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Hox Proteins Have Different Affinities for a Consensus DNA Site That Correlate with the Positions of Their Genes on the *hox* Cluster

ISABELLE PELLERIN,[1,2] CATHERINE SCHNABEL,[1,3] KATRINA M. CATRON,[1,2] AND CORY ABATE[1,2]*

Center for Advanced Biotechnology and Medicine,[1] Department of Neuroscience and Cell Biology, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School,[2] and Program in Cell and Developmental Biology, Rutgers University,[3] Piscataway, New Jersey 08854-5638

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The *hox* genes, members of a family of essential developmental regulators, have the intriguing property that their expression in the developing murine embryo is colinear with their chromosomal organization. Members of the *hox* gene family share a conserved DNA binding domain, termed the homeodomain, which mediates interactions of Hox proteins with DNA regulatory elements in the transcriptional control regions of target genes. In this study, we characterized the DNA binding properties of five representative members of the Hox family: HoxA5, HoxB4, HoxA7, HoxC8, and HoxB1. To facilitate a comparative analysis of their DNA binding properties, we produced the homeodomain regions of these Hox proteins in *Escherichia coli* and obtained highly purified polypeptides. We showed that these Hox proteins interact in vitro with a common consensus DNA site that contains the motif (C/G)TAATTG. We further showed that the Hox proteins recognize the consensus DNA site in vivo, as determined by their ability to activate transcription through this site in transient transfection assays. Although they interact optimally with the consensus DNA site, the Hox proteins exhibit subtle, but distinct, preferences for DNA sites that contain variations of the nucleotides within the consensus motif. In addition to their modest differences in DNA binding specificities, the Hox proteins also vary in their relative affinities for DNA. Intriguingly, their relative affinities correlate with the positions of their respective genes on the *hox* cluster. These findings suggest that subtle differences in DNA binding specificity combined with differences in DNA binding affinity constitute features of the "Hox code" that contribute to the selective functions of Hox proteins during murine embryogenesis.

The development of the murine embryo requires an elaborate program of spatial and temporal signals. These signals direct the activity of transcriptional regulatory proteins which, in turn, guide the expression of target genes that specify cellular phenotypes. Many transcriptional regulatory proteins that function during development are encoded by members of a gene family that contain a conserved sequence motif termed the homeobox (29, 33, 40, 60). The homeobox encodes a DNA binding domain (the homeodomain) that was first identified as a conserved feature of *Drosophila* homeotic genes (47) and subsequently shown to be present in numerous genes whose products regulate transcription during development (3, 22, 31, 33, 60). For mice, more than 60 members of the homeobox gene family have been isolated (33). A majority are members of the *hox* family, which contains 38 genes distributed on four chromosome clusters (Fig. 1A) (3, 33, 48). The *hox* genes are expressed throughout murine embryogenesis (from embryonic day 8.5) in cells contributing to the limbs, mesodermal structures, the neural tube and its derivatives, the neural crest, and the surface ectoderm (15, 29, 33, 65), and studies of both loss and gain of function have demonstrated that these genes are essential for normal development (4, 11, 34, 39, 42, 43, 45, 55).

The *hox* genes have many features in common with the *Drosophila* homeotic genes, including primary sequence, chromosomal organization, and regional expression (Fig. 1A) (3, 48). The homeobox regions of the murine *hox* genes are closely related to the *Drosophila* homeotic genes (60). Moreover, the organization of the murine *hox* genes within chromosome clusters is a feature that has been conserved from their *Drosophila* counterparts (Fig. 1A) (17, 24, 48). The mouse genome contains four *hox* clusters that have presumably arisen by gene duplication of a single ancestral precursor (6). The murine and *Drosophila* genes that are located at the same relative positions within their respective gene clusters share the highest degree of homology, particularly in their homeobox sequences, and are referred to as paralogs (48). The murine *hox* genes and the *Drosophila* homeotic genes have the intriguing property that their positions within the chromosome cluster are colinear with their boundaries of expression during embryogenesis (15, 30, 48, 65). Therefore, genes located more 3′ on the cluster have more anterior boundaries of expression, whereas genes located more 5′ on the cluster have more posterior boundaries of expression (Fig. 1B). This colinearity of chromosomal organization and pattern of expression is presumed to represent a molecular code that specifies positional information along the anterior-posterior body axis during development. It is well established that the *Drosophila* homeotic genes specify positional information during embryogenesis (48), and their extensive similarity to the murine *hox* genes suggests that the murine genes have a similar function. Indeed, targeted disruptions of certain murine *hox* genes produce homeotic transformations during embryogenesis that are reminiscent of the homeotic mutations produced in their *Drosophila* counterparts (39, 55).

Despite their provocative biological roles, comparatively little is known about the biochemical properties of Hox proteins. In particular, the mechanisms that promote selective interactions of related Hox proteins with distinct DNA target

* Corresponding author. Mailing address: CABM, 679 Hoes Lane, Piscataway, NJ 08854-5638. Phone: (908) 235-5161. Fax: (908) 235-4850.

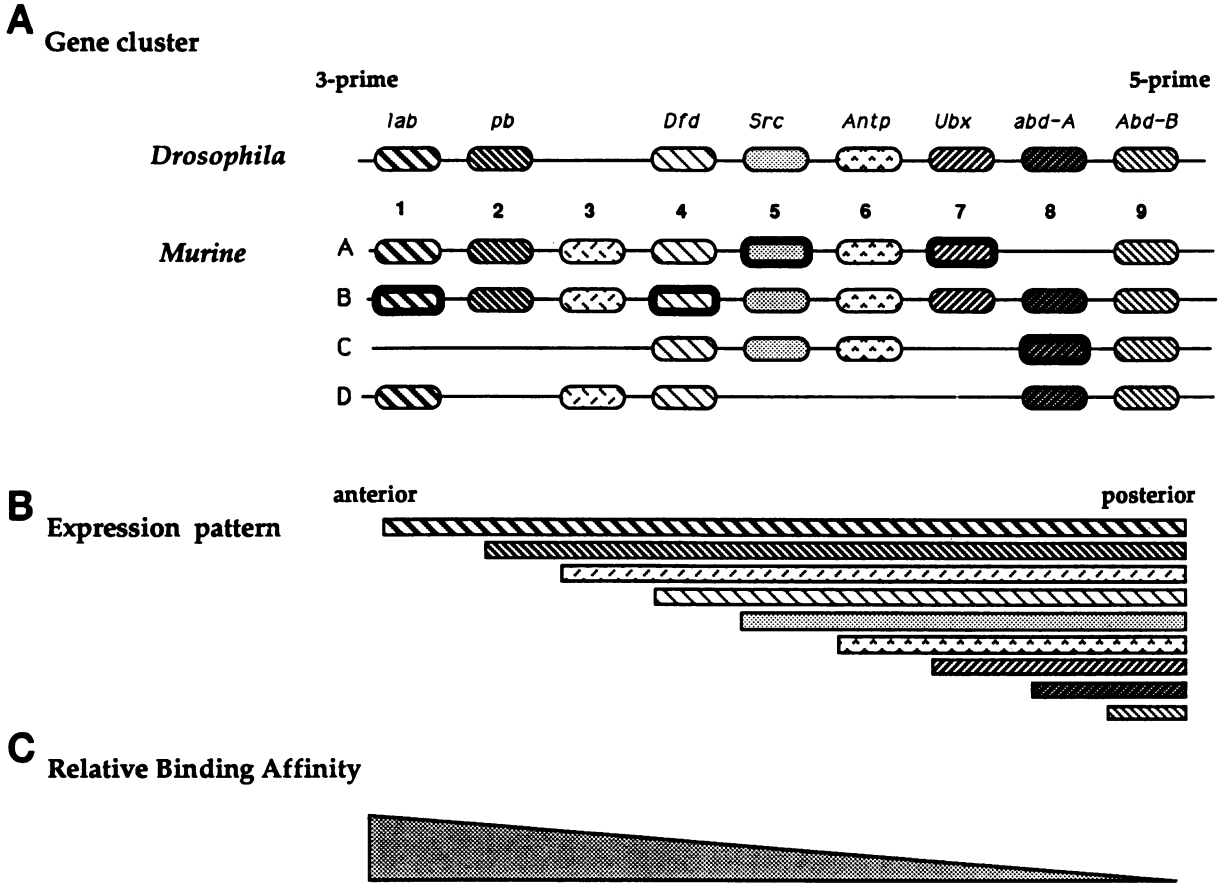

FIG. 1. Relationship of *hox* gene organization, pattern of expression, and relative DNA binding affinities of Hox proteins. (A) Alignment of the *Drosophila* homeotic complex and the murine *hox* complexes (*hoxABCD*). Genes located at the same relative positions within the complexes (i.e., positions 1 to 9 as shown) share a high degree of homology, particularly in the homeobox; these genes are referred to as paralogs and are designated by the similar shading of the ovals. The Hox proteins studied in this report are encoded by the genes outlined in boldface type (i.e., *hoxB1*, *hoxB4*, *hoxA5*, *hoxA7*, and *hoxC8*). (B) Schematic illustration of the pattern of expression of the *hox* genes along the anterior-posterior body axis. Each bar corresponds to the relative domain of expression of each paralogous gene (shading as in panel A). A colinear relationship exists between the positions of the *hox* genes on the clusters and their anterior boundaries of expression. (C) Correlation of the relative affinities of the Hox proteins with the positions of their respective genes on the *hox* gene clusters. As represented by the shaded triangle, the genes located more 3′ on the clusters encode proteins that have a relatively high affinity for DNA, whereas the genes located more 5′ encode proteins that have a relatively low affinity for DNA.

sequences have not been well defined. In this report, we compare the DNA binding properties of five Hox proteins: HoxA5, HoxA7, HoxB4, HoxB1, and HoxC8 (according to the new nomenclature; 59). We demonstrate that these Hox proteins interact with a common consensus DNA site in vitro and in vivo. We further show that these Hox proteins exhibit modest preferences for DNA sites containing variations of the consensus motif. These proteins also vary in their relative affinities for DNA, and their affinities are correlated with the positions of their respective genes on the *hox* cluster. These observations suggest that differential DNA binding properties of closely related Hox proteins contribute to their selective functions during embryogenesis.

