The Homeodomain Protein Barx2 Contains Activator and Repressor Domains and Interacts with Members of the CREB Family*

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Barx1 and Barx2 are homeodomain proteins originally identified using regulatory elements of genes encoding certain cell adhesion molecules (CAMs). In the present study, we characterize regions of Barx2 that bind to regulatory elements of genes encoding three CAMs, L1, neuron-glia CAM (Ng-CAM), and neural CAM (N-CAM), and identify domains of Barx2 that regulate N-CAM transcription. The homeodomain of Barx2 was sufficient for binding to homeodomain binding sites (HBS) from all three CAM genes. The presence of a 17-amino acid Barx basic region resulted in a 2-fold decrease in binding to HBS sequences from the Ng-CAM and L1 genes, whereas it led to a 6.5-fold increase in binding to the HBS from the N-CAM promoter. Thus, the Barx basic region influences the strength and specificity of Barx2 binding to DNA. In co-transfection experiments, Barx2 repressed N-CAM promoter activity. A 24-residue N-terminal region of Barx2 was essential for repression. When this region was absent, Barx2 activated the N-CAM promoter. A 63-residue C-terminal domain was required for this activation. In GST pull-down experiments, Barx2 bound to proteins of the CREB family, CREB1 and ATF2. Overall, these findings provide a framework for understanding developmental and physiological contexts that influence repressor or activator functions of Barx2.

The homeobox was first identified as a common feature of genes of the homeotic complex in Drosophila (1–3). All homeobox genes encode a 61-amino acid DNA binding structure called the homeodomain, and many act as transcription factors that control regional patterning of gene expression (4–6). Phenotypic analysis of homeobox mutants showed that the products of these genes regulate axial patterning and specify segment identity in the Drosophila body plan (7, 8). The vertebrate Hox genes are homologous to the genes of the Drosophila homeotic complex and appear to specify axial location in a manner similar to their fly counterparts (9–11). In addition to homeobox genes of the homeotic complex and Hox clusters, a number of nonclustered homeobox genes have been identified in a wide range of invertebrate and vertebrate phyla (reviewed in Ref. 12) as well as in plants (13). In diverse species, homeobox genes have been shown to be essential for the correct positioning and differentiation of tissues and organs (8, 14–17).

The relationship between the activity of homeodomain transcription factors and cellular processes, such as proliferation, differentiation, and migration, that lead to morphogenesis is not well understood. Molecular targets that may link homeobox gene activity to cellular patterning events are the cell adhesion molecules (CAMs)1 and substrate adhesion molecules. These cell surface glycoproteins and molecules of the extracellular matrix guide cell interactions and influence the formation and subsequent differentiation of cell collectives (18). Relating the activity of particular homeodomain transcription factors to the induction or repression of the expression of particular CAMs during development provides a useful framework for understanding the mechanics of morphogenesis. Homeodomain binding sites (HBSs) have been identified as important regulatory elements in the genes encoding several of these CAMs, particularly those of the immunoglobulin (Ig) superfamily such as neural CAM (N-CAM), neuron-glia CAM (Ng-CAM), and L1 (19–22).

Among the developmentally significant homeodomain proteins that regulate CAM expression are two unusual homeodomain proteins, Barx1 and Barx2. These proteins were both originally identified as factors that bind to the regulatory elements of two different CAM genes. Barx1 was discovered in a Southwestern screening procedure (23) using a regulatory element of the gene encoding the mouse neural cell adhesion molecule (N-CAM) as a probe (24). Barx2 was identified via a similar procedure, using an element from the chicken Ng-CAM (25). Barx1 and Barx2 contain nearly identical homeodomains that are most similar to those encoded by the dual Bar H genes of Drosophila melanogaster (26). A comparison of the homeodomains of Barx1 and Barx2 with other proteins (Fig. 1) indicates that they are most similar to invertebrate homeobox proteins of the Bar subfamily. Homeodomains of Bar subfamily all contain a glutamine residue at position 50 in the third helix of the homeodomain. This property is shared by several other homeodomain proteins including all members of the Antennapedia family and confers a binding preference for target sites containing the core sequence CATTA (27, 28). Bar class homeodomains are further distinguished from those of the Antennapedia family by threonine and tyrosine residues at positions 47

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1 The abbreviations used are: CAM, cell adhesion molecule; N-CAM, neural CAM; Ng-CAM, neuron-glia CAM; HBS, homeodomain binding site; HD, homeodomain; BBR, Barx basic region; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; NHD, amino-terminal region to the homeodomain fragment; HA, hemagglutinin; CRE, cyclic AMP-response element.
and 49, respectively. In addition to the homeodomain, Barx1 and Barx2 share a highly conserved 17-amino acid basic region, designated the Barx basic region (BBR), located immediately downstream of the homeodomain. Barx2 exhibits several other notable features, including a putative leucine zipper and polyalanine tract within the amino-terminal region and an acidic domain within the carboxyl-terminal region (25).

