Binding and transcriptional activation of the promoter for the neural cell adhesion molecule by *HoxC6* (*Hox-3.3*)

(homeobox/gene expression/morphogenesis/place-dependent signaling/N-CAM)

FREDERICK S. JONES*, BRENT D. HOLST*, OSAMU MINOWA*, EDDY M. DE ROBERTIS[†], AND GERALD M. EDELMAN*

*Department of Neurobiology, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037; and [†]Department of Biological Chemistry, University of California School of Medicine, Los Angeles, CA 92004

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ABSTRACT   Scores of homeobox gene-encoded transcription factors are expressed in a definite spatiotemporal pattern during embryogenesis and regulate a series of as yet unidentified target genes to help coordinate the morphogenetic process. We have suggested that homeobox gene products modulate the expression of adhesion molecule genes and have shown in cotransfection experiments that the promoters for the neural cell adhesion molecule (N-CAM) and cytotactin/tenascin genes respond to cues from different homeobox-containing genes. In this study, we show that the *HoxC6* (*Hox-3.3*)-encoded homeoprotein binds to a DNA sequence in the N-CAM promoter CCTAATTATTAA, designated homeodomain binding site I (HBS-I). To test whether *HoxC6* regulated N-CAM promoter activity, we cotransfected the Long and Short reading frame variants of *Xenopus HoxC6* (CMV–*HoxC6-L* and CMV–*HoxC6-S*) driven by the human cytomegalovirus (CMV) promoter together with a chloramphenicol acetyltransferase (CAT) reporter gene driven by the mouse N-CAM promoter (N-CAM-Pro–CAT). Cotransfection of NIH 3T3 cells with either of the CMV–*HoxC6* expression vectors stimulated N-CAM promoter-driven CAT expression. A 47-bp region from the N-CAM promoter that included HBS-I and an adjacent potential HBS, HBS-II, conferred *HoxC6* regulation on a simian virus 40 minimal promoter. HBS-I was sufficient for transactivation of the minimal promoter by CMV–*HoxC6-S*. However, transcriptional activation by CMV–*HoxC6-L* required both HBS-I and HBS-II, inasmuch as mutation of either HBS-I, HBS-II, or both motifs abolished the response. These studies suggest that HBS-I is a target site for binding and transcriptional control of the N-CAM promoter by homeoproteins, although accessory DNA sequences (such as HBS-II) may also be required. Together with previous studies, these results support the notion that N-CAM gene expression may be controlled by different combinations of homeoproteins that appear in a place-dependent manner during embryogenesis.

One of the fundamental riddles of developmental biology is how cells are patterned into tissues and organs in a form characteristic of a given species. Although the ultimate constraints on morphogenesis are coded in the genome, many of the events that give an animal its characteristic shape are epigenetic. The fate of embryonic cells depends on their lineage, on the nature of the immediate environment, and on their potential associations with other cells (1, 2).

Homeobox genes and their protein products have been shown to be important in defining spatial domains of morphogenetic activity in the embryo (3). These transcriptional regulators contain the homeodomain, a helix-turn-helix motif characteristic of a number of DNA binding proteins (4). The DNA core sequence of homeodomain binding sites (HBSs) frequently contains the sequence TAAT (5, 6). Homeobox genes regulate each other in a definite hierarchical and anteroposterior order (7, 8). Only recently have potential target genes for homeobox gene product regulation been identified (9, 10).

To understand better the process of cell patterning during development, a connection must be made between local transcriptional regulatory programs and cell-surface modulation events that coordinate the interactions of cells in time and space. A number of cell and substrate adhesion molecules are expressed in dynamic place-dependent patterns during embryogenesis and regulate the primary cellular processes that shape the animal, such as migration, proliferation, and differentiation (11). We have suggested that homeobox genes control the expression level of the genes encoding such proteins (10, 12, 13). Combinatorial interactions of these morphoregulatory molecules (14) would in turn regulate mechanical associations of cells, eventuating in tissue pattern and form (15).

