Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 1892–1896, March 1996
Neurobiology

Embryonic expression patterns of the neural cell adhesion molecule gene are regulated by homeodomain binding sites

(homeobox/promoter/cell adhesion/transgenic mice/dorsoventral axis/spinal cord)

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ABSTRACT **During development of the vertebrate nervous system, the neural cell adhesion molecule (N-CAM) is expressed in a defined spatiotemporal pattern. We have proposed that the expression of N-CAM is controlled, in part, by proteins encoded by homeobox genes. This hypothesis has been supported by previous *in vitro* experiments showing that products of homeobox genes can both bind to and transactivate the N-CAM promoter via two homeodomain binding sites, HBS-I and HBS-II. We have now tested the hypothesis that the N-CAM gene is a target of homeodomain proteins *in vivo* by using transgenic mice containing native and mutated N-CAM promoter constructs linked to a β-galactosidase reporter gene. Segments of the 5′ flanking region of the mouse N-CAM gene were sufficient to direct expression of the reporter gene in the central nervous system in a pattern consistent with that of the endogenous N-CAM gene. For example, at embryonic day (E) 11, β-galactosidase staining was found in postmitotic neurons in dorsolateral and ventrolateral regions of the spinal cord; at E14.5, staining was seen in these neurons throughout the spinal cord. In contrast, mice carrying an N-CAM promoter–reporter construct with mutations in both homeodomain binding sites (HBS-I and HBS-II) showed altered expression patterns in the spinal cord. At E11, β-galactosidase expression was seen in the ventrolateral spinal cord, but was absent in the dorsolateral areas, and at E14.5, β-galactosidase expression was no longer detected in any cells of the cord. Homeodomain binding sites found in the N-CAM promoter thus appear to be important in determining specific expression patterns of N-CAM along the dorsoventral axis in the developing spinal cord. These experiments suggest that the N-CAM gene is an *in vivo* target of homeobox gene products in vertebrates.**

N-CAM, the neural cell adhesion molecule, mediates cell–cell adhesion in the nervous system as well as in a variety of embryonic tissues (1–3) and has a precise and heritable pattern of place-dependent expression during development (4). It occurs in the proliferating neuroepithelium at an early stage of neural tube formation. At a later stage, N-CAM is expressed by postmitotic neurons, concomitant with the differentiation of neuroblasts along the anteroposterior axis (4–6). Perturbation experiments have shown that blockade of either N-CAM binding or expression leads to alterations in morphogenetic patterning (7–9). Furthermore, in the adult, disruption of the neuromuscular junction by peripheral nerve lesions results in characteristic alterations of N-CAM expression in both the innervating neurons and the denervated muscle (10). These findings indicate that elaborate regulatory mechanisms must govern place-dependent N-CAM expression at specific times of development and regeneration, thereby affecting the guidance of tissue pattern via cell–cell adhesion.

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The dynamic regulation of the expression pattern of N-CAM is likely to involve transcriptional control of the N-CAM gene promoter. Among the candidate genes mediating this control are *Hox* genes. These genes are known to specify transcription factors that are important in establishing specific morphological patterns during embryogenesis. *Hox* genes show place-dependent patterns of expression that overlap those of N-CAM in the hindbrain and spinal cord (11, 12).

To date, few genes have been identified as downstream targets for Hox gene products. We have previously suggested that genes for cell adhesion molecules (CAMs) are direct downstream target genes of *Hox* genes (13). Consistent with this hypothesis, DNA sequences that resemble binding sites for homeodomain transcription factors have been identified in the proximal region of the N-CAM promoter (14–16). We have studied a variety of CAM gene promoters and enhancers to determine whether these elements actually are binding sites for homeobox gene products (15–20). After isolating the promoter for the mouse N-CAM gene, we used promoter activation and binding assays to show that Hox gene products can regulate N-CAM gene expression *in vitro*. Subsequently, we identified two homeodomain binding sites (HBS-I and HBS-II) in the proximal N-CAM promoter that mediated binding and activation by HoxB9 (Hox2.5) and HoxC6 (Hox3.3) (15, 16). Homeodomain proteins Phox2 and Cux (21) have also been shown to regulate N-CAM expression *in vitro*.

