNA from embryonic mouse brain mainly protected a 158-nt fragment of the shorter probe B (Fig. 2B). An intense band and a weaker band underneath indicated heterogeneity of transcription initiation in this region. Protection of a 158-nt fragment would place the major cap site at nt −193 of our sequence. Previously (4), the 5′ end of the NCAM mRNA was determined by primer extension. Its position could be mapped to precisely the same nucleotide by running a sequence ladder of the genomic clone alongside (data not shown). A distinct minor band observed in the S1 nuclease protection assays had the size (208 nt) expected for full protection up to the *Sma*I cloning site, indicating the existence of an additional upstream cap site that had not been observed by primer extension analysis. The longer probe A was used in S1 nuclease protection experiments to map this site in the genomic sequence. The strongest signal at 190 nt corresponded to a transcription start site at position −336 (Fig. 2A). The protected band of 43 nt expected for the major cap site could be observed when the hybridization was done under low-stringency conditions (data not shown). In theory, the colinearity between genomic and mRNA sequences could also be interrupted at nt −336 by the presence of an intron, but no consensus acceptor splice sites were found at or near this point. Using the smaller probe, the 158- and 208-nt fragments occurred in the same relative proportions whether the experiments were done with RNA from embryonic mouse brain, from C2 muscle or N2A neuroblastoma cells (Fig. 2B), or from adult mouse brain (data not shown), indicating that the two transcription start sites are used indiscriminately by different cell types and at different

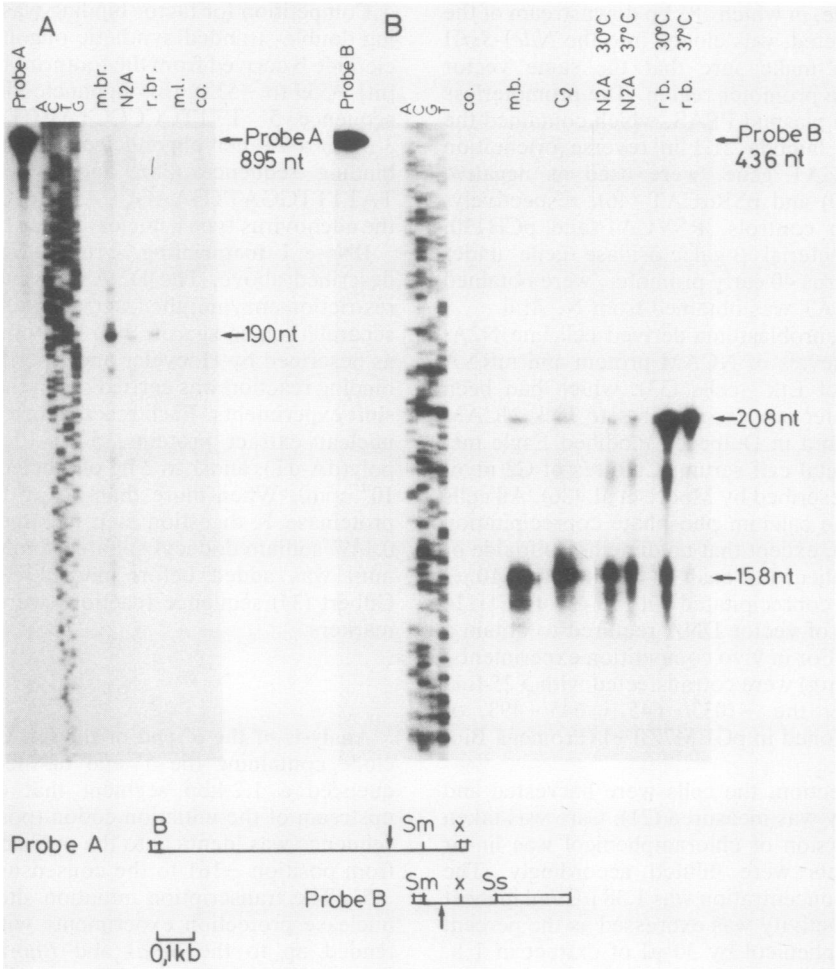



FIG. 2. Mapping of the 5′ termini of NCAM mRNA. The probes used in the S1 nuclease protection assays are diagrammed below. NCAM genomic sequences are indicated by the solid line, the open bars denote the vector sequences contained in the labeled probe, and the arrows indicate the sites for S1 nuclease cutting: B, *Bam*HI; Sm, *Sma*I; X, *Xmn*I; Ss, *Sst*II. Panels A and B show the fragments of probes A and B, respectively, that were protected from S1 nuclease digestion by total RNA from embryonic day-19 mouse brain (m.br. or m.b.), N2A cells, adult rat brain (r.br. or r.b.), and C2 cells. RNA from mouse liver (m.l.), in which NCAM is not expressed, and tRNA (co.) were used as controls. In some cases S1 nuclease digestion was done at 30°C instead of the usual 37°C to see whether the bands underneath the 158-nt fragment were due to S1 nuclease nibbling from the end of this fragment at the higher temperature; the additional bands were still present at 30°C and became even stronger in the rat brain sample. kb, Kilobase pair.