Consistent with these ideas, we have provided evidence that promoters for cell and substrate adhesion molecule genes are targets for regulation by homeobox gene products. In cellular cotransfection experiments, we have shown that the level of neural cell adhesion molecule (N-CAM) gene promoter activity is modulated by different stoichiometric amounts of vectors driving the expression of two *Xenopus Hox* genes, *HoxB9* and *HoxB8* (*Hox-2.5* and *-2.4*). *HoxB9* was a potent activator of N-CAM promoter activity while *HoxB8* was an inhibitor. Base-pair substitutions made within the TAAT motifs of N-CAM promoter HBSs disrupted the ability of *HoxB9* to activate the N-CAM promoter (12). In a separate study, cotransfection of plasmids driving the expression of *Evx-1*, a murine homeobox gene similar to the even-skipped gene product of *Drosophila*, was found to stimulate promoter activity for the gene encoding cytotactin/tenascin, an extracellular matrix protein. Deletion and mutation of a TRE/AP-1 motif, located 276 bp upstream from the RNA start site abolished activation of the cytotactin promoter by *Evx-1* (13).

In the present study, we describe binding and activation of the N-CAM promoter by homeodomain proteins encoded by the *Xenopus HoxC6* (*Hox-3.3*) gene. *HoxC6* was chosen because the expression patterns of *HoxC6* and N-CAM overlap extensively during development of the nervous system and of chick feather buds (16). We found that the *HoxC6* gene product binds to a specific HBS in the N-CAM promoter and that transfection of NIH 3T3 cells with *HoxC6* activates N-CAM promoter activity. Combined with our previous

Abbreviations: CAT, chloramphenicol acetyltransferase; SV40, simian virus 40; CMV, cytomegalovirus; HBS, homeodomain binding site; N-CAM, neural cell adhesion molecule.

results, these observations suggest that a variety of homeobox gene products may regulate N-CAM gene expression and that some homeodomain proteins achieve their modulatory effects on the N-CAM promoter through direct binding of promoter sequences.

MATERIALS AND METHODS

A β-galactosidase–*Xenopus* HoxC6 fusion protein containing the open reading frame of the Short protein minus the first five amino acids was prepared as described (17, 18). Nuclear extracts were prepared by the method of Dignam *et al.* (19). Probes for gel-shift experiments were derived from the 47-bp HBS region located between positions −559 and −512 in the mouse N-CAM promoter (13, 20). The region contains two putative HBSs, designated HBS-I and HBS-II (13). Oligonucleotides were annealed and labeled with the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq) (DuPont/NEN). For DNA/protein binding reactions, either HoxC6 fusion protein or nuclear extract (1–7 μg) was mixed with 3 μl of 7× binding buffer (350 mM Tris·HCl, pH 7.5/1.75 M sucrose/14 mM MgCl₂/175 mM KCl/7 mM dithiothreitol/35 μg of bovine serum albumin per ml/3.5 mM phenylmethylsulfonyl fluoride), 2 μl of poly(dI-dC), and 5 × 10⁴ cpm of probe DNA in a final vol of 20 μl. Competitor oligonucleotides were added and samples were incubated at room temperature for 30 min. Denatured sonicated salmon sperm DNA (1 μl of 5 mg/ml) was added and samples were incubated for an additional 15 min. Samples were applied to 5.5% polyacrylamide gels in 0.5× TBE (1× TBE = 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3), and after electrophoresis the gels were dried and autoradiographed.

Expression vectors containing two protein variants of *Xenopus laevis* HoxC6 were prepared in the human cytomegalovirus (CMV) promoter vector CMV2′ as described (12). The plasmids were designated CMV–*HoxC6*-L and CMV–*HoxC6*-S, for the Long and Short proteins, respectively. The N-CAM promoter/reporter gene plasmid, N-CAM-Pro–CAT (CAT, chloramphenicol acetyltransferase), and HBS enhancer/simian virus 40 (SV40) minimal promoter reporter constructs have been described (12). NIH 3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium with 10% (vol/vol) calf serum. Cells were transfected with 10 μg of *HoxC6* expression vector and 10 μg of N-CAM-Pro–CAT or HBS enhancer/SV40 CAT reporter construct using the calcium phosphate method (21). In addition, 5 μg of CMV–β-galactosidase gene reporter plasmid DNA was cotransfected to provide an internal standard for transfection efficiency. Cells were harvested after 72 hr; extracts were prepared, assayed for β-galactosidase activity, and then assayed for CAT activity (22). The amount of cell extract used for CAT assays was normalized to β-galactosidase activity. Levels of CAT activity were quantitated from TLC plates on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