Barx1 and Barx2 show dynamic expression patterns during development of the central nervous system that overlap with patterns of CAM expression. For instance, between embryonic days 10.5 and 12.5, Barx2 expression is particularly intense in the telencephalon, mesencephalon, and spinal cord, where CAMs such as N-CAM, Ng-CAM, and L1 are also expressed (25). Outside of the nervous system, Barx1 and Barx2 show complementary patterns of expression in the mesenchyme and epithelia during the development of craniofacial tissues. For instance, Barx1 is expressed in the mesenchyme of the mandibular and maxillary processes and in the tooth primordia, while Barx2 is expressed in the oral epithelium prior to tooth development (29). A role for Barx1 in tooth development has recently been suggested by experiments showing that blockade of BMP signaling in the mesenchyme leads to ectopic expression of Barx1 and transformation of incisors into molars (30). Thus, a tentative hypothesis is that Barx proteins influence patterns of cell-cell interaction by regulating the expression of particular cell adhesion molecules. This patterning is also subject to control by inductive signals from secreted differentiation factors, such as BMPs.

In the present study, we examine the binding of Barx1 and Barx2 to the homeodomain binding sites of L1, Ng-CAM, and N-CAM and identify domains in Barx2 involved in transcriptional regulation of the N-CAM promoter. We also demonstrate that Barx2 interacts with two leucine zipper-containing proteins of the CREB family of transcription factors, CREB1 and ATF2. These studies provide the foundation for understanding the role of Barx proteins in regulating the expression of cell adhesion molecules in particular morphogenetic contexts. They also suggest a role for Barx proteins in modulating the function of transcription factors of the CREB family.

**EXPERIMENTAL PROCEDURES**

**Construction of Barx2 Deletion Plasmids**—A Barx1 cDNA fragment was generated by reverse transcription-polymerase chain reaction from embryonic day 10.5 mouse mRNA using primers that flank a region of the Barx1 cDNA that was conceptually translated by Tissier-Seta et al. (24). The Barx1 cDNA was inserted into the Topo2.1 vector, which contains a T7 promoter (Invitrogen). Thirteen different Barx2 expression plasmids (see Fig. 2) were generated by insertion of either the full-length Barx2 cDNA or cDNA fragments into a modified version of the pcDNA3 expression vector (Invitrogen) containing an amino-terminal Myc epitope tag. Barx2 cDNA fragments were generated by polymerase chain reaction from a Bluescript KS subclone of the Barx2 cDNA (25). All cDNA fragments were amplified using T7 polymerase. Plasmid DNA was prepared and purified using Qiagen maxi prep columns (Qiagen).

**Oligonucleotides**—Oligonucleotides corresponding to the region containing the homeodomain binding sites of the mouse L1 gene (called the HBS), of the mouse Ng-CAM gene (called the Ng-CAM HBS) and of the mouse N-CAM gene (called the HBS) were synthesized and used as probes in gel mobility shift experiments. All oligonucleotides were annealed and end-labeled with polynucleotide kinase and [γ-32P]ATP (3000 Ci/mmol) (DuPont). Each DNA probe was purified by elution from an 8% polyacrylamide gel. The relative strengths of binding of Barx2 to wild type L1-HPD, Ng-CAM HBS, and N-CAM HBS probes were assessed in gel mobility shift experiments. All probes were adjusted to a specific activity of 3 × 10^7 cpm/µmol. DNA/protein binding reactions were initiated upon the addition of 5 µl of in vitro translated Barx protein extract with 4 µl of 5% binding buffer (4%
Functional Domains of the Homeodomain Protein Barx2

RESULTS

Binding of Barx Proteins to the Homeodomain Binding Sites of Three CAM Genes—Barx1 and Barx2 were initially isolated in Southwestern screening procedures using CAM gene regulatory elements as probes. To further characterize the binding of these proteins to CAM gene homeodomain binding sites (HBSs), we conducted gel mobility shift analyses. First, full-length and truncated Barx proteins (schematized in Fig. 2) were generated by in vitro transcription and translation of Barx expression plasmids and shown to be of the correct molecular weight (Fig. 3). To estimate the relative binding strength of Barx2 protein to the L1 HPD, Ng-CAM HBS, and N-CAM HBS probes, we measured the intensity of Barx2-probe complexes in gel mobility shift experiments. For these experiments, the specific activity of each probe was 3 × 10^6 cpm/pmol, and 2 × 10^6 cpm (1.5 fmol) of each probe was used in each binding reaction with in vitro synthesized Barx proteins. The relative level of binding of Barx2 to the three probes was then assessed by comparing band intensities of DNA-protein complexes using a PhosphorImager.