developmental stages. In the presence of rat brain RNA, the 208-nt band was relatively more intense. We conclude from these experiments that in rodents, transcription starts mainly at adenosines at position −336 and −193 relative to the initiation ATG. Both are part of (C/T)A(C/T) trinucleotides, as in the majority of eucaryotic genes (7).

The sequence (Fig. 1) contained a TATA box (around nt −994), which was, however, too far from the transcriptional start sites to function as a canonical promoter element (6). Other conspicuous features were an Sp1-factor-binding consensus sequence or CG box (19), a segment in which the coding strand was entirely devoid of pyrimidines (between nt −813 and −671), and an A+T-rich stretch from positions −591 to −519.

**Functional analysis reveals the presence of positive and negative regulatory elements.** To delineate the sequences essential for transcription of the mouse NCAM gene, we fused various portions of the 5′-flanking region to the bacterial CAT gene as a heterologous reporter gene. The constructs (Fig. 3) were transfected into NCAM-expressing N2A neuroblastoma cells and into NCAM-negative L cells. Plasmids that contained the CAT gene transcribed from the strong SRα (pSRαCAT [46]) and RSV (RSVCAT [21]) promoters were used as positive controls. CAT activities were corrected for variations in transfection efficiencies, by cotransfecting the pCH110 plasmid (28) and measuring β-galactosidase activity in the same extracts, and then normalized with respect to those obtained with pSRαCAT.

Transient transfections of the longest construct (PPSS) into N2A cells produced CAT activities that were similar to those seen with RSVCAT and around 17-fold higher than those in the negative control provided by PPAS, which contained the same fragment in antisense orientation (Fig. 3). Shortening the upstream sequences from positions −4680 to −1030 did not produce major changes in CAT activity. However, cells transfected with the next-shorter construct, BgS2, gave twofold lower activities. Deletion to position −645 (NS2) increased the promoter activity fourfold; dele-