The hypothesis that HBS sequences are important in regulating N-CAM gene expression requires corroboration *in vivo*. In the present report, we describe the production of transgenic mice containing the N-CAM promoter linked to a *lacZ* reporter gene. The reporter gene directed by the wild-type promoter had a pattern of expression in the central nervous system that overlapped the known expression pattern of N-CAM. Transgenic mice containing an N-CAM promoter with mutations in both homeodomain binding sites (HBS-I and HBS-II) showed distinct spatiotemporal alterations in *lacZ* expression patterns in the spinal cord. This initial *in vivo* study, taken together with previous work, supports the proposal that N-CAM expression during development is under the control of homeodomain proteins.

MATERIALS AND METHODS

Construction of N-CAM/*lacZ* Gene Constructs and Generation of Transgenic Mice. The N-CAM/*lacZ* genes were constructed as follows: A 6.5-kb *Kpn* I–*Sac* II restriction fragment from the 5′ flanking region of the mouse N-CAM gene was excised from the pEC9.7 plasmid (15) (Fig. 1). The promoter fragment was cloned upstream of the *Escherichia coli lacZ* gene by insertion into the *Kpn* I and *Sma* I sites of a modified version of pnLacF vector (from J. Peschon, Univer-

Abbreviations: CAM, cell adhesion molecule; HBS, homeodomain binding site; CAT, chloramphenicol acetyltransferase; N-CAM, the neural cell adhesion molecule; E*X*, embryonic day *X*; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

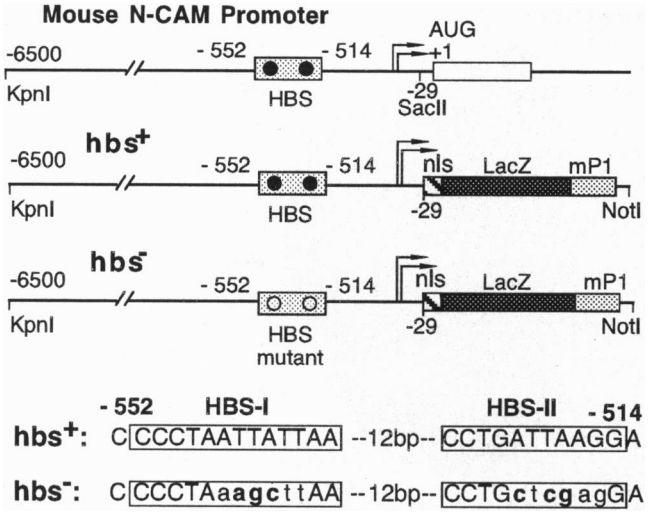

FIG. 1. Diagram of N-CAM promoter/*lacZ* gene constructs. (*Top*) Schematic diagram of the 5′ flanking region of the mouse N-CAM gene and of the hbs$^+$ and hbs$^-$ constructs. The restriction sites used to make the hbs$^+$ and hbs$^-$ constructs are shown. The N-CAM translation initiation site is designated as +1. Arrows represent the transcription initiation sites of the mouse N-CAM gene. The boxes between −552 and −514 represent the region that contains the wild-type (solid circles) and the mutant (open circles) HBS-I and HBS-II sequences. The nuclear localization signal from the simian virus 40 large T antigen (nls), the coding sequence for the *lacZ* gene (dark stippled box), and the 3′ portion of the mouse protamine 1 structural gene (light stippled box designated mP1) in the transgene are also shown. (*Bottom*) Sequences of HBS-I and HBS-II are boxed, and base-pair substitutions in the TAAT or ATTA motifs within HBS-I and HBS-II are indicated in lowercase letters and boldface type.

sity of Washington, Seattle). The pnLacF vector contains a nuclear localization signal sequence from the simian virus 40 T antigen followed by the *E. coli lacZ* gene and the 3′ portion of the mouse protamine structural gene to provide an intron and polyadenylylation signals.