GST Pull-down Assays—A Barx2-GST fusion construct was made by cloning the Barx2 cDNA into the pGEX1T expression vector (Amersham Pharmacia Biotech). Expression of the Barx2 GST fusion protein in Escherichia coli was induced by adding 1 mM isopropyl-β-D-thiogalactoside and incubating overnight at 25 °C. A bacterial cell lysate was prepared by sonication in lysis buffer I (50 mM Tris-Cl, pH 7.5, 2 mM MgCl_2, 25% sucrose, and Complete protease inhibitor (Roche Molecular Biochemicals)). The GST protein was purified from the lysate by incubation with glutathione-Sepharose resin (Amersham Pharmacia Biotech). Glutathione-Sepharose-bound Barx2-GST fusion protein was washed five times with 20 mM Tris-Cl, pH 7.5, 2 mM MgCl_2, 1 mM dithiothreitol. Mouse CREB1 (cAMP-response element-binding protein) was expressed in Neuro 2A cells after transfection of an SRα expression vector containing the CREB cDNA with a amino-terminal hemagglutinin tag. Neuro 2A cells were transfected using Fugene as recommended by the manufacturer (Roche Molecular Biochemicals). Neuro 2A cell lysates were prepared from either wild-type or CREB1-transfected cells (100-mm tissue culture plate) by sonication in 400 μl of lysis buffer II (50 mM HEPES, pH 7.9, 300 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet-P40, and Complete protease inhibitor) (Roche Molecular Biochemicals). Approximately 0.1 μg of purified Barx2 GST protein bound to glutathione-Sepharose resin was combined with 100 μl of Neuro 2A cell lysate and incubated at 4 °C for 4 h. Purified glutathione S-transferase protein was bound to glutathione-Sepharose resin and used in control pull-down assays. Resin-bound protein complexes were washed four times with lysis buffer II for 10 min at 4 °C. Complexes were eluted at 4 °C for 30 min with 25 μl of 0.9 M glutathione, pH 9.6. The eluates were combined with SDS-PAGE sample buffer and resolved by SDS-PAGE on a 10% polyacrylamide gel. Gels were blotted to nitrocellulose and probed with a mouse monoclonal antibody recognizing mouse CREB1 or mouse ATF2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoblots were incubated with secondary antibodies conjugated to horseradish peroxidase and then analyzed by chemiluminescent detection (Amersham Pharmacia Biotech).

FIG. 2. Barx2 expression constructs used in gel mobility shift and cellular cotransfection experiments. Barx2 constructs were generated by insertion of fragments of the Barx1 and Barx2 cDNAs into a modified pGEM3Z expression vector containing an amino-terminal Myc epitope tag. N, amino-terminal domain; HD, homeodomain; 17, 17-amino acid BBR; C, carboxyl-terminal domain. Constructs DelA-HD, DelB-HD, and DelC-HD encode proteins containing truncated amino-terminal domains and the homeodomain. Constructs DelA, DelB, and DelC encode the same amino-terminal deletion fragments with the addition of the BBR and the carboxyl-terminal domain. Two significant features of the amino-terminal domain, a leucine zipper and a polyalanine tract, are indicated by open and filled boxes, respectively.

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experiments.

The isolated Barx2 homeodomain (HD) was sufficient for binding to each of the three probes (Fig. 4, lanes 3). However, the addition of the amino-terminal region to the homeodomain fragment (NHD) reduced binding relative to the HD fragment (Fig. 4, lanes 2) indicating that the amino-terminal domain inhibited binding of the homeodomain to all of the CAM sequences.