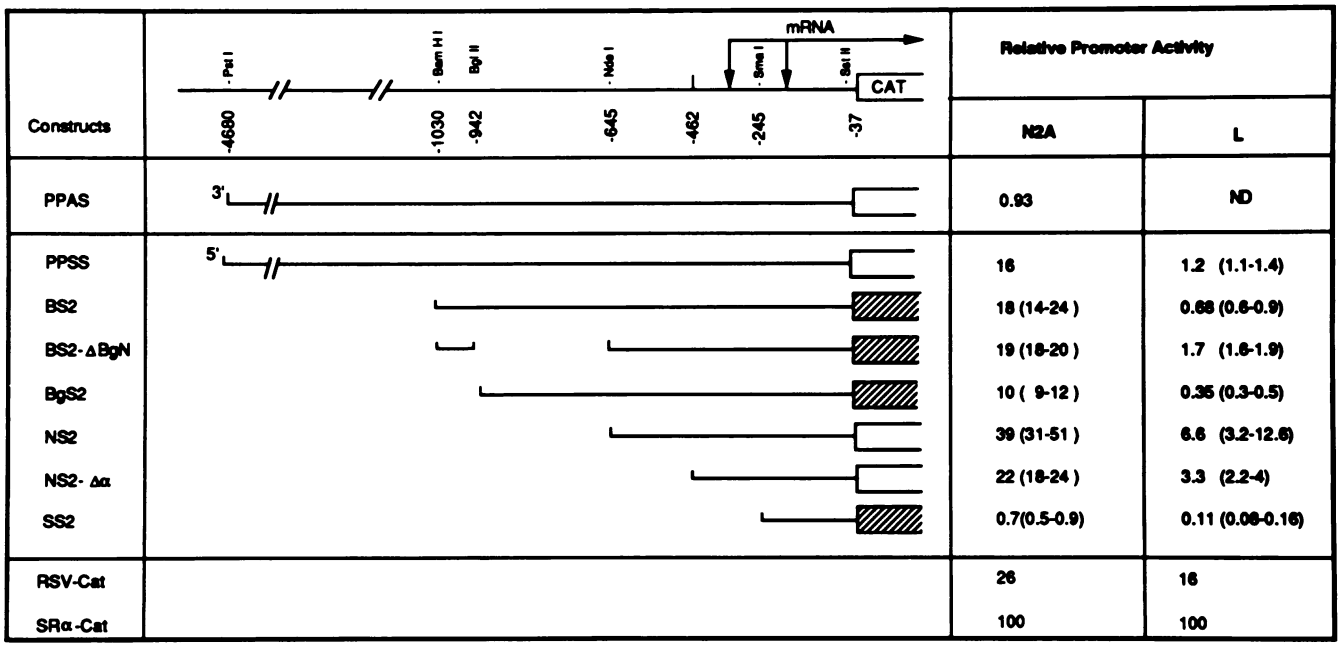



FIG. 3. Promoter activities of different fragments from the 5′ end of the NCAM gene. The NCAM-*cat* fusion genes used are diagrammed at the left of the figure. They are aligned with the longest NCAM sequence numbered relative to the translational initiation site. In PPAS, the NCAM genomic sequence is present in reverse orientation. The promoterless *cat* genes from pBLCAT3 and pconaCAT are represented by open and hatched boxes, respectively. *cat* genes driven by the Rous sarcoma virus (RSV-Cat) and a composite simian virus 40–human T-cell leukemia virus type 1 promoter (SRα-Cat) were used as positive controls. Equimolar amounts of each construct were transfected into N2A and L cells together with plasmid pCH110 containing the β-galactosidase gene driven by the simian virus 40 early promoter. CAT activities were corrected for variations in transfection efficiency by normalizing them with respect to β-galactosidase expression and are expressed as a percentage of that obtained for SRα-Cat in the same experiment (right). The values shown represent the means and the range (in brackets) for 6 to 12 independent experiments, except for RSV-Cat and PPSS (in N2A cells), for which the average is given for duplicate transfections within the same series of experiments. The relative promoter activities of the different deletion constructs measured after transfection into the same cell line are strictly comparable. However, comparison between N2A and L cells depends on the assumption that the SRα promoter is equally efficient in both cell types; this seems highly likely in view of the composite nature of this promoter and the results obtained by Takebe et al. (46) with different cell lines. ND, Not determined.

tion mutant NS2-Δα (starting at position −462) produced a twofold lower expression of CAT activity than did NS2. Further deletion down to position −245, which cuts between the two transcriptional start sites, reduced the promoter activity to background levels. An internal deletion in BS2 that removed the segment at nt −942 to −645 yielded a CAT activity similar to that in BS2. Although these variations in CAT expression were relatively small, they were reproducibly observed in many different experiments, and qualitatively similar changes were also seen after transfection into L or C2 cells. To verify that the transfected NCAM promoter was correctly initiated, S1 nuclease protection experiments were done with RNA from N2A cells transfected with NS2, NS2-Δα, and BS2. The protected bands corresponded in size to initiation at or near positions −193 and −336 and were present in the same relative amounts as the bands protected by the RNA produced from the endogenous gene and revealed in the same gel (data not shown).

After transfection into L cells, NS2 produced relative CAT activities that were sixfold lower than in N2A cells, but still clearly over background and only two- to threefold less than those seen with RSVCAT (Fig. 3). However, when the sequences up to the *Bgl*II site and beyond were present in the constructs, the CAT activities dropped to very low levels, comparable to those found with the antisense construct PPAS and with the promoterless CAT gene (data not shown), as expected for a cell line in which the NCAM gene is not transcribed. A stronger negative effect of the segment at nt −942 to −645 in L cells as compared with that in N2A cells was also shown by the clear increase in CAT expression resulting from its deletion.

In another series of experiments, several of the NCAM promoter constructs were transfected into C2 myoblasts, which express moderate amounts of NCAM mRNA (36). CAT activities were normalized relative to those produced by NS2, the fragment that showed the strongest promoter activity in the previous experiments. C2 myoblasts were transfected with 10 μg of four CAT fusion genes. The CAT activities, normalized with respect to β-galactosidase activity provided by the contransfected pCH110 plasmid, were as follows: PPSHS, 26%; BS2, 20%; NS2, 100%; SS2, <0.2%. Also in C2 cells, NS2 produced the highest levels of CAT activity, and inclusion of longer upstream sequences resulted in a severalfold drop in CAT expression.