MATERIALS AND METHODS

Production of HoxA5, HoxA7, HoxC8, HoxB1, and HoxB4. The sequences corresponding to the homeobox regions of *hoxA5*, *hoxA7*, and *hoxC8* were isolated from 9.5-day-old P.C. mouse embryonic RNA (a generous gift from J. McMahon and A. McMahon, Harvard University) by reverse transcription-PCR. The sequences corresponding to the homeobox regions of *hoxB1* and *hoxB4* were isolated from the full-length cDNAs (kindly provided by R. Krumlauf, National Institute for Medical Research) by PCR amplification. The oligonucleotides used for PCR amplification corresponded to the 5′ and 3′ regions of the respective homeobox sequences. These oligonucleotides also contained unique restriction sites (*Bam*HI and *Hin*dIII) to facilitate cloning into the *Escherichia coli* expression vector pDS56 in frame with an initiator methionine codon and six histidine codons (Fig. 2A) (1, 9). The recombinant *hox* genes encode the homeodomain regions corresponding to amino acids 172 to 270 of HoxA5 (50), amino acids 110 to 190 of HoxA7 (35), amino acids 131 to 209 of HoxC8 (7), amino acids 95 to 297 of HoxB1 (21), and amino acids 134 to 250 of HoxB4 (25). The production of Hox proteins in *E. coli* and purification from bacterial lysates by nickel affinity chromatography were performed as previously described (1, 9).

DNA binding assays. Gel mobility shift assays were performed as described by Catron et al. (9). In brief, oligonucleotides were radiolabeled with T4 polynucleotide kinase in the

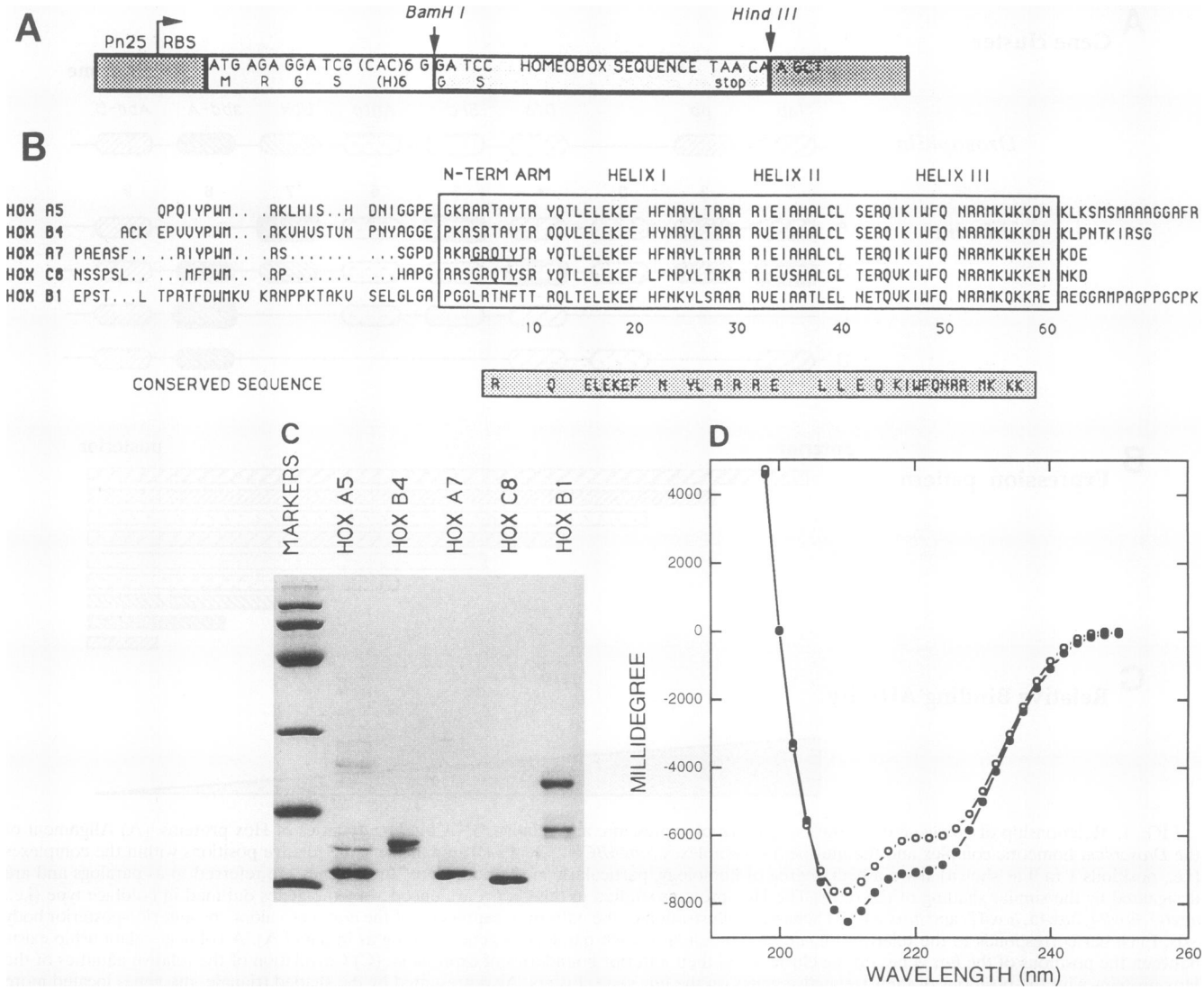

FIG. 2. Expression and purification of HoxA5, HoxB4, HoxA7, HoxC8, and HoxB1. (A) The homeobox sequences of *hoxA4*, *hoxB4*, *hoxA7*, *hoxC8*, and *hoxB1* were cloned into the *Bam*HI and *Hin*dIII sites of vector pDS56. This vector contains codons for the indicated residues, including six histidine codons [(H)6]. The expression of the recombinant *hox* genes is driven by promoter Pn25. A ribosome binding site (RBS) in close proximity to the transcription start site (indicated by the arrow) facilitates efficient translation. (B) Primary structures of the recombinant Hox proteins, as represented by the single-letter code. The homeodomain regions of the Hox proteins are boxed, and the positions of the N-terminal arm (N-TERM ARM) and helices (HELIX) I, II, and III are indicated. The amino acids conserved among the five Hox homeodomains are shown in the shaded box. The numbers (10 to 60) indicate the relative positions of amino acids within the homeodomains. (C) The Hox proteins were produced in *E. coli* as hexahistidine fusion proteins and purified by nickel affinity chromatography as detailed in Materials and Methods. The purified proteins (2.5 μg) were resolved on an SDS–13.5% polyacrylamide gel and visualized by staining with Coomassie brilliant blue. Markers correspond to molecular mass standards (Bio-Rad) in kilodaltons (bovine serum albumin, 68 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 20 kDa; and lysozyme, 14 kDa). (D) Circular dichroism analysis showing that Hox proteins have α-helical structures; the α-helix content (71%) indicates that a majority of the protein is appropriately folded. Spectra are shown for HoxB1 (●) and HoxC8 (○); similar spectra were obtained for the other Hox proteins. Spectra were obtained with 0.1 mg of protein per ml in buffer containing 10 mM potassium phosphate (pH 7.0). Spectra were recorded in a 1-mm jacketed cuvette maintained at 25°C. Data were collected in triplicate from 260 to 198 nm, with a step size of 0.25 nm.

presence of [γ-^{32}P]ATP and annealed at 37°C at equimolar concentrations. Binding reaction mixtures contained 10 mM Tris-HCl (pH 7.5), 50 mM sodium chloride, 1 mM EDTA, 7.5 mM $MgCl_2$, 5% (vol/vol) glycerol, 5% (wt/vol) sucrose, 0.1% Nonidet P-40, 0.5-mg of bovine serum albumin per ml, 10 mM dithiothrietol, and 0.025 mg of dI-dC per ml. Equilibrium dissociation experiments were performed as described previously (9). The equilibrium dissociation constant (K_d) was calculated by Lineweaver-Burk analysis and, alternatively, by Scatchard analysis (9). All DNA binding assays were repeated a minimum of four times, and representative data are presented.