Alterations in the HBS sequences of the N-CAM promoter (15) were made by site-directed mutagenesis (Mutagene kit, Bio-Rad). A 65-nt segment of the N-CAM promoter containing the homeodomain binding sites was modified to replace the TAAT and ATTA motifs in HBS-I and HBS-II with *Hin*dIII and *Xho* I linkers, respectively (Fig. 1). Each of the mutations was identified by restriction digestion and confirmed by DNA sequencing.

The wild-type or mutant N-CAM/*lacZ* gene constructs shown in Fig. 1 were excised from the vector by digestion with *Kpn* I and *Not* I and introduced into the RC6 mouse genome by standard oocyte microinjection techniques (22). F_0 embryos were taken at E11 and subjected to histochemical analysis. To identify transgenic mice, progeny were screened by Southern blot hybridization or PCR analysis (23). For Southern analyses, genomic DNA isolated from mouse tails was digested with *Eco*RI and probed with a ^{32}P-labeled 3.6-kb *Kpn* I–*Hin*dIII fragment containing the *lacZ* gene from the pnLacF plasmid. Transgene-positive samples were identified by the presence of a 3.0-kb band in autoradiographs. For PCR analysis, the 5′ primer was complementary to the −168 to −189 region of the N-CAM promoter, and the 3′ primer was the M13 (−40) sequencing primer located near the polylinker region of the pnLacF vector. The expected PCR product was 305 bp in length and was visualized on an ethidium bromide-stained agarose gel after electrophoresis. Transgenic animals were bred with either negative littermates or C57BL6 mice to establish individual lines and to obtain F_1 and F_2 transgenic embryos.

Histochemical Analysis of Mouse Embryos for *lacZ* Expression. F_0 or transgenic embryos were collected from pregnant females at embryonic day (E) 11 or E14.5, considering 12 noon of the day on which a copulatory plug was found as E0.5. The embryos were rinsed with phosphate-buffered saline (PBS) and fixed in 1% freshly prepared paraformaldehyde/0.2% glutaraldehyde/2 mM $MgCl_2$/5 mM EGTA/0.02% Nonidet P-40 at 4°C for 90 min (for E11 embryos) or in 4% paraformaldehyde/PBS overnight at 4°C (for E14.5 embryos). After fixation, the E11 embryos were then washed three times for 30 min in PBS/0.02% Nonidet P-40 at 25°C. The E14.5 embryos were submerged in PBS/24% sucrose, frozen, and sectioned on a cryostat. To detect β-galactosidase activity, the embryos or sections were stained in 2 mM $MgCl_2$/15 mM NaCl/44 mM Hepes, pH 7.4/3 mM potassium ferrocyanide/5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) (0.5 mg/ml) for 16 hr in the dark at 37°C. The stained whole-mount embryos were washed 3 times with 70% ethanol. The stained sections were washed in PBS and covered with 10% glycerol/PBS. Processed embryos were photographed under a stereomicroscope (Zeiss) and then either were embedded in paraffin and sectioned on a microtome or were frozen and sectioned on a cryostat. All processed sections were photographed under a light microscope (Zeiss) using differential interference contrast optics.

RESULTS

N-CAM/*lacZ* Gene Constructs. The HBS region of the N-CAM promoter has been found to bind and mediate responses to homeobox gene products *in vitro* (15, 16, 21). To determine the role of these elements in the neural patterning of N-CAM gene expression *in vivo*, we prepared two N-CAM gene constructs (Fig. 1) to produce transgenic mice as described in *Materials and Methods*. The first gene construct, designated hbs$^+$, contained ≈6.5 kb of 5′ flanking sequence and most of the 5′ untranslated region of the mouse N-CAM gene (15). The second gene construct, designated hbs$^-$, contained mutations in both the HBS-I and the HBS-II sequences of the N-CAM promoter. These mutations disrupt the TAAT motifs known to be essential for recognition of homeodomain proteins (24).