The relative levels of binding of each of the Barx fragments to the three CAM gene probes was measured in several experiments and expressed as a percentage of the binding strength of the HD fragment to each probe (Table I). The addition of the BBR to the homeodomain (HD17) reduced the level of binding to the native L1 and Ng-CAM gene sequences to 58 and 31%, respectively, whereas it increased binding to the N-CAM sequence by 6.5-fold, relative to the HD alone. Barx2 fragments containing the amino and carboxyl-terminal domains also showed significantly less binding than HD17. The addition of the amino-terminal domain to HD17 led to a further reduction in the level of binding (down to 13%, 11%, and 2.5-fold, respectively), and the addition of the carboxyl-terminal domain to HD17 also resulted in decreased binding (down to 33%, 23%, and 6.02-fold, respectively) as compared with the level of binding to L1, Ng-CAM, and N-CAM probes by the HD fragment.

This quantitative assessment of binding reflects the qualitative observations in individual gel mobility shift experiments, indicating that the BBR and both the amino- and carboxyl-terminal domains influence the overall level of binding of the Barx2 homeodomain to all of the CAM gene target sequences.

Activity of Barx2 Protein Regions in Cellular Transfection Experiments—To determine the domains of Barx2 that regulate N-CAM promoter activity, several of the Barx2 deletion constructs shown in Fig. 2 were co-transfected with synthetic or native N-CAM HBS luciferase reporter plasmids in Neuro 2A neuroblastoma and NIH3T3 fibroblast cell lines. Neuro 2A and NIH3T3 cells have been shown in previous experiments to express high and moderate levels of N-CAM mRNA and protein, respectively. The two HBS-containing synthetic promoters used in these studies were HBS\textsubscript{2}/SV40\textsubscript{pro}/luc and HBS\textsubscript{1}/SV40\textsubscript{pro}/luc. The HBS+ promoter construct contained the 47-base pair HBS region of the mouse N-CAM promoter upstream of a minimal SV40 early promoter. The HBS− promoter construct contained a similar cassette from the N-CAM promoter in which the ATTA motifs within each HBS were mutated (Fig. 5).

As shown in Fig. 6A, the HBS+ promoter construct showed a high level of basal activity in Neuro 2A cells in the absence of any co-transfected homeobox gene expression plasmid. In contrast, the HBS− construct showed a background level of luciferase activity that was comparable with that produced by the pGL3 promoter vector. These data indicate that mutation of the ATTA motifs within the homeodomain binding sites eliminated activation of the promoter by endogenous homeodomain proteins in Neuro 2A cells. Co-transfection of four different Barx2 expression plasmids (Barx2, NHD, HD, and HD17) repressed the activity of the HBS+ promoter construct. This repressive effect on the HBS+ promoter by Barx2 was in contrast to that of HoxB9, which strongly activates this promoter (19). Full-length Barx2 induced a 3-fold repression of the HBS+ reporter, whereas NHD (encoding the amino-terminal region and the homeodomain) induced an approximately 6-fold repression (Fig. 6). The constructs HD, encoding the homeodomain, and HD17, encoding the homeodomain as well as the 17-amino acid BBR, induced a similar level of repression of HBS+ reporter activity as native Barx2.

Overall, these results indicate that binding of the Barx2 homeodomain to the HBS blocks activation of the HBS+ promoter by endogenous homeodomain pro-
relative luciferase activity

Table I
Differential binding of Barx proteins to CAM gene HBS sequences

Relative strengths of binding for different Barx protein fragments to each CAM gene probe are shown. Data were compiled from seven different gel mobility shift experiments similar to those shown in Fig. 4. The amount of radiolabeled probe in each of the DNA complexes was quantified using a PhosphorImager. The binding of the HD fragment of Barx2 to each of the three probes was assigned a value of 1. Binding strengths of the other Barx protein fragments were then expressed as ratios of their band intensities relative to that of the HD fragment. S.E. values for binding values are shown.

![Graph](http://www.jbc.org/Downloadedfrom)

**Fig. 6.** Co-transfection of Barx2 expression plasmids reduces the activity of both synthetic and native N-CAM promoter constructs in Neuro 2A and NIH3T3 cells. A, expression plasmids encoding either full-length Barx2 or the Barx2 protein fragments NHD, HD, and HD17 (see Fig. 2) were co-transfected with synthetic N-CAM promoter/luciferase constructs in Neuro 2A cells. An expression plasmid encoding HoxB9, which is known to activate the N-CAM promoter, was used as a positive control. The synthetic reporter plasmids contain the −552 to −514 HBS region of the mouse N-CAM promoter driving a minimal SV40 early promoter and a luciferase gene cassette. HBS-I and II are shown, and the ATTA motifs that represent the core binding sequences are indicated by boxes. The base pair substitutions in the ATTA motifs are indicated by gray circles. The native N-CAM promoter construct (N-CAM1.0luc) was generated by inserting a 1-kb 