LacZ–HoxC6 Fusion Protein Binds to N-CAM Promoter HBS-I. The *X. laevis* HoxC6 gene (previously designated *Xenopus-Hox-3.3* and *XlHbox-1*) was the first homeobox-containing gene to be isolated from vertebrates (23). The gene has been renamed in accordance with the improved nomenclature for the *Hox* genes (24). Transcription of *Xenopus HoxC6* is initiated from two separate promoters spaced 9 kb apart in the genome, producing two mRNA transcripts during early development, 2.2 and 1.8 kb long. These transcripts encode HoxC6-L and -S protein variants, both of which contain a DNA binding homeodomain of the Antennapedia
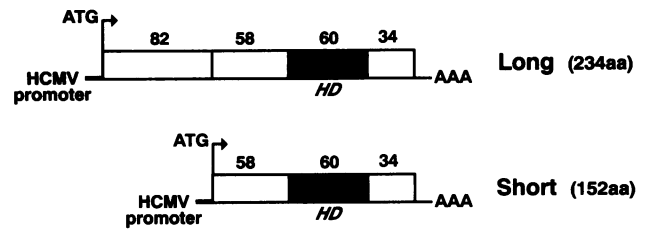

FIG. 1. Diagram of the two *Xenopus HoxC6* variants and expression vectors. Expression of both variants is driven by the human (H) CMV promoter. Protein-coding regions of *HoxC6* cDNA (boxed segments) are preceded by a short segment of 5′ leader sequence from the *Xenopus* β-globin gene (dark line preceding the ATG initiator). Both *HoxC6* variants contain an Antennapedia class homeodomain (HD) (solid box) and segments of 34 amino acids C-terminal and 58 amino acids N-terminal to it. The long protein has an N-terminal segment of 82 residues not found in the short variant.

class (17). Both proteins share the same reading frame but the Long protein contains an 82-amino acid N-terminal domain not found in the Short protein (Fig. 1).

We found previously that a 47-bp segment of DNA, containing two TAAT motifs, designated HBS-I and HBS-II, and located upstream of the mouse N-CAM gene, mediated responsiveness of the N-CAM promoter to *HoxB9* and *HoxB8* (*Hox-2.5* and *Hox-2.4*) gene products (12). Previous studies also showed that a LacZ–HoxC6-S fusion protein bound to the *Xenopus HoxB7* (*Hox-2.3*) promoter (17), which contains the DNA sequence TAAT—a motif known to be the core of a number of homeodomain binding sites (5, 6).

To determine whether the N-CAM promoter could bind *Hox* gene products, we assayed the 47-bp HBS region for binding to the HoxC6-S fusion protein in gel-shift experiments. We observed a single shifted band when the double-stranded ^{32}P-labeled HBS DNA probe was mixed with the HoxC6-S fusion protein and electrophoresed on a native polyacrylamide gel (Fig. 2B, lane 2). We then tested four double-stranded oligonucleotides for their ability to compete for HoxC6-S fusion protein binding to the ^{32}P-labeled HBS probe. Competitors included the wild-type 47-bp HBS region of the N-CAM promoter containing HBS-I and HBS-II (designated HBS-I⁺/II⁺) or three different mutants with base-pair substitutions in the TAAT core sequences of either HBS-I (HBS-I⁻/II⁺), HBS-II (HBS-I⁺/II⁻), or both sites (HBS-I⁻/II⁻) (Fig. 2A). A 100-fold molar excess of the wild-type HBS DNA (HBS-I⁺/II⁺) competed effectively for binding to the labeled HBS probe; the band representing the DNA–protein complex disappeared almost completely (Fig. 2B, compare lane 3 to lane 2). Excess unlabeled HBS-I⁻/II⁻ DNA, with mutations in both HBS-I and HBS-II, did not compete with the HBS probe for binding of the fusion protein (lane 5). The HBS-I⁺/II⁻ DNA, containing a mutation in HBS-II, competed for HoxC6-S fusion protein binding to HBS (compare lane 6 to lane 2) although not as effectively as HBS-I⁺/II⁺ DNA. The HBS-I⁻/II⁺ oligonucleotide, with a mutation in HBS-I, did not compete for HoxC6-S fusion protein binding to the probe (compare lane 5 to lane 2). Taken together, the binding and competition experiments suggest that the HoxC6-S fusion protein binds to the HBS-I motif in the N-CAM promoter.