1,10-Phenanthroline–copper (Cu^{2+}-OP) footprinting was performed with a 45-bp DNA fragment that contained the consensus DNA site (61). The fragment was ^{32}P end labeled by PCR amplification with one radiolabeled primer and one unlabeled primer (61). Protein-DNA complexes were formed and resolved from free DNA by polyacrylamide gel electro-

phoresis. Subsequent to electrophoresis, the gel was immersed in 50 mM Tris-HCl (pH 8.0) (100 ml). DNA cleavage was initiated by the addition of 2 mM Cu^{2+}-OP (10 ml) and 58 mM 3-mercaptopropionic acid (10 ml) (62). Cleavage reactions proceeded for 10 min and were stopped by the addition of 28 mM 2,9-dimethyl-1,10-phenanthroline (10 ml). Following autoradiography, free and bound DNAs were isolated, cleavage products were precipitated, and DNA fragments were resolved on a 10% polyacrylamide–6 M urea gel.

Methylation interference experiments were performed with a 45-bp end-labeled DNA fragment (as above) that was partially methylated with dimethyl sulfate (46, 66). Protein-DNA complexes were formed, and bound DNA was resolved from free DNA by gel electrophoresis. Bound and free DNAs were identified by autoradiography, eluted from the gel, and cleaved with 1 M piperidine (46, 66). DNA fragments were resolved on a 10% polyacrylamide–6 M urea gel.

Transient transfection assays. NIH 3T3 cells were seeded 16 to 24 h prior to transfection at 10^5 cells per 35-mm dish in Iscove's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 50 U of penicillin per ml, 50 μg of streptomycin per ml, and 2 mM L-glutamine. Transfection assays were performed by the calcium phosphate procedure essentially as described previously (57). The luciferase reporter plasmid (pGL2 promoter vector; Promega) was modified to contain three copies of the consensus DNA site (site 6) or a mutated DNA site (site 6Q) cloned upstream of the simian virus 40 (SV40) promoter. Sequences encoding the homeobox of *hoxA7* were cloned alone or in frame with sequences encoding the VP16 activation domain (56) (a generous gift from C. Kunsch and C. Rosen, Human Genome Sciences) into the mammalian expression vector pCb6+ (52). A cytomegalovirus–β-galactosidase vector (63) (a generous gift from J. Morris and F. Rauscher, Wistar Institute) was used as an internal control to monitor transfection efficiency. Cells were harvested 48 h after transfection with 1× reporter lysis buffer (Promega). β-Galactosidase assays were performed according to the manufacturer's instructions (Promega). Luciferase activity was measured by scintillation counting with the luciferase assay system (Promega), and counts were normalized to levels of β-galactosidase activity.

RESULTS

Expression and purification of Hox proteins. Although Hox proteins share a high degree of homology in their DNA binding domains, they have distinct functions in vivo that are presumed to be mediated in part by differences in DNA binding specificities (10, 14, 23, 37, 41, 44). Therefore, a comparison of the DNA binding properties of Hox proteins is likely to provide insight into the molecular bases of their functional specificities. As representatives of the Hox family, we selected HoxA5, HoxA7, HoxB1, HoxB4, and HoxC8 for DNA binding studies. These proteins are encoded by genes distributed in the 3′ (*hoxB1*), middle (*hoxB4* and *hoxA5*), and 5′ (*hoxA7* and *hoxC8*) regions of their respective *hox* clusters (Fig. 1A). To study their DNA binding properties, we engineered polypeptides that contained the homeodomain and the amino acids directly flanking this domain (Fig. 2B). The polypeptides also contained an N-terminal hexahistidine fusion to facilitate purification by nickel affinity chromatography (Fig. 2A). The proteins were produced in *E. coli*, extracted from bacterial cells with 6 M guanidine, and purified in the presence of guanidine on a nickel affinity resin (1, 9). The purified proteins were renatured by extensive dialysis against 25 mM sodium phosphate (pH 7.4)–50 mM sodium chloride–5 mM magnesium chloride–10% (vol/vol) glycerol–1 mM dithiothreitol. This procedure yielded proteins that were approximately 90% homogeneous, as illustrated by Coomassie brilliant blue staining of a sodium dodecyl sulfate (SDS)-polyacrylamide gel (Fig. 2C). The renaturation procedure was highly efficient, since circular dichroism analysis showed that greater than 90% of each Hox protein was appropriately folded (Fig. 2D).

Hox proteins interact with a common consensus DNA site and exhibit modest preferences for DNA sites that contain variations of this motif. Numerous biochemical studies have demonstrated that homeodomain proteins interact with DNA sites that contain the core nucleotide sequence TAAT (reviewed in reference 38), and have shown that nucleotides flanking this motif contribute to DNA binding specificity (9, 14, 18, 19). In a previous study, we characterized the DNA binding specificities of three divergent homeodomain proteins, one of which, HoxA3, was a member of the Hox family (9). We identified a common DNA site [(C/G)TAATTG] that was recognized by all three proteins and showed that these homeodomain proteins interacted differentially with DNA sites that contained variations of the common DNA site (9). Therefore, to characterize the DNA binding specificities of HoxA5, HoxB4, HoxA7, HoxC8, and HoxB1, we studied their interactions with the common DNA site (Fig. 3, site 6) and with DNA sites that contained substitutions of the nucleotides within the common DNA site (Fig. 3, sites 6-1 to 6-22). Binding activity was assessed by quantitative electrophoretic mobility shift assays with an equimolar concentration of each DNA site (5 nM) and various concentrations of the Hox proteins (1 and 3 μM). Protein-DNA complexes were resolved from free DNA by polyacrylamide gel electrophoresis, and the percentage of bound complex was quantitated with a PhosphorImager (Molecular Dynamics). DNA binding activity was linear over the range of protein concentrations tested.

Analysis of the DNA binding activities of HoxA5, HoxA7, HoxB4, HoxB1, and HoxC8 revealed that each Hox protein interacted preferentially with the previously described common DNA site (with the exceptions noted below) (Fig. 3A). On the basis of these observations and our previous study (9), we conclude that this DNA site (site 6) is a consensus DNA site for members of the Hox family. Although each Hox protein interacted optimally with the consensus DNA site, each exhibited subtle, but distinct, preferences for nucleotides flanking the TAAT core (Fig. 3A). Therefore, DNA sites that contained substitutions of the nucleotides 5′ or 3′ of the TAAT core (sites 6-1 to 6-19) were bound with various degrees of efficiency by each Hox protein (Fig. 3). For example, at position 4, a substitution of C with G resulted in reduced binding activities of HoxA5, HoxB4, and (to a lesser extent) HoxB1 but did not alter the binding activity of HoxA7 or HoxC8 (Fig. 3A, cf. site 6 with site 6-4). In contrast, a substitution with A or T at position 4 reduced the binding activity of each Hox protein (Fig. 3A, cf. site 6 with sites 6-5 and 6-6).

The Hox proteins also exhibited differences in their ability to interact with DNA sites that contained substitutions of the nucleotides 3′ of the TAAT core (Fig. 3A, sites 6-9 to 6-18). For example, at position 9, a substitution of T with G was well tolerated by HoxB4 but not by the other Hox proteins (Fig. 3A, cf. site 6 with site 6-11). In contrast, an A or C at position 9 reduced the binding activity of each Hox protein (Fig. 3A, cf. site 6 with sites 6-9, 6-10, 6-17, and 6-18). At position 10, a substitution of G with A reduced the binding activity of HoxA7 but not the other Hox proteins, whereas a substitution with T or C reduced the binding activity of each Hox protein (Fig. 3A, cf. site 6 with sites 6-12 to 6-14). Interestingly, even substitutions of nucleotides several positions from the TAAT core (i.e.,