**Fig. 5.** Four synthetic HBS-containing and native N-CAM promoter luciferase constructs used in co-transfection experiments with Barx2. Synthetic promoter constructs designated HBS+/SV40pro/luc and HBS−/SV40pro/luc contain the −552 to −514 HBS region of the mouse N-CAM promoter driving a minimal SV40 early promoter and a luciferase gene cassette. HBS-I and II are shown, and the ATTA motifs that represent the core binding sequences are indicated by boxes. The base pair substitutions in the ATTA motifs are indicated by gray circles. The native N-CAM promoter construct (N-CAM1.0luc) was generated by inserting a 1-kb BamHI/SacI fragment from the mouse N-CAM promoter into the plasmid pGL3 basic. The two HBS sites are indicated by hatched circles. A version of this reporter plasmid (N-CAM1.0lucHBS−) was generated by deletion of HBS-I and II.

Co-regulation of the native N-CAM promoter by Barx2 constructs

Functional Domains of the Homeodomain Protein Barx2

In vitro translated Barx protein

| Sequence | In vitro translated Barx protein |
|----------|---------------------------------|
| L1       | HD     | HD17   | NHD    | NHD17  | Barx2  | Barx1  |
| Ng-CAM   | 1.0 ± 0.10 | 0.33 ± 0.03 | 0.22 ± 0.04 | 0.13 ± 0.02 | 0.19 ± 0.05 | 0.21 ± 0.04 | 0.26 ± 0.07 |
| N-CAM    | 1.0 ± 0.10 | 0.23 ± 0.02 | 0.19 ± 0.01 | 0.11 ± 0.01 | 2.46 ± 0.38 | 4.11 ± 0.10 | 5.09 ± 0.64 |

Table I
Differential binding of Barx proteins to CAM gene HBS sequences

Relative strengths of binding for different Barx protein fragments to each CAM gene probe are shown. Data were compiled from seven different gel mobility shift experiments similar to those shown in Fig. 4. The amount of radiolabeled probe in each of the DNA complexes was quantified using a PhosphorImager. The binding of the HD fragment of Barx2 to each of the three probes was assigned a value of 1. Binding strengths of the other Barx protein fragments were then expressed as ratios of their band intensities relative to that of the HD fragment. S.E. values for binding values are shown.

![Graph](http://www.jbc.org/Downloadedfrom)

**Fig. 6.** Co-transfection of Barx2 expression plasmids reduces the activity of both synthetic and native N-CAM promoter constructs in Neuro 2A and NIH3T3 cells. A, expression plasmids encoding either full-length Barx2 or the Barx2 protein fragments NHD, HD, and HD17 (see Fig. 2) were co-transfected with synthetic N-CAM promoter/luciferase constructs in Neuro 2A cells. An expression plasmid encoding HoxB9, which is known to activate the N-CAM promoter, was used as a positive control. The synthetic reporter plasmids contain the −552 to −514 HBS region of the mouse N-CAM promoter driving a minimal SV40 early promoter and a luciferase gene cassette. HBS-I and II are shown, and the ATTA motifs that represent the core binding sequences are indicated by boxes. The base pair substitutions in the ATTA motifs are indicated by gray circles. The native N-CAM promoter construct (N-CAM1.0luc) was generated by inserting a 1-kb BamHI/SacI fragment from the mouse N-CAM promoter into the plasmid pGL3 basic. The two HBS sites are indicated by hatched circles. A version of this reporter plasmid (N-CAM1.0lucHBS−) was generated by deletion of HBS-I and II.