Based on these data, the 5′-flanking region of the NCAM gene may be divided as follows: the upstream region, which appears to be devoid of regulatory elements in the cell lines tested; the segment at nt −1030 to −645, which harbors both positive and negative regulatory elements; the region between nt −645 and −462, the deletion of which results in a twofold drop in promoter activity; and the region at nt −462 and −245, which confers strong promoter activity to the CAT gene. In N2A cells, but not in L cells, the negative effect of the segment at nt −1030 to −645 was partially compensated by a positive regulatory element located between nt −1030 and −942.

TABLE 1. In vivo competition analysis by cotransfection[a]

| Reporter plasmid | Competitor plasmid | CAT activity |
|---|---|---|
| BS2 | pGem5Z | 12.8 |
| BS2 | −1030/−645 | 6.6 |
| NS2 | pGem5Z | 39.1 |
| NS2 | −645/−245 | 14.5 |
| NS2 | −645/−462 | 43.8 |
| NS2 | −498/−245 | 13.7 |

[a] N2A cells were cotransfected with a mixture of three components: (i) 1 μg of the CAT fusion gene as the reporter plasmid, either BS2 or NS2 (Fig. 3), (ii) a 25-fold molar excess of competitor DNA or of pGem5Zf+ DNA as a control, and (iii) 5 μg of pCH110 as a control for transfection efficiency. The competitor DNA consisted of the test fragment cloned into pGem5Z. Each fragment is designated by its position in the 5′-flanking region of the NCAM gene. CAT activities, determined as percent conversion into acetylated forms, were corrected by the β-galactosidase activities measured in the same extracts. The mean values are given for two experiments; maximum variation between duplicates was 7.5%.

**Competition experiments and mobility shift assays provide evidence for specific factor binding.** To test whether the regulatory effects observed in the functional assays were mediated by cellular *trans*-acting factors, we performed a series of in vivo competition experiments. Reporter plasmids, either BS2 or NS2, were cotransfected into N2A cells with competitor DNA that contained different fragments of the region at nt −1030 to −37. Cotransfection of a 25-fold excess of plasmid −498/−245 with NS2 as the reporter plasmid reduced CAT activity to 35% of the control activity provided by cotransfection of vector sequences only (Table 1). Similarly, with BS2 as the reporter plasmid and plasmid −1033/−645 as the competitor, an approximately twofold drop in CAT activity was observed. By contrast, the −645/−498 fragment did not impair the levels of CAT expression generated by NS2. These results show that *trans*-acting factors binding to the segments at nt −1030 to −645 and nt −498 to −245 are responsible for the positive regulatory effects that have been localized in these two regions of the NCAM gene.

One reason for our failure to provide evidence for the binding of factors to the −645/−498 segment by in vivo competition may be that their concentration is not limiting in N2A cells. We therefore sought to demonstrate the specific binding of nuclear proteins to this region by mobility shift experiments. When the end-labeled −645/−498 *Nde*I-*Sau*3AI fragment was incubated with nuclear extracts from N2A cells in the presence of poly(dI-dC) as a nonspecific competitor, two major retarded bands were observed (marked C1 and C2 in Fig. 4A). This binding was specific, since addition of a fourfold excess of the unlabeled fragment, but not a similar amount of vector DNA, displaced both complexes. The protein-DNA complexes seen in these assays were sensitive to poly(dA-dT) added as a nonspecific competitor (results not shown), suggesting that they involve the A+T-rich segment in the 3′ part of this region.

Within the A+T-rich segment of fragment −645/−498, three footprints (a, b, and c in Fig. 1) were revealed by the DNase I protection experiments described below. Footprint b contained a centrally located 5′ TAATTATTAAA 3′ motif that resembled binding sites for the *Drosophila* engrailed and fushi tarazu and the mouse Hox1.3 homeodomain proteins (11, 38). To show the importance of the ATTA motif for factor binding, competition experiments with double-stranded oligonucleotides were carried out. A domain b oligonucleotide completely displaced complex C2, but not C1, at the lowest concentration tested, whereas oligonucleotide b0, in which the ATTATT core motif had been mutated to ACTATC, was very inefficient as a competitor (Fig. 4B). A 10-nt stretch within oligonucleotide b matches precisely the consensus recognition sequence for Hox1.3 protein (38). We thus considered the possibility that formation of C2 may involve Hox1.3 or a closely related protein. Although a 26-mer derived from the authentic Hox1.3-binding site performed somewhat better than oligonucleotide b0, it was a much less efficient competitor than oligonucleotide b. The same sequence has been shown to compete readily for Hox1.3 binding (38). Thus, the results, although underscor-