To provide additional support of HBS-I binding to the HoxC6-S fusion protein, we examined binding of three tandem copies of the N-CAM HBS-I motif (HBS-I⁺×3). A number of wild-type and mutant multimers of the HBS-I and HBS-II sites were used to provide competition. The multimers contained three copies of a 12-bp fragment with either the wild-type or mutant HBS-I or HBS-II sequence (see bottom of Fig. 2A) and were designated HBS-I⁺×3, HBS-II⁺×3, HBS-I⁻×3, and HBS-II⁻×3, respectively. When the

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A

HBS-I⁺: CCTA ATTATTA A
        GGAT TAATAAT T

HBS-II⁺: CCTG ATTA AGGA
         GGAC TAAT TCCT

HBS-I⁻: CCTA ACTATCA A
        GGAT TGATAGT T

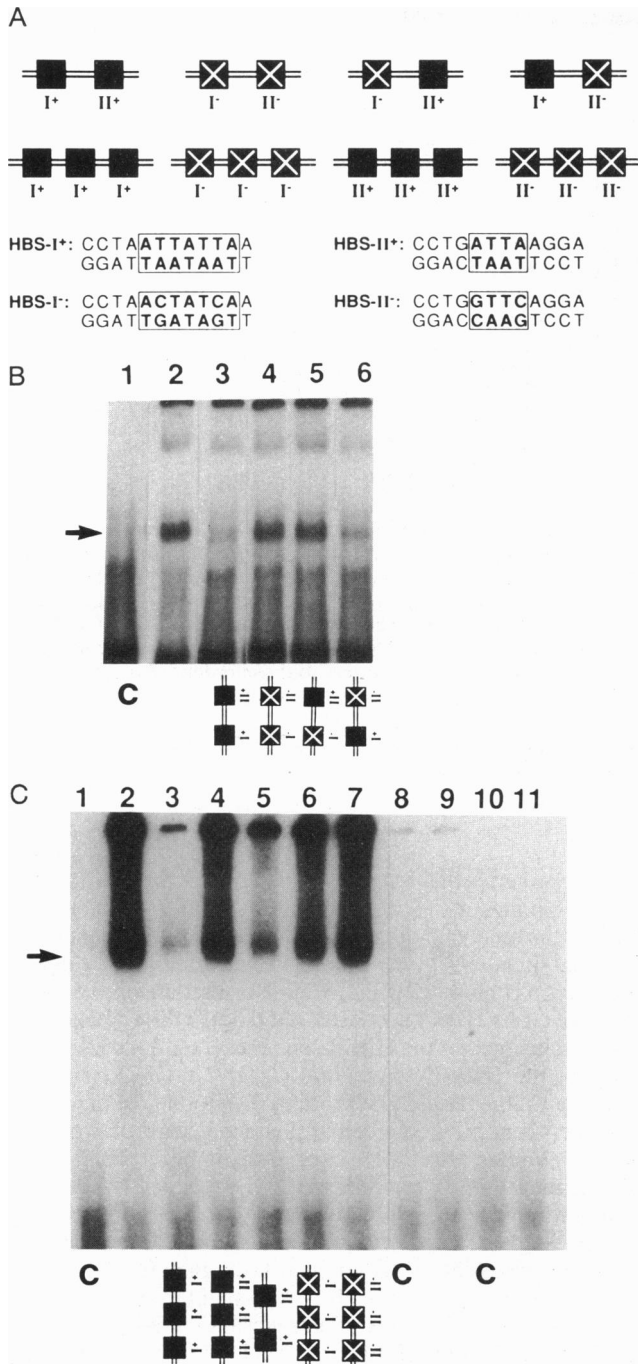HBS-II⁻: CCTG GTTC AGGA
         GGAC CAAG TCCT