Co-regulation of the native N-CAM promoter by Barx2 constructs

Functional Domains of the Homeodomain Protein Barx2

In vitro translated Barx protein

| Sequence | In vitro translated Barx protein |
|----------|---------------------------------|
| L1       | HD     | HD17   | NHD    | NHD17  | Barx2  | Barx1  |
| Ng-CAM   | 1.0 ± 0.10 | 0.33 ± 0.03 | 0.22 ± 0.04 | 0.13 ± 0.02 | 0.19 ± 0.05 | 0.21 ± 0.04 | 0.26 ± 0.07 |
| N-CAM    | 1.0 ± 0.10 | 0.23 ± 0.02 | 0.19 ± 0.01 | 0.11 ± 0.01 | 2.46 ± 0.38 | 4.11 ± 0.10 | 5.09 ± 0.64 |
were prepared and examined for their effect on N-CAM promoter activity in co-transfection experiments of Neuro 2A cells. Schematic representations of the Barx2 constructs are shown on the left; the corresponding activity levels of reporter constructs in co-transfection experiments with each Barx2 construct are shown on the right. Barx2, NHD, HD, and HD17 constructs (which were used in the set of experiments shown in Fig. 6) were also used in experiments with additional Barx2 deletion constructs to provide a reference standard to which the activities of the new Barx2 constructs could be compared. The new deletion constructs include DelA-HD, DelB-HD, and DelC-HD, which encode Barx2 proteins having deletions of the amino-terminal domain. These constructs extend only to the homeodomain. Constructs DelA, DelB, and DelC have the same amino-terminal deletions but also extend to the carboxyl terminus. Relative luciferase activities of N-CAM1.0uc and N-CAM1.0ucHBS reporter constructs are shown in gray and white, respectively. LZ, leucine zipper; PT, polyalanine tract; AD, acidic domain. Data shown are derived from five independent experiments, performed in triplicate.

**Fig. 7.** Barx2 contains domains that mediate activation and repression of the N-CAM promoter. Twelve expression plasmids encoding either native Barx2 or truncated variants of Barx2 were co-transfected with the N-CAM1.0uc and N-CAM1.0ucHBS reporter plasmids in Neuro 2A cells. Schematic representations of the Barx2 constructs are shown on the left; the corresponding activity levels of reporter constructs in co-transfection experiments with each Barx2 construct are shown on the right. Barx2, NHD, HD, and HD17 constructs (which were used in the set of experiments shown in Fig. 6) were also used in experiments with additional Barx2 deletion constructs to provide a reference standard to which the activities of the new Barx2 constructs could be compared. The new deletion constructs include DelA-HD, DelB-HD, and DelC-HD, which encode Barx2 proteins having deletions of the amino-terminal domain. These constructs extend only to the homeodomain. Constructs DelA, DelB, and DelC have the same amino-terminal deletions but also extend to the carboxyl terminus. Relative luciferase activities of N-CAM1.0uc and N-CAM1.0ucHBS reporter constructs are shown in gray and white, respectively. LZ, leucine zipper; PT, polyalanine tract; AD, acidic domain. Data shown are derived from five independent experiments, performed in triplicate.

**Interaction of Barx2 with Proteins of the CREB Family**—In view of the observation that the amino-terminal region of Barx2 contains a putative leucine zipper, we investigated whether Barx2 is capable of interacting with other leucine zipper proteins. In particular, we analyzed interactions of Barx2 with the most prominent members of the CREB and Fos/Jun families of factors that contain leucine zipper domains. To identify transcription factors that interact with Barx2, a GST-Barx2 fusion protein was produced in *E. coli* and tested for binding to proteins from Neuro 2A cells in co-precipitation (pull-down) experiments. glutathione-Sepharose resin with bound Barx2-GST fusion protein was incubated with protein extracts from Neuro 2A cells or Neuro 2A cells that had been transfected with an expression plasmid that encodes human CREB1 with an amino-terminal hemagglutinin (HA) peptide tag (HA-hCREB1). Incubated proteins were washed in a high salt buffer to remove nonspecifically bound proteins. Glutathione-Sepharose-bound protein complexes were then eluted from the resin and resolved by SDS-PAGE, and the identities of the proteins that co-precipitated with Barx2 were examined by immunoblot analyses using antibodies to particular leucine zipper-containing transcription factors.

As shown in Fig. 8, Barx2 formed complexes with two members of the CREB family, CREB1 and ATF2. These proteins were identified in immunoblot analyses using antibodies to particular leucine zipper-containing transcription factors.

As shown in Fig. 8, A (lane 3) and B, respectively. To further substantiate the interaction with CREB1, Neuro 2A cells were transfected with an expression plasmid encoding human CREB1 and containing an amino-terminal hemagglutinin tag (HA-hCREB1),
and extracts from these cells were used in pull-down assays with the Barx2-GST fusion protein. In HA-hCREB1-transfected Neuro 2A cells, both the endogenous mouse CREB1 and the HA-tagged human CREB1 proteins were recognized in immunoblot analyses with the mouse anti-CREB antibody (Fig. 8A, left panel, lane 4). In addition, the HA-hCREB1 protein that was bound to Barx2 was also identified in immunobots using an anti-HA antibody (Fig. 8A, right panel, lane 4). In these experiments, the HA-tagged CREB protein recognized by both anti-CREB and anti-HA antisera showed a greater molecular weight than the endogenous CREB due to the presence of the HA epitope tag. These experiments indicate that Barx2 interacts with members of the CREB family, possibly via leucine zipper motifs. Similar pull-down experiments of Neuro 2A proteins using antibodies to c-Fos and c-Jun proteins revealed only a very low level of interaction of these transcription factors with Barx2 (V10,000 of the signal intensity obtained with the anti-CREB and ATF antibodies; data not shown).