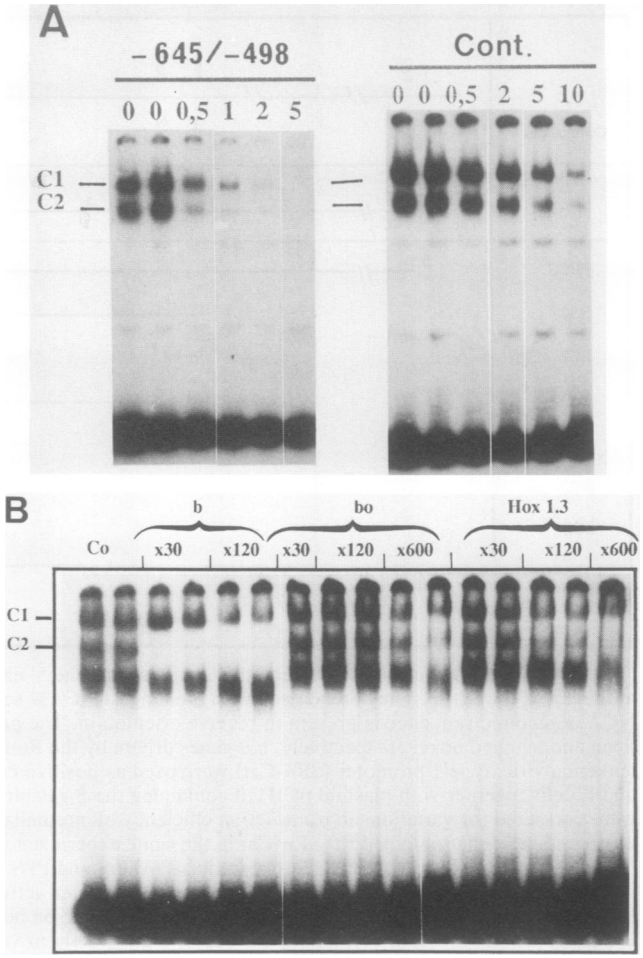



FIG. 4. Specific binding of nuclear factors to the −645/−498 fragment. Samples of approximately 0.5 ng (A) and 0.8 ng (B) of end-labeled −645/−498 (*Nde*I-*Sau*3AI) fragment were incubated with 3.5 μg (A) or 2.5 μg (B) of N2A cell nuclear proteins, and protein-DNA complexes were revealed by gel retardation assays. (A) The binding reactions were done in the presence of competitor DNA (0 to 10 ng, as indicated) with either the unlabeled −645/−498 fragment or vector DNA as a control (Cont.). (B) Synthetic double-stranded oligonucleotides were used as competitors in a 30- to 600-fold molar excess as indicated. Oligonucleotide b is derived from the footprint b sequence. In oligonucleotide b0 the ATTATTA core motif has been changed to ACTATC. Oligonucleotide Hox1.3 is a 26-mer that has been shown to bind the Hox1.3 protein (38). The position of complexes C1 and C2 is indicated. In panel A, longer exposure of the gels revealed additional, more rapidly migrating bands; they are enhanced in panel B, since a lower amount of poly(dI-dC) was used (1 instead of 3 μg). These additional bands cannot be competed for by the unlabeled fragment and probably represent nonspecific binding.

ing the importance of the ATTA motif for factor binding, do not support the possibility that the N2A protein that recognizes this sequence contains a Hox1.3 homeobox as DNA binding domain. However, it remains formally possible that a Hox1.3-related protein present in N2A cells binds the NCAM promoter site with higher affinity than the reference site, perhaps due to protein-protein interactions. The factor involved in the formation of complex C1 seems to bind outside domain b.

**DNase I footprinting reveals multiple interactions with nuclear factors.** To define the sequences involved in the binding of nuclear proteins, we performed DNase I protection experiments using as probes 3'-end-labeled fragments that were incubated with nuclear extracts from N2A or L cells. Typical examples are shown in Fig. 5; the data from these and a number of similar experiments are summarized in Fig. 1.

In the fragment at positions −645 to −498, the deletion of which reduced promoter activity by half, the A+T-rich region between nt −592 and −514 was protected from DNase I digestion by N2A-cell nuclear extracts. A series of enhanced bands marked the G stretch upstream of it (Fig. 5C and D). The protection was interrupted at two sites, leading to the subdivision of the region into three footprinted domains, labeled a, b, and c. These sequences were only weakly protected by equal amounts of nuclear extracts from L cells. However, binding of L-cell proteins to domain a was indicated by the presence of enhanced bands at its borders.