A

Fold binding activity

	HOX A5	HOX B4	HOX A7	HOX C8	HOX B1
6	1.00	1.00	1.00	1.00	1.00
6-1	0.53+/-.01	0.66+/-.05	0.56+/-.03	0.59+/-.04	0.56+/-.06
6-2	0.74+/-.05	0.91+/-.06	0.82+/-.04	0.73+/-.06	0.76+/-.06
6-3	0.70+/-.03	0.73+/-.02	0.60+/-.02	0.54+/-.06	0.54+/-.06
6-4	0.53+/-.01	0.63+/-.05	0.85+/-.05	0.96+/-.02	0.76+/-.07
6-5	0.28+/-.01	0.38+/-.06	0.45+/-.04	0.48+/-.09	0.38+/-.06
6-6	0.43+/-.04	0.59+/-.03	0.59+/-.03	0.49+/-.08	0.60+/-.04
6-7	0.76+/-.05	0.88+/-.06	0.89+/-.03	1.00+/-.08	0.95+/-.04
6-8	0.60+/-.01	0.72+/-.03	0.82+/-.09	1.13+/-.05	0.96+/-.02
6-9	0.45+/-.01	0.60+/-.06	0.51+/-.05	0.53+/-.08	0.54+/-.01
6-10	0.44+/-.03	0.43+/-.04	0.28+/-.05	0.32+/-.05	0.35+/-.01
6-11	0.64+/-.04	0.92+/-.02	0.60+/-.05	0.55+/-.02	0.58+/-.04
6-12	0.91+/-.02	1.01+/-.07	0.72+/-.02	1.03+/-.08	1.01+/-.10
6-13	0.54+/-.06	0.54+/-.07	0.44+/-.04	0.49+/-.03	0.43+/-.03
6-14	0.40+/-.03	0.40+/-.04	0.37+/-.05	0.36+/-.01	0.31+/-.01
6-15	0.85+/-.03	1.15+/-.14	1.03+/-.02	0.85+/-.06	1.06+/-.02
6-16	0.85+/-.03	1.06+/-.02	0.68+/-.06	0.54+/-.06	0.88+/-.02
6-17	0.23+/-.05	0.17+/-.02	0.31+/-.01	0.34+/-.02	0.30+/-.03
6-18	0.24+/-.03	0.25+/-.08	0.37+/-.05	0.43+/-.02	0.38+/-.01
6-19	< 0.03	<0.03	0.06+/-.01	0.15+/-.02	0.07+/-.01
6-20	0.11+/-.02	0.12+/-.02	0.30+/-.01	0.29+/-.04	0.28+/-.02
6-21	0.04+/-.01	0.09+/-.01	0.08+/-.02	0.09+/-.05	0.08+/-.01
6-22	0.23+/-.02	0.26+/-.05	0.33+/-.07	0.32+/-.04	0.25+/-.01

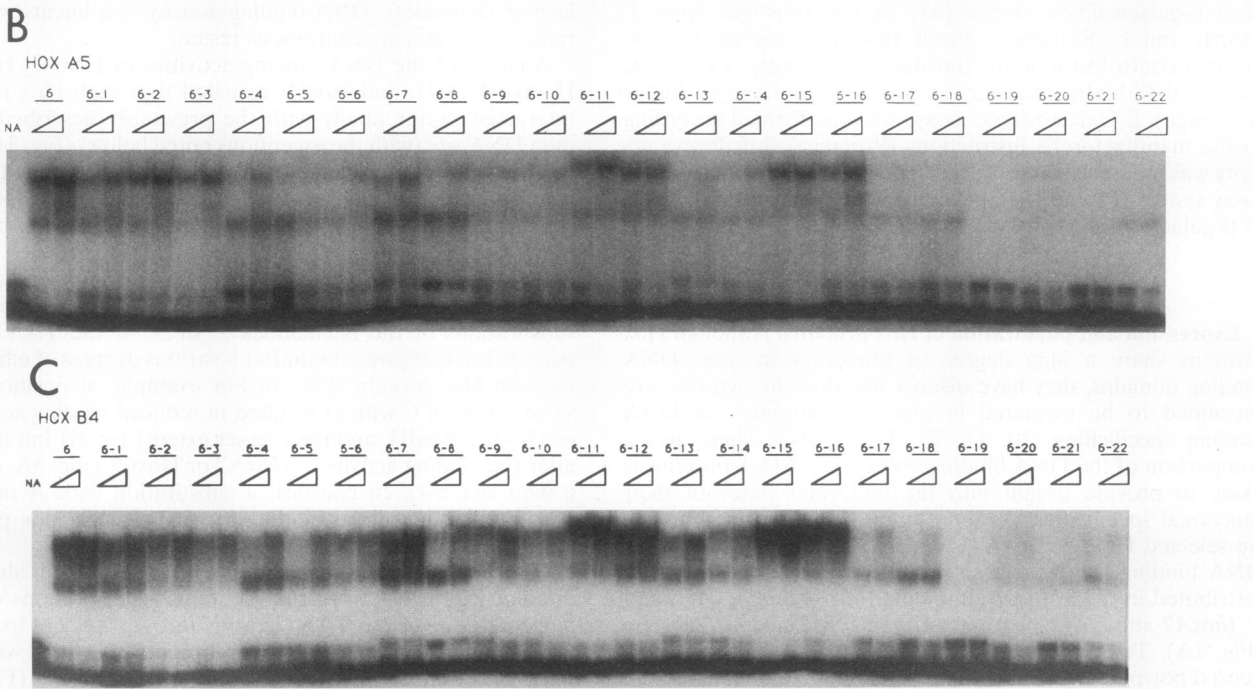

FIG. 3. Characterization of the DNA binding specificities of Hox proteins. DNA binding specificities were tested with a consensus DNA site (site 6) or DNA sites that contained variations of this site (sites 6-1 to 6-22), as indicated by the shaded boxes in panel A. Gel retardation assays were performed with 5 nM each DNA site and 1 or 3 μM each Hox protein, as indicated by triangles (HoxA5 [B], HoxB4 [C], HoxA7 [D], HoxC8 [E], and HoxB1 [F]). DNA binding activity was quantitated with a PhosphorImager. Activity was calculated as the percentage of bound DNA relative to total DNA [bound/(bound plus Free)]. DNA binding activity obtained for each Hox protein with site 6 was arbitrarily designated 1.00, and the activities obtained with the other sites were expressed relative to this value. Differences among proteins are highlighted by underlining and open boxes. The data in panel A represent the average of four independent experiments; the standard deviations are indicated. A value of <0.03 indicates that binding activity was negligible. NA in panels B to F indicates that no protein was added.

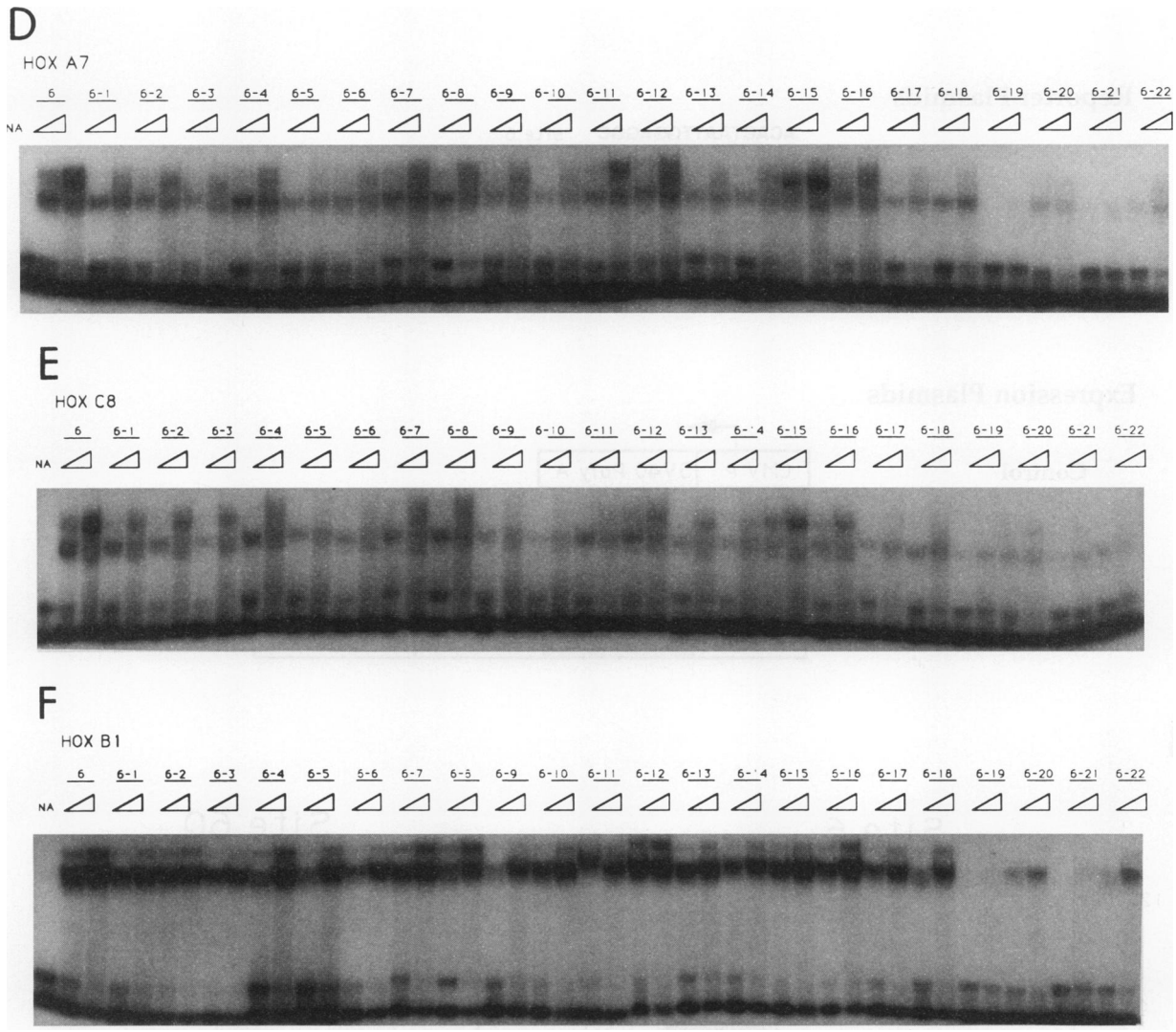

FIG. 3—*Continued.*

positions 13 and 14) affected the binding activities of HoxA7 and HoxC8, although the activities of the other Hox proteins were not altered (Fig. 3A, cf. site 6 with site 6-16). In contrast to the modest perturbations of binding activity observed with substitutions of the flanking nucleotides, substitutions of nucleotides within the TAAT core markedly reduced the binding activity of each Hox protein (Fig. 3A, cf. site 6 with sites 6-19 to 6-22). In summary, these results demonstrate that Hox proteins recognize a common consensus DNA site: (C/G)TA ATTG. The TAAT core is essential for binding activity, and nucleotides flanking the core contribute to subtle differences in the binding specificities of the various Hox proteins.