**DISCUSSION**

Our ongoing studies have shown that homeobox genes, which regulate regional patterns of gene expression, have as targets certain CAMs that mediate interactions leading to morphogenesis (22). In particular, we have investigated the regulation of genes encoding three CAMs of the immunoglobulin superfamily, L1, Ng-CAM, and N-CAM. All of these genes contain HBSs that are composed of a conserved ATTA core motif but vary with respect to base pairs flanking this motif. The sequence composition of the HBS determines which homeodomain proteins can interact with these CAM gene regulatory sequences. HBS sequences have been shown to regulate the expression of CAM genes in cultured cells (19, 32), and mutation of these sequences leads to aberrant patterning of CAM gene expression in transgenic mice (20, 21). Very little is known about the functional domains of the proteins that bind to these elements and how they affect transcriptional activation or repression of target genes.

Barx1 and Barx2 were originally identified in Southwestern screening procedures as novel factors that interact with HBS sequences of the N-CAM and Ng-CAM genes, respectively (24, 25). The present study was carried out to identify functional domains within the Barx2 protein using CAM gene regulatory elements as model targets. We first identified domains of Barx2 that are involved in binding to DNA elements from three CAM genes and then defined domains within Barx2 that mediate both transcriptional repression and activation of the mouse N-CAM gene.

**Regions of Barx2 That Mediate Sequence-specific DNA Binding—**In gel mobility shift analyses using in vitro translated proteins and probes derived from the three CAM gene HBSs, binding complexes formed between native Barx2 and the N-CAM HBSs were considerably more intense (11- and 22-fold, respectively) than those formed with either the L1-HPD or the Ng-CAM HBS sequences. The homeodomain of Barx2 was sufficient for binding to each of these sequences. The binding preference of the homeodomain in isolation differed greatly from that of native Barx2, whereas a protein fragment of Barx2 containing the homeodomain and adjacent 17-amino acid BBR recapitulated the binding pattern of native Barx2. Moreover, these results indicate that the BBR can modulate binding of the Barx2 homeodomain to DNA targets in a sequence-specific manner. Since our results were obtained with in vitro-translated proteins, modulation of binding by the BBR does not require interaction with other cellular factors. Therefore, modulation of Barx2 binding to DNA by the BBR is likely to involve either direct binding of the peptide to DNA or a change in the conformation of the homeodomain that alters its ability to bind DNA. The proximity of the BBR to the DNA-binding helix of the homeodomain lends support for both of these possible mechanisms. The BBR contains consensus serine phosphorylation sites, and our recent experiments indicate that phosphorylation of Barx2 reduces binding to all three CAM gene regulatory sequences. Thus, it appears that altering the net positive charge of the BBR is likely to affect the DNA binding function of Barx2 proteins. Such modulation of DNA binding by an adjacent peptide domain has been observed in other homeodomain proteins. For instance, the POU domain can bind independently to DNA and also change the DNA target specificity of the POU type homeodomain (12).

The amino-terminal domain of Barx2 also had an effect on DNA binding. The addition of this region to either the homeodomain or the homeodomain plus the BBR reduced the level of binding to all three HBS sequences of the CAM genes used in this study. The negative effect of the amino-terminal region on DNA binding may also be important for the function of this domain in the transcriptional regulation of target genes. Modulatory effects of amino-terminal domains on DNA binding and

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R. Meech, D. B. Edelman, and F. S. Jones, unpublished results.
transcriptional regulation have been noted for a number of other homeodomain proteins including Bicoid, extradenticle, MEC-3, UNC-86, and the yeast mating-type protein α2 (33–36). Barx2 Domains That Mediate Repression and Activation of Transcription—In an earlier study that examined the regulation of the L1 cell adhesion molecule, it was concluded that Barx2 contains domains that mediate both repression and activation (25). These conclusions were based on the observation that L1 constructs containing the HPD element were activated, whereas similar constructs without the HPD were repressed by Barx2. In the present study, we have defined domains of Barx2 that have activator or repressor functions by examining amino-terminal and carboxyl-terminal Barx2 deletion constructs in co-transfection experiments using either synthetic HBS-containing promoters or native N-CAM promoter constructs. In our minimal HBS-containing promoter construct, activation of transcription is contingent on proteins binding to the HBS. However, in experiments involving the native N-CAM promoter, the HBS element represents only one of many elements that are known to be involved in the regulation of this complex promoter.