The N-CAM Promoter Directs Neural Expression of the Reporter Gene in Transgenic Embryos. The N-CAM promoter/*lacZ* gene constructs were introduced into the mouse genome to produce transgenic lines or F_0 embryos. We analyzed three lines each of hbs$^+$ and hbs$^-$ mice at both E11 and E14.5. In addition, we examined six F_0 embryos from hbs$^+$ mice and five F_0 embryos from hbs$^-$ mice. Examples of individuals from transgenic lines of normal or mutant mice are presented in Fig. 2. A summary of all analyses of the expression patterns of transgenic lines is given in Table 1.

Embryos were examined for patterns of *lacZ* expression by whole-mount staining and subsequently by tissue sectioning. As seen in the example in Fig. 2*A*, whole mounts of the hbs$^+$ transgenic embryos showed *lacZ* expression in the central nervous system including the forebrain, midbrain, hindbrain, spinal cord, and, in some cases, in dorsal root ganglia. As indicated in Table 1, while spinal cord patterns did not vary, patterns in other sites varied among different transgenic embryos. We therefore chose the spinal cord for further analysis particularly because there was a consistent pattern of β-galactosidase staining across multiple transgenic lines.

To analyze the distribution of the neural expression pattern at the cellular level, coronal sections were examined throughout the anteroposterior axis of X-Gal-stained embryos. The hbs$^+$ transgenic mice showed β-galactosidase staining external to the ventricular zone in both dorsolateral and ventrolateral regions of the spinal cord, which, at this stage, are known to consist mainly of postmitotic neurons. This staining is shown for one embryo at E11 in Fig. 2*C*. At a later stage of neuronal development, E14.5, there was persistent and elevated expres-