Hox proteins activate transcription through the consensus DNA site in vivo. These DNA binding studies defined a consensus DNA site that was recognized by several related Hox proteins in vitro. To determine whether the consensus DNA site was also recognized by Hox proteins in vivo, we performed transient cotransfection assays (Fig. 4). Since the purpose of the transfection assays was to test the interaction of the Hox proteins with the consensus DNA site in vivo, we constructed a chimeric *hox* gene that contained the homeobox sequence of *hoxA7* fused to sequences encoding the transcriptional activation domain of VP16 (Fig. 4A). Expression plasmids were cotransfected with a reporter plasmid containing three copies of the consensus DNA site (site 6) or a mutated version of this DNA site (site 6Q) (Fig. 4A). The expression of *hoxA7*-VP16 but not *hoxA7* alone resulted in a six- to eightfold level of activation of the reporter gene containing the consensus DNA site (site 6). In contrast, the reporter gene containing the mutated DNA site (site 6Q) was activated only twofold by *hoxA7*-VP16 (Fig. 4B). Similar results were obtained with each of the other *hox*-VP16 fusion genes (58).

Hox proteins contact similar nucleotides within the consensus DNA site. The findings described above demonstrate that Hox proteins recognize a common consensus DNA site in vitro and in vivo. To define the nucleotides contacted by the Hox proteins, we performed chemical modification footprinting experiments (Fig. 5). As shown by Cu^{2+}-OP footprinting, each Hox protein contacted a similar region of DNA encompassing the consensus DNA site (Fig. 5A). The extents of the footprinted region were 11 nucleotides on the bottom strand (summary in Fig. 5C; data are shown in Fig. 5A) and 13 nucleotides on the upper strand (summary in Fig. 5C; data not shown). Methylation interference analysis demonstrated that a

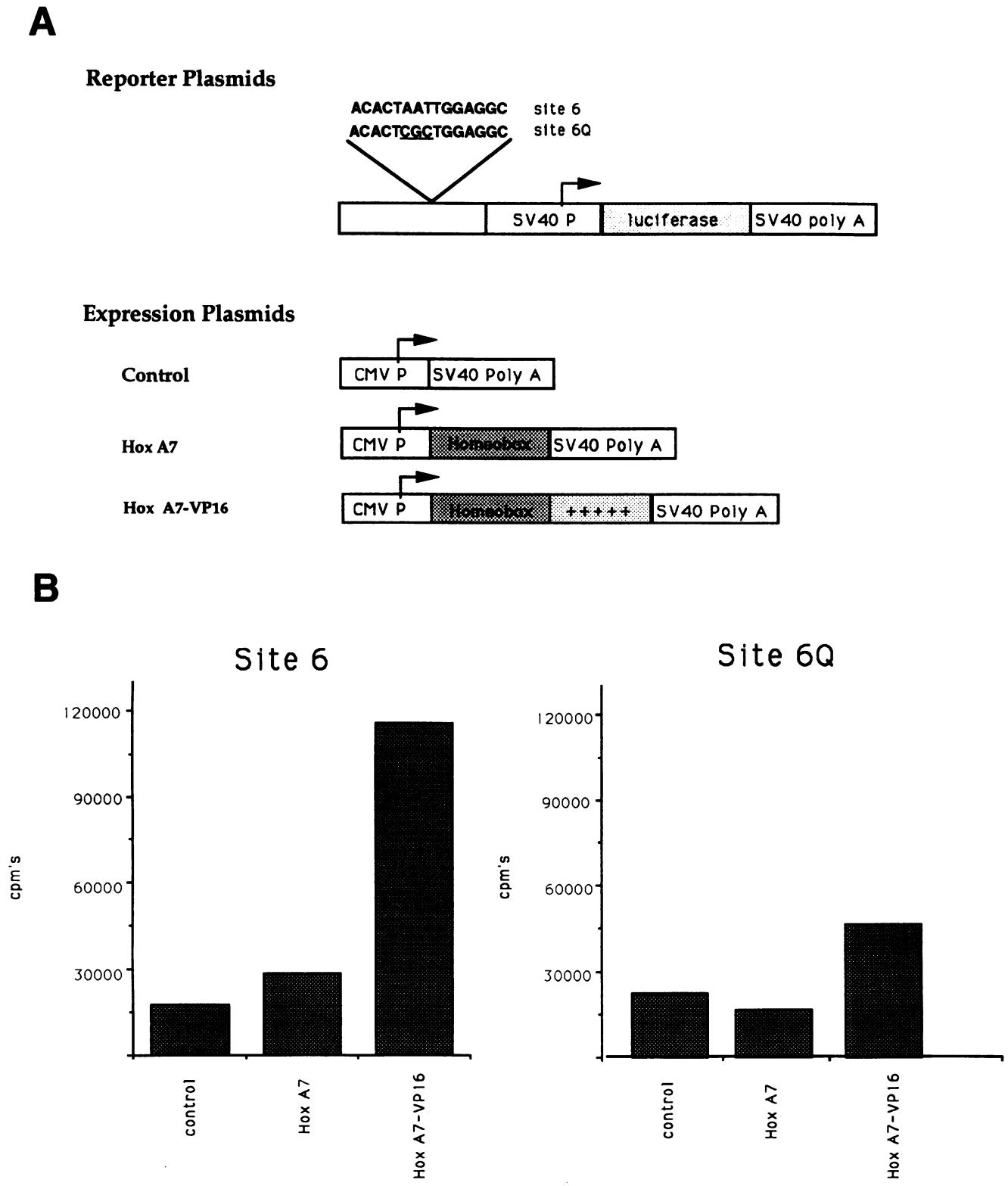

FIG. 4. HoxA7 activates transcription through the consensus DNA site in vivo. (A) The luciferase reporter plasmids contained three copies of the consensus DNA site (site 6) or a mutated version of this DNA site (site 6Q) as indicated. The DNA sites were cloned upstream of the SV40 promoter (SV40 P) driving the expression of the luciferase gene. The position of the SV40 polyadenylation signal (SV40 poly A) is shown. The expression plasmids contained sequences corresponding to the homeobox alone (HoxA7) or to the homeobox plus the sequences encoding the VP16 activation domain (HoxA7-VP16). These sequences were cloned downstream of the cytomegalovirus promoter (CMV P), and a vector without any insert was used as a control. (B) The reporter and expression plasmids were cotransfected into NIH 3T3 cells (as detailed in Materials and Methods). Luciferase activity was determined by scintillation counting and expressed in counts per minute. The assay was performed four times, and a representative experiment is shown. Variability among assays was in the range of 15%.