Two protected domains were mapped in the proximal fragment, which contributed most of the promoter activity (Fig. 5A and B). One, designated d, was only protected by N2A-cell extracts. Hence, the proteins that bind to it may be responsible for the higher promoter activity of this region in N2A cells. The occurrence of discrete hypersensitive sites between positions −250 and −240 led to a further division into two subdomains. Protection between the hypersensitive sites could not be assessed due to the lack of DNase cuts in the absence of protein. Strong hypersensitive sites were also revealed at both borders of domain d. Several sequence elements in domain d resemble the recognition sites for proteins of the NFI family (26, 37, 39, 41): 5' AGCCAA 3' and 5' GCCA 3' sequences on the upper strand and a 5' TGGA 3' sequence on the lower strand; more extended similarities were seen with the NFI-binding sites from adenovirus type 2 (nt −262 to −248), the albumin promoter (nt −260 to −248), and the SL3-3 virus enhancer (nt −246 to −235). We therefore tested whether factor binding could be blocked by adding an excess of an authentic NFI-binding sequence. Indeed, an oligonucleotide containing the adenovirus type 2 NFI recognition site (26) competed for the binding of N2A proteins to domain d (Fig. 5A). Surprisingly, the second domain (CG box), which contained the potential SpI-factor-binding site, was only protected when the d domain was not occupied. In the presence of poly(dA-dT), which impaired factor binding to site d, the CG box was protected by both N2A- and L-cell extracts (Fig. 5B), as expected from the ubiquitous occurrence of this protein (5); in the presence of poly(dI-dC), this footprint could be revealed in L cells, where domain d is not footprinted, and in N2A cells only when binding to domain d was blocked by the competing oligonucleotide (Fig. 5A).

Three potential binding sites for nuclear proteins occurred in the upstream region, which exerted both positive and negative effects on the expression of CAT activity. Two sites were mapped near the 5' end of the sequence shown in Fig. 1. The distal site, designated e, contained the TATA box. It was much better protected by N2A-cell extracts than by L-cell extracts. The adjacent footprint f was only weakly protected, more so by L-cell nuclear proteins. Its proximal border was marked by DNase I-hypersensitive sites (Fig. 5E). A third site, designated g, occupied a GGA repeat in the region at nt −942 to −645 shown to harbor a repressor activity (Fig. 5F). The protein(s) that filled this site appeared to be more abundant in L cells, consistent with our observation of a stronger negative effect in this cell type.