B

C

FIG. 2. (*A*) Schematic representation of the eight double-stranded oligonucleotides used in gel mobility-shift experiments. The two potential HBSs, HBS-I and HBS-II, from the N-CAM promoter are indicated by solid boxes. Mutants in the TAAT motifs are marked with an X. DNA sequences of the wild-type and mutant HBSs and the 12-bp segments repeated in synthetic HBS triplicates are shown below. (*B*) Binding of HoxC6 fusion protein to the 47-bp N-CAM HBS region. Autoradiograph of native polyacrylamide gel showing ³²P-labeled HBS probe alone (lane 1) or binding to HoxC6-S fusion protein (lane 2). Competition of the HBS/HoxC6-S binding reaction was performed with the oligonucleotides indicated below each lane (lanes 3–6). (*C*) Binding of HoxC6-S fusion protein to HBS-I⁺×3. Autoradiograph of gel with ³²P-labeled HBS-I⁺×3 probe either alone (lane 1) or mixed with HoxC6-S fusion protein (lane 2). Competition of HBS-I⁺×3/HoxC6-S binding reaction was performed using the oligonucleotides specified below each lane (lanes 3–7). Binding reactions were also performed with ³²P-labeled HBS-I⁻×3 and HBS-II⁻×3 probes without (lanes 8 and 10, respectively) or with (lanes 9 and 11, respectively) addition of HoxC6-S fusion protein.

HoxC6-S fusion protein was incubated with a ³²P-labeled HBS-I⁺×3 DNA, we observed an intense band (Fig. 2*C*, lane 2; component indicated by arrow). The intensity of the band was reduced when a 50-fold excess of unlabeled HBS-I⁺×3 was added to the binding reaction mixture as a competitor (compare lane 3 to lane 2), but it was not reduced by 50-fold excess unlabeled HBS-II⁺×3 (lane 4), HBS-I⁻×3 (lane 6), or HBS-II⁻×3 (lane 7). Binding of HoxC6-S fusion protein to HBS-I⁺×3 was moderately blocked by competition with a 50-fold excess of the native N-CAM HBS (compare lane 5 to lane 2). No binding of HoxC6-S fusion protein to ³²P-labeled HBS-I⁻×3 or HBS-II⁻×3 probes (lanes 9 and 11, respectively) was observed.

Activation of N-CAM Promoter by *HoxC6* in Cotransfection Experiments. To test whether the *HoxC6* gene products could modulate N-CAM promoter activity, we first constructed vectors that would drive the expression of both the Long and Short variants (designated CMV–*HoxC6-L* and CMV–*HoxC6-S*, respectively; see Fig. 1). We were able to detect both the long and short *HoxC6* mRNA transcripts by RNase protection after transfection of these vectors in NIH 3T3 cells (data not shown). To determine whether the two *HoxC6* expression vectors could produce proteins capable of binding the HBS-I motif, we tested nuclear extracts prepared from NIH 3T3 cells transfected with CMV–*HoxC6-L* and CMV–*HoxC6-S* for binding to a ³²P-labeled N-CAM promoter HBS-I⁺×3 probe. As shown in Fig. 3, extracts from cells transfected with either CMV–*HoxC6-L* (lane 3), CMV–*HoxC6-S* (lane 4), or an equal mixture of both vectors (lane 5) bound to the HBS-I⁺×3 sequence and produced a prominent band. This band was absent when nuclear extracts prepared from mock-transfected NIH 3T3 cells were used in the DNA binding reaction (lane 2).

To determine whether expression of *HoxC6* variants in cells affected N-CAM promoter activity, we cotransfected the CMV–*HoxC6-L* and CMV–*HoxC6-S* plasmids in NIH 3T3 cells together with the mouse N-CAM promoter/CAT gene reporter plasmid, N-CAM-Pro–CAT. Cotransfection of cells with either CMV–*HoxC6-L* or CMV–*HoxC6-S* plasmids induced CAT reporter gene expression driven by the N-CAM promoter (Fig. 4*A*). Cotransfection of the N-CAM-Pro–CAT construct with CMV–*HoxC6-L* produced 7.6-fold greater CAT activity than the background level produced by the vector control, pCAT basic, which had no N-CAM promoter sequences. Similarly, after cotransfection with CMV–*HoxC6-S*, N-CAM-Pro–CAT yielded 6.8-fold greater CAT activity than pCAT basic. In the presence of an equal mixture
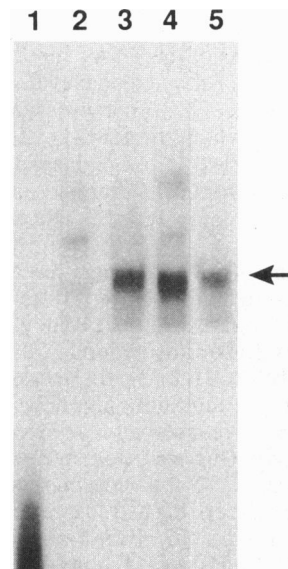