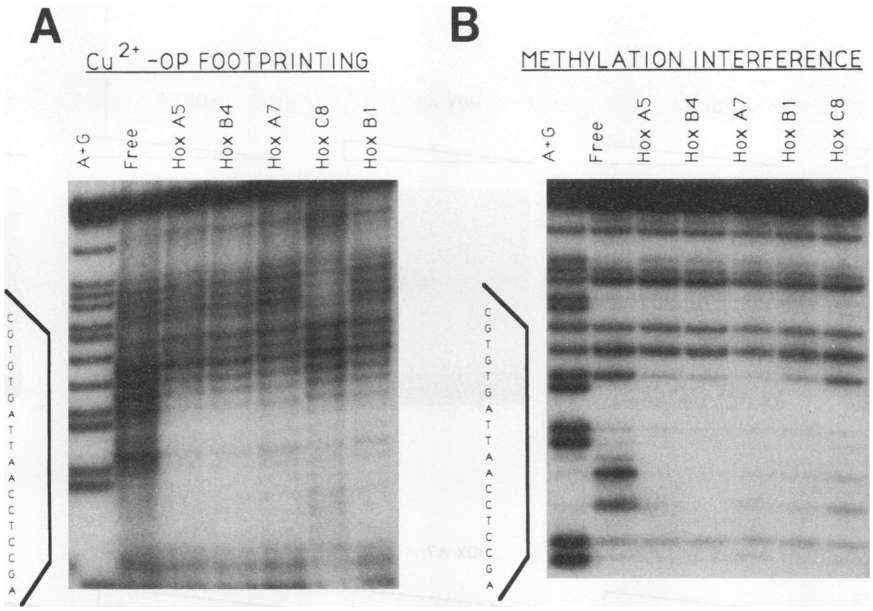

FIG. 5. Hox proteins contact similar nucleotides within the consensus DNA site. (A) Cu^{2+}-OP footprinting was performed with a 45-bp DNA fragment containing the consensus DNA site (^{32}P end labeled on the bottom strand). The radiolabeled DNA fragment was incubated with the indicated Hox proteins. Protein-DNA complexes were formed and resolved from free DNA by gel electrophoresis. DNA cleavage was initiated in situ by the addition of Cu^{2+}-OP (as described in Materials and Methods). Cleavage products were resolved on a 10% polyacrylamide–6 M urea gel and visualized by autoradiography. A+G is a Maxam-Gilbert (46) sequencing reaction. (B) Methylation interference analysis was performed with a ^{32}P-end-labeled DNA fragment (as in panel A) that was partially methylated with dimethyl sulfate. The methylated DNA was incubated with the indicated Hox proteins, and protein-DNA complexes were resolved from free DNA by gel electrophoresis. The free and bound DNAs were extracted and cleaved with 1 M piperidine. As in panel A, cleavage products were resolved on a 10% polyacrylamide–6 M urea gel and visualized by autoradiography. A+G is a Maxam-Gilbert (46) sequencing reaction. (C) Summary of Hox protein interactions with the consensus DNA site. Shown is the region of the 45-bp DNA fragment that encompasses the consensus DNA site. The bracketed region indicates the extent of the Cu^{2+}-OP footprint on both top and bottom strands of the DNA fragment. The arrowhead indicates the single G nucleotide contacted by most of the Hox proteins, as determined by the methylation interference assay.

single G nucleotide was contacted on the bottom strand of the DNA site by most of the proteins (at position 4) (Fig. 5B; summary in Fig. 5C). Interestingly, in contrast to the other Hox proteins, HoxC8 did not contact the G nucleotide at position 4 (Fig. 5B), suggesting that HoxC8 may differ in its mode of interaction with DNA.

Hox proteins exhibit differences in their relative affinities for DNA. In the course of our in vitro DNA binding analysis, we observed that the Hox proteins varied in their degree of binding activity when tested at equimolar concentrations, suggesting that these proteins differ in their relative affinities for DNA (see, e.g., Fig. 3B to F). To test this possibility, we compared the relative binding affinities of HoxA5, HoxA7, HoxB4, HoxC8, and HoxB1 by using the consensus DNA site (site 6) and two other DNA sites (sites 6-8 and 6-15) that were bound as well as or more efficiently than the consensus DNA site (Fig. 3A). Gel retardation assays were performed with a constant amount of DNA (1×10^{-10} M) and various concentrations of each Hox protein (from 5×10^{-10} to 1×10^{-7} M). As was readily apparent in the gel retardation assays, the Hox proteins exhibited differences in their relative DNA binding affinities (Fig. 6). Therefore, measurable DNA binding activity (>5%) was obtained with the consensus DNA site at the lowest concentration of HoxB4 and HoxB1 (5×10^{-10} M), whereas HoxA7 and HoxC8 required at least 1×10^{-9} or 5×10^{-9} M for equivalent DNA binding activity (Fig. 6A). Similar results were obtained with sites 6-8 and 6-15, although HoxA5 had a greater apparent affinity for these sites than for the consensus DNA site (Fig. 6B and C, respectively).

We further examined the relative DNA binding affinities of the Hox proteins by competition analysis (Fig. 7). Protein-DNA complexes were formed with 1×10^{-9} M radiolabeled consensus DNA site (site 6) and 1×10^{-7} M each Hox protein. Following incubation for 20 min, increasing concentrations of unlabeled consensus DNA site (6×10^{-14} to 2×10^{-11} M) were added, and incubation was continued for an additional 5

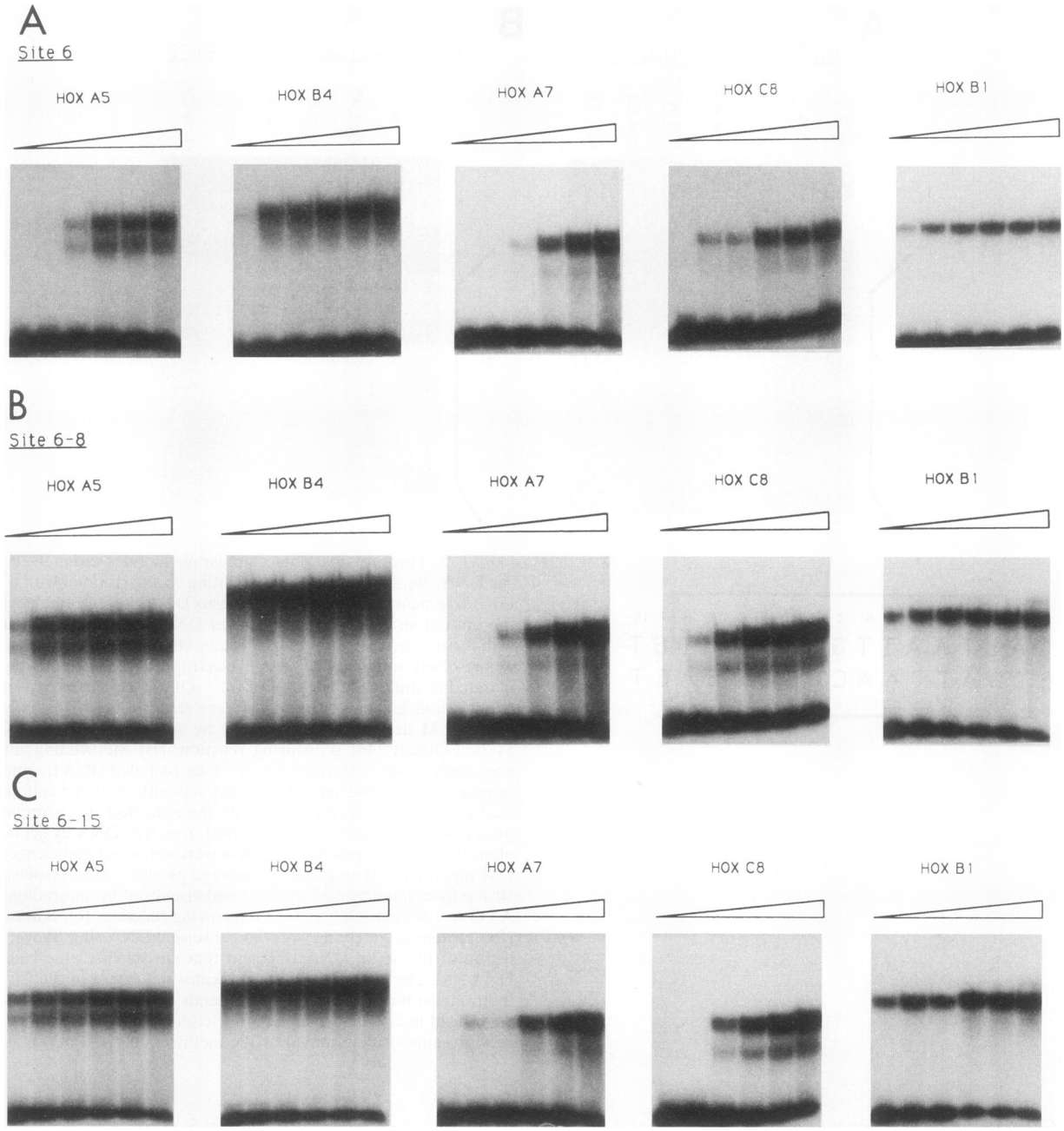

FIG. 6. Hox proteins exhibit different relative affinities for DNA sites. Gel retardation assays were performed with a constant amount of labeled DNA (1×10^{-10} M) and increasing concentrations of each Hox protein (5×10^{-10} to 1×10^{-7} M, as indicated by triangles). DNA sites were the consensus DNA site (site 6) (A), site 6-8 (B), and site 6-15 (C) (as in Fig. 3A). Binding reaction mixtures contained DNA binding buffer (9) without dI-dC, and protein-DNA complexes were formed at room temperature for 20 min to allow reactions to reach equilibrium (9). Protein-DNA complexes were resolved from free DNA by gel electrophoresis and visualized by autoradiography. All assays were performed a minimum of three times, and representative data are shown.

min. The addition of the unlabeled consensus DNA site (site 6) but not an unlabeled mutated DNA site (site 6-19, as in Fig. 3A) effectively inhibited by competition the binding of each Hox protein to the radiolabeled consensus DNA site (Fig. 7). Moreover, DNA binding by HoxC8 and HoxA7 was effectively inhibited by competition at a relatively low concentration (6×10^{-12} M) of unlabeled site 6, whereas HoxA5, HoxB1, and HoxB4 required a relatively high concentration of unlabeled site 6 (2×10^{-11} M). These results, in combination with the experiments described above, indicated that Hox proteins varied in their relative affinities for DNA.