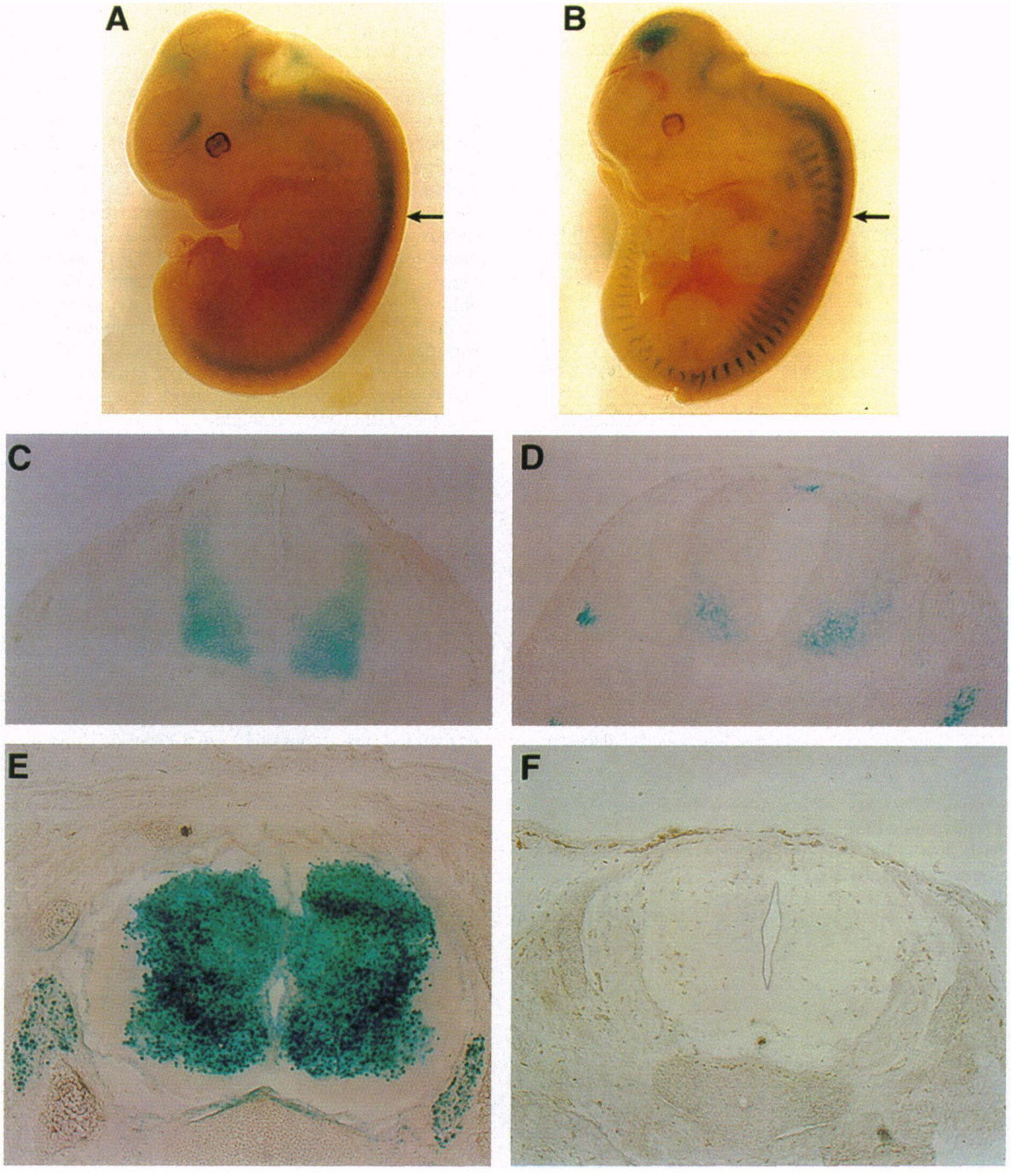

FIG. 2. Expression patterns of native and mutated N-CAM/*lac-Z* gene constructs in transgenic embryos. Stained whole-mount E11 embryos from hbs$^+$ (*A*) and hbs$^-$ lines (*B*). Coronal sections of the lumbar region of the spinal cord (see arrows in *A* and *B*) were obtained from hbs$^+$ embryos staged at E11 (*C*) and at E14.5 (*E*) or from hbs$^-$ embryos staged at E11 (*D*) and at E14.5 (*F*). Sections were stained with X-Gal as detailed. As shown in *E*, β-galactosidase staining was seen in a subset of dorsal root ganglia cells. Note the additional β-galactosidase staining in the roof plate of the spinal cord and in the laterally located pre-muscle masses in *D*. Unlike the staining pattern in the spinal cord, this pattern was not consistent among multiple transgenic and F_0 embryos. (*A* and *B*, ×7.5; *C–F*, ×55.)

sion of β-galactosidase throughout the spinal cord in hbs$^+$ embryos (Fig. 2*E*).

Mutation of the HBS Sequences Results in Altered Neural Expression Patterns. At E11 (Fig. 2*B*), hbs$^-$ transgenic mice showed *lacZ* expression in the central nervous system, including the spinal cord. In contrast to mice with the unmutated gene construct, β-galactosidase staining was concentrated in the ventrolateral region and was absent in the dorsolateral region of the spinal cord at E11 (Fig. 2*D*). This expression pattern was observed throughout the spinal cord, regardless of position along the anteroposterior axis.

In all three hbs$^-$ lines at E14.5, staining for β-galactosidase disappeared in the spinal cord across the entire anteroposterior axis (Fig. 2*F*). Thus, the mutations in the HBS region of the N-CAM promoter eliminated transgene expression in the dorsolateral area in the spinal cord at E11.0 and resulted in complete failure of expression at E14.5.

Constancy and Variability of Expression in Transgenic Lines and F_0 Embryos. In all transgenic mice carrying the hbs$^+$ construct at E11, spinal cord patterns were the same or only slightly variable. These patterns of *lacZ* expression in the spinal cord agreed well with the reported native expression pattern

Table 1. Expression patterns of the *lacZ* gene in separate lines of transgenic mice

Construct	Spinal cord*		Other sites†
	Dorsal	Ventral	
E11			
hbs$^+$			
Line 1	+	+	Brain (f,m,h), DRG
Line 2	+	+	Brain (h), DRG
Line 3	+	+	Brain (m,h), kidney
hbs$^-$			
Line 1	−	+	DRG, premuscle, limbs, brain (f)
Line 2	−	+	DRG, premuscle, limbs, heart, gut
Line 3	−	+	DRG, premuscle
E14			
hbs$^+$			
Line 1	+	+	DRG
Line 2	+	+	DRG
Line 3	+	+	Brain (m,h), kidney
hbs$^-$			
Line 1	−	−	None
Line 2	−	−	Heart, lung, gut
Line 3	−	−	Facial mesenchyme, muscle