FIG. 3. Nuclear extracts prepared from NIH 3T3 cells transfected with *HoxC6* expression vectors bind to HBS-I⁺×3. ³²P-labeled HBS-I⁺×3 probe (lane 1) was incubated in binding reactions with nuclear extracts prepared from mock-transfected NIH 3T3 cells (lane 2) or from NIH 3T3 cells transfected with either 10 μg of CMV–*HoxC6-L* (lane 3), 10 μg of CMV–*HoxC6-S* (lane 4), or a mixture of 5 μg of both vectors (lane 5).
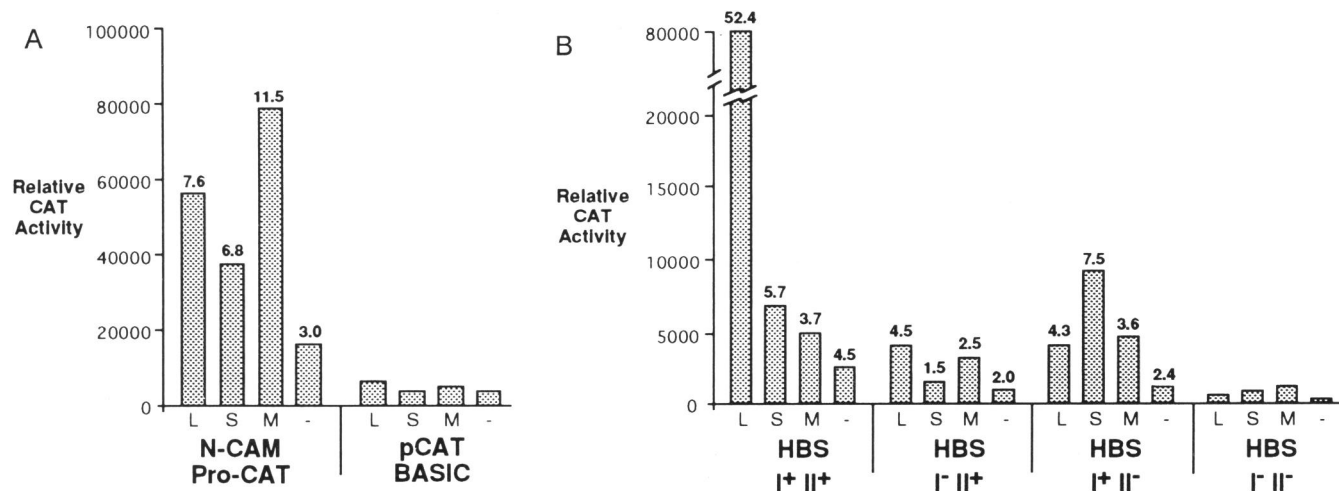
FIG. 4. Activation of CAT gene expression driven by the N-CAM promoter (*A*) or the N-CAM HBS enhancer/SV40 minimal promoter (*B*) constructs upon cotransfection with *HoxC6* expression vectors. NIH 3T3 fibroblasts were cotransfected with 10 μg of either pCAT basic, N-CAM-Pro–CAT, HBS-I$^+$/II$^+$, HBS-I$^-$/II$^+$, HBS-I$^+$/II$^-$, and HBS-I$^-$/II$^-$ reporter vectors and 10 μg of either CMV–*HoxC6-L* (L), CMV–*HoxC6-S* (S), an equal mixture containing 5 μg of both vectors (M), or no expression vector at all (−). CAT activity levels were quantitated on a PhosphorImager (Molecular Dynamics) from scans of CAT assay samples applied to TLC plates. The degree of induction (*n*-fold) is indicated over appropriate histograms and is derived from comparison of levels of CAT activity produced by N-CAM-Pro–CAT to levels produced by pCAT basic (*A*) or levels of CAT activity produced by HBS-I$^+$/II$^+$, HBS-I$^-$/II$^+$, and HBS-I$^+$/II$^-$ reporters to those produced by HBS-I$^-$/II$^-$ (*B*).