To quantitate the apparent differences in DNA binding affinities among the various Hox proteins, we determined the equilibrium dissociation constants (K_ds) of the protein-DNA complexes by Lineweaver-Burk analysis. The K_ds (Table 1) reflected the relative differences in DNA binding affinities among the Hox proteins. HoxA7 and HoxC8 had relatively low affinities (7 and 8 nM, respectively) for the consensus site,

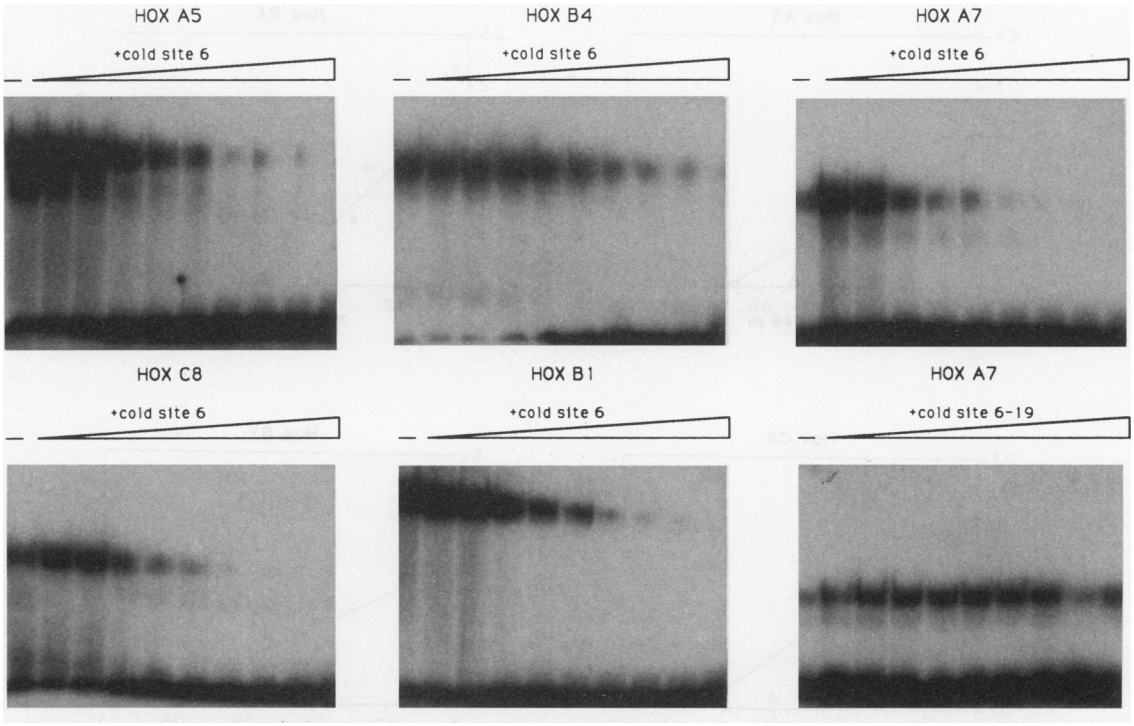

FIG. 7. Competition experiments showing that Hox proteins have different affinities for the consensus DNA site. Protein-DNA complexes were formed with a constant concentration of labeled site 6 DNA (1×10^{-9} M) and a constant concentration of protein (1×10^{-7} M). Following incubation for 20 min, samples were further incubated with no cold competitor DNA (−) or increasing concentrations (indicated by triangles) of cold competitor DNA (site 6 or site 6-19). The concentrations of cold competitor DNA were 0, 6×10^{-14}, 1×10^{-13}, 3×10^{-13}, 6×10^{-13}, 1×10^{-12}, 3×10^{-12}, 6×10^{-12}, 1×10^{-11}, and 2×10^{-11} M. Protein-DNA complexes were resolved from free DNA by gel electrophoresis and visualized by autoradiography. Assays were performed a minimum of three times, and representative data are shown.

HoxA5 had an intermediate affinity (5 nM), and HoxB1 and HoxB4 had relative high affinities (2.6 and 1 nM, respectively). The differences in the K_ds were also apparent by Scatchard analysis, as reflected by the different slopes obtained with each of the various Hox proteins (Fig. 8). On the basis of these kinetic data and the circular dichroism analysis, which showed that the proteins are appropriately folded (Fig. 2D), we conclude that the Hox proteins have different affinities for DNA.

DISCUSSION

In this report, we characterized the DNA binding properties of several Hox proteins, namely, HoxA5, HoxA7, HoxB1, HoxB4, and HoxC8. We demonstrated that these proteins interacted with a common consensus DNA site in vitro [(C/G)TAATTG] and activated transcription through this site in vivo. Although the Hox proteins interacted optimally with the consensus DNA site, they also bound with subtle, but distinct, preferences to DNA sites that contained variations of the nucleotides within the consensus motif. We further showed that Hox proteins varied in their relative affinities for DNA. These data demonstrate that closely related Hox proteins exhibit subtle differences in DNA binding specificities and affinities. These differences are likely to contribute to the selective interactions of Hox proteins with target DNA sites in vivo.

TABLE 1. K_ds of the DNA-protein complexes[a]

Site	K_d (nM) of:				
	HoxA5	HoxB4	HoxA7	HoxC8	HoxB1
6	5.0	1.0	7.0	8.0	2.6
6-8	2.0	2.0	8.5	4.0	2.0
6-15	2.3	1.0	4.0	7.0	2.6

[a] Data were derived by Lineweaver-Burk analysis.

A common theme for homeodomain proteins is that they share similar DNA binding specificities in vitro (see, e.g., 9, 12, 13, 28, and 38). Paradoxically, these proteins have diverse functions in vivo that are mediated by the homeodomain (10, 14, 23, 37, 41, 44). The results presented in this study are consistent with this theme. We have identified a common consensus DNA site that is recognized by several Hox proteins. This consensus DNA site is identical to one that we previously described for a related murine homeodomain protein, HoxA3, and for the divergent murine homeodomain proteins Msx-1 and Engrailed (9). This DNA site is also similar to one identified for HoxA5 with cell extracts that expressed the full-length protein (50). Moreover, similar consensus DNA sites have been described for a related *Drosophila* homeodomain protein, Ultrabithorax (Ubx) (19), and for a divergent *Drosophila* homeodomain protein, Fushi Tarazu (Ftz) (20). These studies indicate that the motif (C/G)TAATTG is a common consensus DNA site for many homeodomain proteins, including members of the Hox family.

The consensus DNA site contains two features that promote Hox protein-DNA interactions: (i) a TAAT core, which is requisite for DNA binding activity, and (ii) nucleotides flanking the core, which contribute to subtle differences in DNA binding site preferences among the various Hox proteins. The essential contribution of the TAAT core to homeodomain-DNA interactions has been well documented (38). The amino