*In the hbs$^+$ embryos, the β-galactosidase staining was in both alar and basal plates of the spinal cord at stage E11 and was uniformly distributed in the spinal cord at E14.5 (see Fig. 2 for examples).
†f, forebrain; m, midbrain; h, hindbrain; DRG, dorsal root ganglia. A minimum of five embryos per transgenic line were examined.

for the mouse N-CAM gene (5, 6). Expression of the hbs$^+$ and hbs$^-$ transgenes also occurred at sites outside the nervous system in the myocardium and the wall of the descending aorta, renal epithelium, submucosal layer of the stomach, pre-muscle masses, and the limb bud, all tissues and regions in which N-CAM is normally expressed (5). As shown in Table 1, expression in neural sites other than the spinal cord as well as in other nonneural sites varied in different lines. However, in no case was expression of any of the constructs ectopic to that seen (5, 6) for native N-CAM expression patterns.

To extend our observations, we also examined six F_0 embryos carrying the hbs$^+$ gene construct. All showed expression in the dorsal and ventral alar plates of the spinal cord at E11. Two showed expression in the spinal cord only; the others also showed expression in midbrain, hindbrain, and dorsal root ganglia. In addition, one showed expression in the renal epithelium and another in pre-muscle masses. Five F_0 embryos carrying the hbs$^-$ gene construct showed patterns of ventrolateral staining in the spinal cord at E11 consistent with those seen in Fig. 2*D*. Three of the F_0 embryos showed expression in midbrain and hindbrain, and of these, two showed expression in dorsal root ganglia, limb-bud, myocardium, and pre-muscle masses. As in the case of the transgenic lines carrying the unmutated gene construct, the expression patterns of the hbs$^-$ F_0 embryos showed no ectopic sites when compared to the native patterns of N-CAM expression.

DISCUSSION

The 6.5 kb of the 5′ flanking region of the N-CAM gene was sufficient to confer expression of the reporter gene in postmitotic neurons of the developing spinal cord in a pattern consistent with that of the endogenous N-CAM gene. Mutations introduced in the HBS elements within the N-CAM proximal promoter region resulted in the loss of reporter gene expression in the dorsolateral regions of the spinal cord at E11 and a complete loss of expression at E14.5. These differences between the patterns generated by the wild-type and HBS-mutated N-CAM promoters were consistent among all of the multiple independent transgenic lines (Table 1) and among F_0 embryos carrying these gene constructs. The data suggest that HBS elements within the N-CAM promoter are functional cis elements *in vivo* that are necessary for patterning N-CAM expression along the dorsoventral axis in the developing spinal cord. Moreover, the elimination of all β-galactosidase staining from hbs$^-$ lines at E14.5 suggests that the HBS sequences and associated trans factors are essential for the continuation of N-CAM expression in the spinal cord between E11 and E14.5.

Several different homeodomain proteins are capable of recognizing homeodomain binding sequences containing TAAT motifs (25). Moreover, different *Hox* genes from the paralogous clusters exhibit colinear expression patterns (26) that overlap with zones of N-CAM expression during spinal-cord development. The findings of the present study do not indicate, however, which of the various Hox gene products are responsible for place-dependent expression of N-CAM. It is known that the products of the *HoxB* (*Hox-2*) complex (including HoxB9 and HoxB8) are expressed predominantly in the dorsal half of the spinal cord (11), while expression of *Hox*C (*Hox-3*) clusters (including HoxC5 and HoxC6) is restricted to the ventral region (11, 27, 28).