In experiments using the synthetic promoter constructs, native Barx2 and three other Barx2 deletion constructs (NHD, HD, and HD17) reduced promoter activity. These results suggest a simple mechanism for repression in which the homeodomain of Barx2 prevents the binding of endogenous activators to the HBS. However, in our experiments with the native N-CAM promoter, only constructs containing the amino-terminal domain (constructs designated Barx2 and NHD) reduced the level of N-CAM promoter activity, suggesting that binding of the homeodomain alone is not sufficient to repress N-CAM promoter activity. In order for Barx2 to repress the N-CAM promoter, it must affect the function of activators bound at the HBS as well as those bound by other cis elements located within this promoter. The data therefore support the idea that the amino-terminal domain of Barx2 reduced N-CAM promoter activity by interaction either with activator proteins bound to the N-CAM promoter or components of the basic transcription machinery.

Co-transfection experiments using Barx2 deletion constructs revealed that a minimal region of 24 amino acids at the extreme amino terminus was required for repression of the N-CAM promoter. A significant feature of this region is a leucine zipper motif. Leucine zippers are found in many transcription factors but also the function of the Barx2 activation domain. The activation domain is located within the carboxyl-terminal 63 amino acids of the protein and was unmasked only when the amino-terminal repressor domain was removed from Barx2. In the context of the N-CAM promoter, the repressor domain has the capacity to block not only the function of exogenous activators but also the function of the Barx2 activation domain. The capacity of Barx2 to act as activator or repressor is likely to depend on the nature of its interactions with other proteins.

Interaction of Barx2 with Transcription Factors of the CREB Family—Binding and co-transfection experiments supported the hypothesis that protein-protein interactions are likely to play a role in the ability of Barx2 to repress the activity of the N-CAM promoter. The amino-terminal region of the Barx2 cDNA encodes a region of 50 amino acids that resemble a leucine zipper that might bind to other transcription-factor-containing transcription factors. Moreover, in GST pull-down experiments, we found that Barx2 bound to two members of the CREB family, CREB1 and ATF2. These findings suggest that Barx2 and CREB proteins might modulate each other’s function in the cellular contexts in which these proteins are co-expressed.

CREB proteins are transcription factors that bind to cyclic AMP-response elements (CREs) found in a large number of genes and play important roles during development of neural and nonneural tissues and in synaptic plasticity (for a review, see Ref. 37). These factors are commonly referred to as constitutive transcription factors because they are ubiquitously and constitutively expressed and can be bound to DNA in inactive forms that are activated by phosphorylation (38). Heterodimerization of CREB family members with other transcription factors leads to the formation of complexes that have unique functions and DNA binding properties. For instance, CREB1 heterodimerizes with ATF1 to form a protein complex that has an altered binding affinity for the CRE sequences (39). ATF2 heterodimerizes with ATF3, NF-xB, and c-Jun, and complexes with c-Jun can recognize TRE/AP-1 elements in addition to CREs (40, 41). Future experiments with both HBS- and CRE-containing promoters will be necessary to determine whether Barx2 can modulate the function of CREB family members and whether complexes containing Barx2 and CREB proteins are involved in transcriptional repression by Barx2.

The hypothesis that the function of Barx2 might be modulated by interactions with other factors, specifically members of the CREB family, could be further investigated in the context of genes that contain binding sites for both of these factors. One such gene is that encoding the cell adhesion molecule Ng-CAM. Its promoter contains both HBS and CRE sequences that are important for its regulation (42). The interaction of Barx2 and CREB proteins might also provide a means of indirect regulation by Barx2 of genes that contain CRE sequences but do not contain HBS sequences. Conversely, CREB may be able to regulate genes that do not contain a CRE but contain homeodomain binding sites and are bound by Barx2. The challenge for future experiments is to define developmental and physiological contexts in which Barx and CREB proteins interact. Functional integration of these various factors is likely to take place during development of the nervous system where regional regulation of neuronal differentiation and axonal path-finding by homeobox proteins and activity-dependent transcriptional regulation by CREB proteins converge.

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