of both CMV–*HoxC6-L* and CMV–*HoxC6-S*, N-CAM-Pro–CAT had 11.5-fold greater activity than that produced from pCAT basic. In the absence of any *HoxC6* expression vector N-CAM-Pro–CAT had only 3.0-fold higher CAT activity than pCAT basic (Fig. 4*A*).

To assess whether the HBS region of the N-CAM promoter was capable of mediating transcriptional activation by *HoxC6*, we tested reporter vectors containing wild-type and mutant HBS-I and HBS-II sequences for their ability to confer inducibility by *HoxC6* to a SV40 minimal promoter in cotransfection experiments. As shown in Fig. 4*B*, cotransfection of CMV–*HoxC6-L* and CMV–*HoxC6-S* increased CAT activity from a reporter gene construct containing the wild-type HBS region (HBS-I$^+$/II$^+$) 52.4-fold and 5.7-fold, respectively, over that produced by a reporter construct containing mutations in the TAAT motifs of both HBS-I and HBS-II (HBS-I$^-$/II$^-$). In contrast to N-CAM-Pro–CAT, the HBS-I$^+$/II$^+$ construct did not show a greatly elevated CAT activity when cotransfected with a mixture of the two *HoxC6* expression plasmids (3.7-fold) (Fig. 4*B*). Thus, for the HBS-I$^+$/II$^+$ reporter construct, the presence of *HoxC6-S* seems to inhibit the much greater activation produced by *HoxC6-L* alone. A construct containing mutations of the TAAT motifs in both HBS-I and HBS-II (HBS-I$^-$/II$^-$) showed no elevated CAT activity levels after cotransfection of *HoxC6* expression plasmids. CAT activity from cells in which the HBS-I$^+$/II$^+$ reporter had been cotransfected with CMV–*HoxC6* plasmids was significantly higher than activity from cells that had not received the *HoxC6* plasmids (Fig. 4, compare L, S, and M with −).

Experiments with reporter constructs containing mutations in either HBS-I or HBS-II were able to discern differences in the HBS motifs required for activation of promoter activity by the two variants of *HoxC6*. Cotransfection of cells with CMV–*HoxC6-L* and either HBS-I$^-$/II$^+$, HBS-I$^+$/II$^-$, or HBS-I$^-$/II$^-$ reporter constructs (with mutations in HBS-I, HBS-II, or both HBS-I and HBS-II, respectively) showed little, if any, increase in CAT activity (Fig. 4*B*). In contrast, cotransfection of cells with CMV–*HoxC6-S* showed a marked elevation in CAT activity driven by the HBS-I$^+$/II$^-$ reporter (containing wild-type HBS-I and a mutated HBS-II motif) and no increase from HBS-I$^-$/II$^+$ or HBS-I$^-$/II$^-$ reporter

vectors (containing mutations in HBS-I and both HBS-I and HBS-II, respectively).

DISCUSSION

Through a series of DNA mobility-shift experiments, we have shown binding of a HoxC6-S homeoprotein to a region of the N-CAM promoter that contains two potential homeodomain binding sites. Wild-type HBS-I oligonucleotides competed for *HoxC6-S* binding, while HBS-II or mutants in the TAAT cores of either HBS-I and HBS-II did not. These data support the conclusion that the HBS-I sequence CCTAATTATTAA, and not the HBS-II sequence CCTGATTAGGA, mediates binding to the HoxC6-S protein. Components of nuclear extracts from NIH 3T3 cells transfected with plasmids expressing either HoxC6-L, HoxC6-S, or both proteins also bound the N-CAM promoter HBS-I sequence, while those from untransfected cells did not.