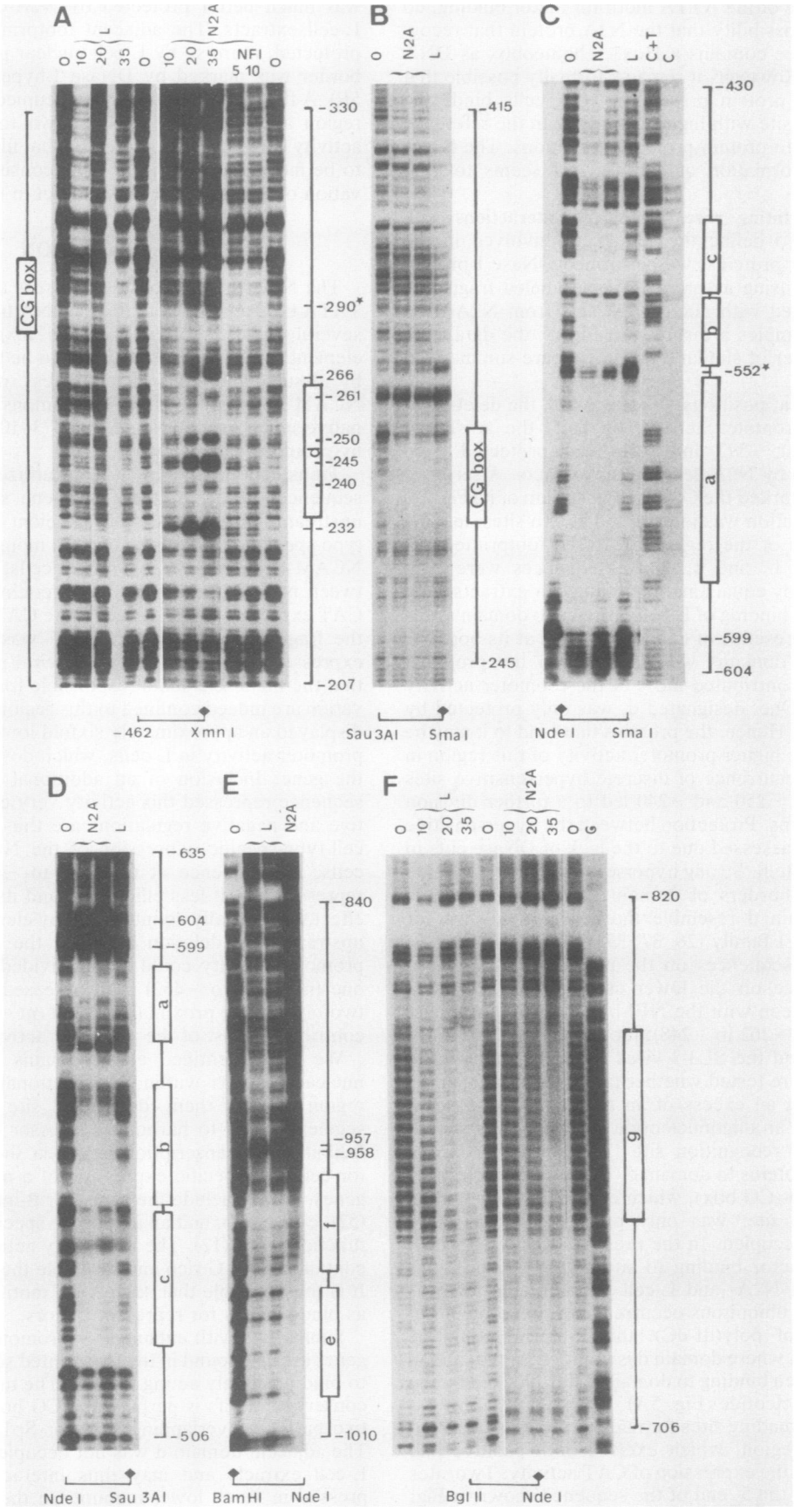
## DISCUSSION

The NCAM gene does not possess a typically positioned TATA box, and the initiation of RNA transcription occurs at several sites, as often observed in genes that lack this element (14). These sites appear to be used indiscriminately by tissues and cells that express differently processed NCAM transcripts (8, 18, 36), demonstrating that the alternative splicing of the NCAM gene (3, 10, 50) is not controlled by promoter choice.

Transfection experiments that utilize different 5'-flanking sequences linked to the CAT gene show that 840 bp of upstream sequence contain sufficient information for cell-type-specific expression of the mouse NCAM gene. In NCAM-expressing N2A and C2 cells, sequences lying between nt −645 and −245 were required for high levels of CAT expression. In N2A cells, the CAT activity directed by the fragment at nt −645 to −37 was comparable to that expressed from two strong retroviral promoters, indicating that the major elements responsible for transcriptional activation are indeed confined to this region. The same fragment displayed an approximately sixfold lower but still significant promoter activity in L cells, which do not normally express the gene. Inclusion of an additional 300 bp of upstream sequence repressed this activity very efficiently. Both positive and negative regulations are thus required to achieve cell-type-specific expression of the NCAM gene. In N2A cells, the sequence at nt −942 to −645 acted also as a repressor, albeit less efficiently, and its negative regulatory effect was partially counteracted by elements located further upstream. By deletion analysis, the region of maximum promoter activity could be subdivided into two segments, one (nt −645 to −462) that increased promoter efficiency twofold and the proximal segment (nt −462 to −245), which contributed most of the promoter activity.

We have identified eight domains of interactions with nuclear proteins within the functionally important 840-bp region. One of them, designated site g, is located in the segment found to harbor a repressor or silencer element. Inhibition of transcription has been shown to be important for cell-type-specific expression of a number of eucaryotic genes. These include the genes for β-interferon (20), HSP70 (52), c-*myc* (27), and an adipocyte-specific gene of unknown function (aP2) (12). The negatively acting *cis* elements they contain are A+G rich and resemble the domain g sequence. It is thus possible that such A+G motifs play a general role as binding sites for repressor factors.

Similarities with enhancer or promoter elements of other genes are also found in the footprinted sequences that appear to bind positively acting factors. The most obvious of these conserved motifs is perhaps the CG box, which consists of two partially overlapping sites for Sp1 factor binding (19). The adjacent domain d was not occupied in the presence of L-cell extracts and may thus interact with proteins expressed in much lower amounts in these cells. It contains motifs that resemble the recognition sites for proteins of the


A
B
C
D
E
F
CG box
-330
-290*
-266
-261
-250
-245
-240
-232
-207
-462
Xmn I
-415
-245
Sau3AI
Sma I
-430
-552*
-599
-604
Nde I
Sma I
-635
-604
-599
-506
Nde I
Sau 3AI
-840
-957
-958
-1010
BamHI
Nde I
-820
-706
Bgl II
Nde I
0
10
20
35
L
N2A
NFI
5 10
C+T
C
G

NFI family (26, 39, 41). An oligonucleotide containing the Ad2 NFI-binding site competed for binding to domain d, supporting our proposal that this domain is recognized by a protein(s) with NFI activity. Surprisingly, occupation of domain d interfered with the binding of nuclear protein, presumably Sp1, to the CG box, since the latter was only protected when the NFI site was not occupied. Hence, binding of SpI and NFI to their respective sites may be mutually exclusive, and the higher promoter activity of the proximal segment in N2A cells may be due to the presence of NFI activity in these cells.