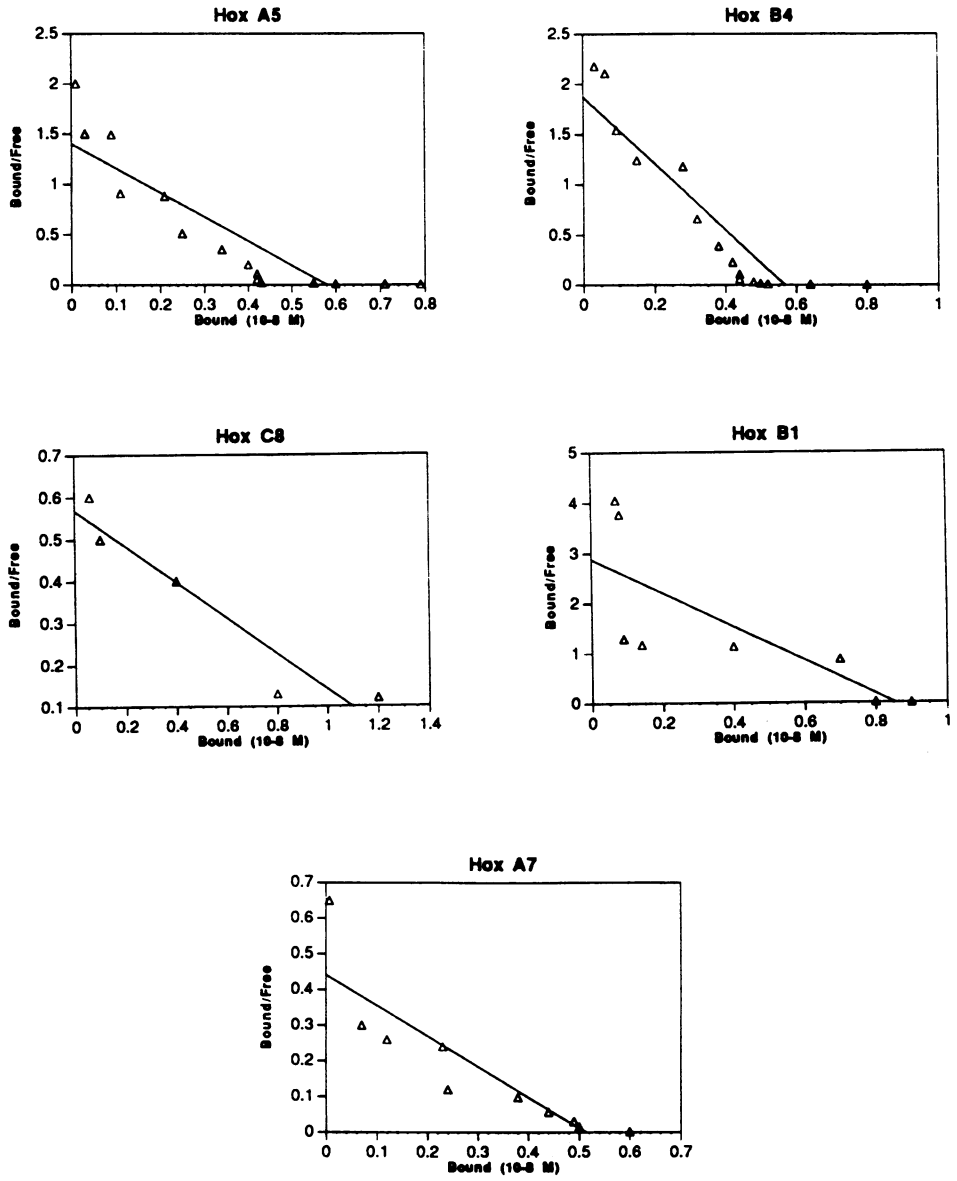

FIG. 8. Scatchard analysis of DNA binding experiments. Gel retardation assays were performed as described in the legend to Fig. 6 with the consensus DNA site (site 6). Data are plotted as bound/free versus bound, and the K_ds were obtained from the negative reciprocal of the slope (slope = $-1/K_d$). The values obtained were as follows: HoxA5, 4.8 nM; HoxB4, 3 nM; HoxA7, ≥10 nM; HoxC8, ≥0 nM; and HoxB1, 3 nM. These values are in agreement with the values obtained by Lineweaver-Burk analysis.

acid residues that make base-specific DNA contacts with the TAAT core have been identified by analysis of the three-dimensional structures of the Engrailed-DNA complex and the Antennapedia-DNA complex (36, 51). These residues are highly conserved among all homeodomain proteins, including the Hox proteins characterized in the present study. In the crystal structure, Engrailed contacts DNA in both the major and the minor grooves. The residues that contact DNA in the major groove (i.e., Ile-47, Gln-50, Asn-51, and Met-54) are invariant among all of the Hox proteins tested (Fig. 2B). These are located in helix III and make various base contacts with the TAAT core. Additional contacts made with the phosphate backbone (i.e., Arg-31, Tyr-25, Trp-48, Arg-53, and Lys-57) are made by residues that are also conserved among these Hox proteins (Fig. 2B). In contrast, the residues that contact DNA in the minor groove in the Engrailed-DNA complex (i.e., Arg-3 and Arg-5) are not invariant (Fig. 2B). In fact, HoxC8 contains a serine at position 3, and HoxB1 contains a glycine at this position. One possibility is that variations at position 3 contribute to differences in DNA binding specificity or affinity. Alternatively, the interaction of R-3 with DNA may be nonessential for DNA binding activity and may be compensated for by contacts of other residues with DNA. It is noteworthy that HoxC8, which lacks R-3, differs from the other Hox proteins in its mode of interaction with the consensus DNA site, as inferred by methylation interference analysis.

Whereas the TAAT core is essential for binding activity, nucleotides flanking the core promote selective interactions of Hox proteins with DNA sites in vitro (summary in Table 2). It is possible that the modest differences in DNA binding site

TABLE 2. Comparison of preferences for flanking nucleotides

Protein	Nucleotides preferred 5′		Core	Nucleotides preferred 3′	
HoxA5	A > G, C > T	C > G, T > A	TAAT	T > G > A, C	G, A > T, C
HoxB4	A, G > C, T	C > G, T > A	TAAT	T, G > A > C	G, A > T, C
HoxA7	A, G > C, T	C, G > T, A	TAAT	T > G, A > C	G > A > T, C
HoxC8	A > G > C, T	C, G > T, A	TAAT	T > A, G > C	G, A > T, C
HoxB1	A > G > C, T	C > G > T, A	TAAT	T > A, G > C	G, A > T, C

preference observed in vitro are enhanced in vivo and therefore contribute to the selective interactions of Hox proteins with target sites in vivo. Indeed, Dfd and Ubx have modest differences in DNA binding specificity in vitro that correlate with their selective functions in vivo (14, 37). Selectivity for specific target sites in vivo may be enhanced by positioning of multiple DNA sites in tandem within regulatory elements. In fact, a recent study has shown that Ubx interacts cooperatively with multiple DNA sites and that this interaction greatly enhances the stability of the Ubx-DNA complex (5). An additional mechanism that is likely to promote target site selection is the interaction of Hox proteins with other protein factors bound at overlapping or adjacent regulatory elements (26, 27, 49, 67).

In addition to subtle differences in DNA binding specificities, Hox proteins also vary in their relative affinities for DNA. HoxB1 and HoxB4 have a relatively high affinity for DNA, HoxA5 has an intermediate affinity, and HoxA7 and HoxC8 have a relatively low affinity. Moreover, HoxA3 (previously characterized in reference 9) also has a relatively high affinity for DNA, similar to that of HoxB1 and HoxB4 (53). A comparison of the homeodomain sequences of these Hox proteins reveals that the N-terminal arm is the most divergent region among the otherwise closely related homeodomains (Fig. 2B). Previous studies showed that the DNA binding affinities of other homeodomain proteins are modulated by the N-terminal arm (2, 20, 54) and that the N-terminal arm is essential for functional specificity in vivo (68). Therefore, it is likely that the N-terminal sequences of the Hox homeodomain proteins contribute to their differential DNA binding affinities. Interestingly, HoxA7 and HoxC8, which both have a relatively low affinity for DNA, share a similar motif in their N-terminal sequences (from position 4 to position 8 [GRQTY]) (Fig. 2B), and the motif is conserved among their paralogs (i.e., HoxB7, HoxB8, and HoxD8; Fig. 2B). This motif may contribute to the relatively low affinity of these Hox proteins for DNA. Although it is likely that the N-terminal arm modulates DNA binding affinity, the proteins used in this study contain additional amino acids flanking the homeodomain (Fig. 2B); therefore, we cannot eliminate the possibility that these other residues also contribute to differences in DNA binding affinity. However, experiments performed with Hox proteins that contain the homeodomain plus various flanking sequences showed that the homeodomain makes the most significant contribution to DNA binding affinity and specificity (8, 53).

The relative affinities of the Hox proteins for DNA correlate with the positions of their respective genes on the *hox* cluster (Fig. 1). The chromosomal organization of the *hox* genes is highly conserved, and the biological significance of this organization is evident from the colinear relationship between the pattern of *hox* gene expression and the positions of these genes within the chromosome cluster (Fig. 1). Here we have defined an additional relationship inherent in this organization, namely, that the genes located more 3′ in the *hox* cluster encode proteins that have a relatively high affinity for DNA, whereas the genes located more 5′ in the *hox* cluster encode proteins that have a relatively low affinity for DNA (Fig. 1). The implication of this relationship is that the Hox proteins expressed in the more anterior regions of the developing embryo are limited to those which have a relatively high affinity for DNA. In contrast, in the more posterior regions, numerous Hox proteins are expressed, and these have a range of affinities for DNA. This result suggests that a gradient is established along the anterior-posterior body axis, wherein, at different positions, there are different combinations of Hox proteins with various affinities for DNA. The significance of relative protein concentrations for the Hox proteins is evident. In fact, the levels of *hox* gene expression are highest at the most anterior boundaries (48), suggesting that the concentrations of particular Hox proteins may indeed vary along the anterior-posterior body axis. The establishment of gradients is a common mechanism of regulation during development (32). Gradients of transcriptional regulators occur via controlling subcellular localization, as in the case of the *Drosophila* morphogen Dorsal (64), or via differentially stabilizing mRNA levels, as in the case of another *Drosophila* morphogen, Bicoid (16, 31). Here we suggest an alternative mechanism to establish a gradient that proceeds via the coordinate expression of proteins with different affinities for DNA. Indeed, distribution along the anterior-posterior body axis of Hox proteins that have various binding affinities may modulate their selective interactions with target DNA sites and thus contribute to their specific functions. Differential DNA binding affinities are likely to provide an important parameter of the "combinatorial code" by which Hox proteins specify positional information during development.

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