We tested vectors driving the expression of Long and Short protein variants of the *Xenopus HoxC6* gene for their ability to modulate activity of the N-CAM promoter. When cotransfected into NIH 3T3 cells, either variant stimulated N-CAM promoter activity. Mixture of the two variants yielded a greater induction of N-CAM promoter activity than did transfection with either variant alone. This result supports the notion that combinatorial expression of the *HoxC6* variants has a synergistic effect on transactivation of the N-CAM promoter. During vertebrate development, both HoxC6 protein forms are expressed in the neural tube, although with different anterior borders of expression, at a time when N-CAM is also expressed (18).

The HBS-I and HBS-II motifs were able to confer regulation by both *HoxC6-L* and *HoxC6-S* to a heterologous SV40 promoter. However, unlike the native N-CAM promoter, the synthetic reporter (HBS-I$^+$/II$^+$) was unable to mediate the synergistic activation by the mixture of *HoxC6-L* and *HoxC6-S*. In fact, mixture of the two *HoxC6* variants resulted in lower activation levels. These results suggest that HBS-I and HBS-II, when taken out of context, are not sufficient to reproduce the same responses to homeoproteins observed in the native promoter, and that other sequences in the N-CAM

promoter may facilitate the synergy of activation normally observed.

We tested mutations in the TAAT motifs of HBS-I and HBS-II for their ability to mediate promoter activation by the *HoxC6* variants. We found that HBS-I, the motif bound by the LacZ/HoxC6-S fusion protein in gel-shift experiments, was sufficient to confer activation of the minimal promoter by *HoxC6-S* and that mutation of HBS-II did not reduce activation. However, mutations in the TAAT motifs of either HBS-I, HBS-II, or both motifs abolished the ability of the HBS region to mediate activation by *HoxC6-L*. These data suggest that both HBS-I and HBS-II are required for regulation of promoter activity by *HoxC6-L*, while activation by *HoxC6-S* requires only a single HBS-I motif. The 82-amino acid N-terminal domain of *HoxC6-L* may engage in cooperative associations with other transcriptional regulatory proteins (which may include other homeoproteins) bound to accessory DNA sites at other locations in the promoter. HBS-II may be an example of such an accessory site. Such cooperative interactions and synergistic transcriptional regulation have been shown to occur between a variety of homeodomain proteins (25–27). The present results suggest that the Long and Short versions of *HoxC6* (17, 28) can activate target genes differentially.

In a previous study using the same cotransfection system, we found that HBS-II mediated activation of the N-CAM promoter by *HoxB9* (*Hox-2.5*) (12). Hence, HBS-I and HBS-II may mediate binding and transcriptional effects of different homeoproteins. Genes of the *HoxB* and *HoxC* (*Hox-2* and *Hox-3*) clusters have been shown to be expressed in distinct regions in the dorsoventral axis of the developing neural tube and spinal cord (29, 30). Our results suggest that combinations of homeoproteins found at different positions in the embryo may determine appropriate levels of N-CAM gene expression during development.

In addition to the results of our studies, there is a growing body of evidence that also supports the idea that there is a tight link between homeotic gene function and cell adhesion. An immunoprecipitation strategy for uncovering chromatin actively bound by homeodomain protein *in vivo* (31) has recently revealed a regulatory region in the *Drosophila* connectin gene that is bound by the *Ubx* homeotic gene product (32). The connectin gene product has been shown to be a cell adhesion molecule (33). Mutations in the *mab-5* gene, which encodes an Antennapedia class homeobox gene product in *Caenorhabditis elegans*, leads to abnormal neural pathfinding during development (34). Members of the N-CAM, cadherin, and integrin families of cell and substrate adhesion molecules have been implicated in cell axonal guidance phenomena in both invertebrate and vertebrate species (11, 35–38). Furthermore, it has been reported that uptake of an Antennapedia homeodomain peptide and the binding of target DNA sequences by primary cultures of neurons alters their morphology and neurite process extension properties (39, 40). In view of the accumulated evidence, it is an attractive hypothesis that a variety of cell and substrate adhesion molecules are targets for homeobox gene regulation during development. Different subsets of homeoproteins may be used for the many contexts of place-dependent adhesion molecule gene expression (15, 41) during development. Such a diversity of transcriptional cues may be essential in local specification of neuronal migration pathways and tissue modeling.

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