Footprints a, b, and c are clustered in a segment that contains four partially overlapping ATTA motifs on both strands. We demonstrated the importance of this motif by competition experiments, showing that a mutated oligonucleotide competes very inefficiently for complex formation. All homeodomain proteins of the *Antennepedia* (*antp*) class studied, but also several proteins with more distantly related homeoboxes, recognize sequences sharing the ATTA motif (11, 38, 45). Furthermore, a 10-nt stretch in domain b conforms to the consensus sequence for binding of the mouse homeodomain protein Hox1.3 (38). One of the factors that modulate the transcriptional activity of the NCAM promoter may thus belong to the family of gene regulators that contain an *antp*-like DNA-binding domain and are supposed to play important roles in determining pattern formation and cell fate during development (1, 13). However, the authentic Hox1.3 sequence was much less efficient than the cognate sequence in competing for factor binding. One should thus consider the possibility that proteins with homeodomains of a different type but similar target specificity (31) are involved in regulating NCAM promoter activity. One such class comprises the Oct-1, Oct-2, and Pit-1/GHF-1 proteins (for a review, see reference 31) as well as recently identified additional proteins, which are all expressed in the developing nervous system in a region- and developmental-stage-specific manner (22).

Deletion of domains a to c resulted only in a twofold drop in promoter activity in N2A cells. It remains to be seen whether this segment exerts a greater influence in other cell types. On the other hand, a twofold modulation in transcription efficiency may be functionally important, since small changes in NCAM concentration have been reported to result in overproportionally large effects on NCAM-mediated adhesion (25).

The results presented here define the upstream region of the NCAM gene that is responsible for high promoter activity in NCAM-expressing cells and low or negligible activity in nonexpressing cells and delineate the sequences involved in factor binding. Future experiments will be required to determine whether the same or different sequence elements control NCAM expression in other cell types and at different stages of development. The NCAM promoter appears to have a rather unique organization, with the juxtaposition of Sp1- and NFI-factor-binding sites, the occupation of which appears to be mutually exclusive, an extended A+T-rich footprinted domain that shares sequence similarity with the recognition sites of homeodomain proteins, and a negative control element consisting of a GGA repeat. We have initiated studies to molecularly define the transcription factors involved in its regulation, some of which may turn out to play a general role in directing vertebrate development.

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FIG. 5. DNase I footprint analysis of the upstream region of the NCAM gene. The end-labeled fragments used are indicated below each panel, the labeled end marked with a diamond. Their positions in the sequence are as follows: (A) nt −462 to −143, (B) nt −498 to −245, (C) nt −645 to −245, (D) nt −645 to −498, (E) nt −1033 to −645, (F) nt −941 to −645. The different probes were incubated in the absence of protein (0) or in the presence of nuclear extracts from N2A or L cells as indicated. The approximate extent of each protected domain is indicated by an open box. Their boundaries were determined by running Maxam-Gilbert sequencing reactions of the same fragments alongside the DNase I digests, examples of which are shown in panels C and F (lanes marked C, C+T, and G). DNase I-hypersensitive sites are marked alongside; the sites marked with an asterisk occurred only in the presence of L-cell nuclear proteins (nt −552) or only when the CG box was not protected (nt −290). In panels A and F, increasing amounts of extract protein were used, and they are indicated above each lane in micrograms; the amounts of protein in the other panels were as follows: (B) 10 μg; (C) 15 μg, (D) 5 μg, (E) 20 μg. In panel A, competition experiments are shown using 5 and 10 ng of a double-stranded oligonucleotide corresponding to the adenovirus type 2 NFI factor-binding site (lanes marked NFI 5 and NFI 10) in the presence of 35 μg of protein. In other experiments (data not shown), 10 ng of an irrelevant oligonucleotide did not suppress footprint d. Poly(dI-dC) was used as a nonspecific competitor in all experiments but the ones in panels B and F, in which it was replaced by poly(dA-dT). Poly(dA-dT) blocked factor binding to domain d in panel B. This effect was also observed when the probe from panel A was used; similarly, footprints a to c were suppressed by poly(dA-dT) (data not shown). Note that the CG box is revealed only when domain d is not occupied (panel A).

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