In our previous studies of transactivation of the N-CAM promoter by Hox gene products, we defined two HBSs 12 bases apart. An analysis of binding of the homeodomain proteins specified by *HoxC6* indicated that HBS-I was essential, but HBS-II was not (16). Moreover, transfection experiments indicated that transactivation by *HoxB9* was mediated by HBS-II and was counteracted by *HoxB8*, a neighboring gene in the *HoxB* cluster (15). This competition and other complex interactions of various Hox gene products are likely to be involved in determining the final *in vivo* pattern of N-CAM expression. Identifying the particular homeodomain proteins responsible for induction of N-CAM expression in the spinal cord will require a detailed temporal analysis of the disappearance of *lacZ* expression and correlation with known patterns of transcription factors during earlier and later periods of spinal cord development. Breeding the N-CAM promoter/*lacZ* transgenic mice with other genetically engineered mice in which specific members of the *Hox* gene complex are either ectopically expressed or are inactivated by homologous recombination should also be revealing.

In this initial study of *Hox* and N-CAM gene activation *in vivo*, we deliberately restricted detailed analysis to the developing spinal cord, which showed consistent patterns from line to line of transgenic mice. During development, N-CAM is expressed in many different tissues outside of the nervous system. The N-CAM promoter fragments in the gene constructs used here directed *lacZ* expression mainly to the central nervous system; to a lesser extent these constructs also directed *lacZ* expression to nonneural tissues that are known to express N-CAM (e.g., heart, lung, kidney, gut, and muscle; Table 1). The expression of the gene constructs in nonneural sites varied among the different transgenic lines. In no case, however, did we observe expression ectopic to the normal expression patterns of N-CAM. The observed variation may result, in part, from differences in insertion of the gene construct in different chromosomal locations. Alternatively, the constructs used in our experiments may not have contained all the cis elements necessary to replicate the complete embryonic expression pattern of the endogenous N-CAM gene. Additional regulatory elements located elsewhere in the N-CAM gene may be required for the appropriate expression of native N-CAM in different tissues. In accord with this notion, we have shown that elements containing an HBS in the L-CAM gene enhancer can direct expression to appropriate tissues in transgenic mice when combined with a heterologous promoter (18, 29).

While a connection between *Hox* genes and regulation of N-CAM expression is strongly supported by our studies, it is likely that other gene products may also regulate N-CAM expression via the HBS sequences in the N-CAM promoter.

Candidates include proteins with paired type homeodomains—for example, the *Pax-3* gene, which is also expressed in dorsal regions of the spinal cord (30, 31). *Pax* genes are important regulators of neural differentiation and are known to contribute to the control of neural migration (32, 33). All members of the *Pax* gene family contain a common DNA-binding structure of 128 amino acids called the paired domain. Transcription factors containing this structure are likely to be important *in vivo* in affecting place-dependent N-CAM expression in the central nervous system. Recently, we have identified a region of the N-CAM promoter containing two paired domain binding site (PBS) sequences and have shown that they bind to the paired domains of Pax-1, Pax-6, and Pax-8 proteins (32, 34). In the case of Pax-8 (34) and Pax-3 (ref. 35; our unpublished results), we have demonstrated that these Pax proteins control transactivation of the N-CAM promoter *in vitro*. Analyses of the *in vivo* effects of mutations in paired domain binding site regions and comparisons with the HBS regions analyzed in the present study are obviously required.

All observations reviewed above are consistent with the hypothesis that elements within the N-CAM promoter act combinatorially to determine normal place-dependent expression in the nervous system. At different places in the embryo, different combinations of trans-acting factors encoded by homeobox, as well as by *Pax*, genes may bind to HBS and paired domain binding site sequences of the N-CAM promoter and either activate or repress N-CAM gene transcription in a synergistic fashion. Moreover, as indicated by variation in expression of the hbs^+ and hbs^- constructs in the present study, other regions of the N-CAM promoter, as well as enhancers at other sites, may be required to generate the native expression pattern. To determine the relevant combinations of cis and trans elements important for N-CAM expression in the nervous system and in other tissues during development, it will be necessary to produce a variety of transgenic mice with appropriate alterations in both cis- and trans-acting regulatory components.

We are grateful to Farid Karimi for excellent animal care assistance and to George L. Gabor Miklos and J. Michael Salbaum for critical reading of the manuscript. This work was supported by U.S. Public Health Service Grants HD33576 and HD09635 and a grant from the G. Harold and Leila Y. Mathers Charitable Foundation to G.M.E. G.M.E. is a consultant to Becton Dickinson.

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