Differential DNA binding properties of three human homeodomain proteins

Maria T. Corsetti1, Paola Briata1, Lorenza Sanseverino1,2, Antonio Daga1, Irma Airoldi1, Antonio Simeone3, Giulio Palmisano1, Cristiano Angelini1, Edoardo Boncinelli4 and Giorgio Corte1,2*

1IST—Istituto Scientifico per la Ricerca sul Cancro, 16132 Genoa, 2Istituto di Chimica Biologica, Università di Genova, 16132 Genoa, 3IIGB, via Marconi 10, 80125 Naples and 4Istituto Scientifico H.S.Raffaele, 20132 Milan, Italy

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ABSTRACT

The products of three human homeobox containing (HOX) genes, 2C, 3C and 4B, were produced in insect cells using the Baculovirus expression system and purified to near homogeneity. In this system we observed that the DNA binding forms of the three proteins are not glycosylated. HOX 3C and 4B are phosphorylated in insect cells, while HOX 2C is not. The three HOX proteins bind to a DNA sequence known to be a target site for Antennapedia protein with a very similar affinity (Kd = 1-2 x 10^-9 M). We then measured their binding properties to four human sequences present in the HOX 3D, 4C, 1C and 4B promoters. Two of these sequences have been reported to be binding sites for HOX proteins. HOX 2C, 3C and 4B behaved quite differently, showing low affinity for promoters of genes located upstream from their own gene in the HOX clusters and a higher affinity for regulatory sequences of their own gene and downstream HOX genes.

INTRODUCTION

Homeobox containing genes are a class of regulatory genes coding for nuclear proteins, termed homeoproteins, that most probably act as transcription factors (1). The protein domain encoded by the homeobox, the homeodomain (HD), is capable of recognizing and binding to specific DNA sequences (2). Through the recognition property of the HD, homeoproteins are believed to regulate the expression of batteries of target genes. Among homeobox genes of vertebrates, stand out those belonging to the Hox gene family, primarily involved in controlling the development of the body plan during early embryogenesis (3).

They occur in gene clusters confined in compact genomic regions since the remote evolutionary past (4). Evidence for such clustering came first from the study of Drosophila homeotic genes (5), located in two genomic regions called the Antennapedia (ANT-C) and Bithorax (BX-C) complexes (4, 5, 6). The two complexes present in flies in turn appear to have arisen from a single complex, called HOM-C, early in insect evolution (reviewed in 4). The ancestral cluster corresponding to the HOM-C of insects, probably containing not less than 4 different homeoboxes, has undergone duplications in the evolutionary lineage leading to vertebrates (7, 8). Higher vertebrates have four such homologous clusters, termed HOX loci in humans (9 for a review) and Hox loci in the mouse (3 for a review). Within each cluster, all Hox genes show the same transcriptional orientation (10).

Hox genes were originally identified on the basis of the primary sequence of their HD (11 for a review). In fact, most Hox genes encode proteins containing a HD, often referred as a class I HD, most closely related to the archetypal Antennapedia (Antp) HD. However, since the similarity is not so high for all genes in the four clusters (9), it now appears more convenient to define Hox genes on the basis of their physical location on chromosomes.

The genomic organization of Hox gene clusters is reflected in their expression pattern. All Hox genes are developmentally regulated in mouse (3 and references therein), frog (12 and references therein) and human (13) embryos, where they cooperate in providing positional information along the anteroposterior (A/P) body axis (3). Hox genes are expressed in partially overlapping domains along the embryonic A/P axis. The anterior boundaries of the expression domains of the various genes belonging to a given cluster follow a 5'-posterior/3'-anterior rule (14, 15, 16). This phenomenon was first observed for the homeotic genes in the BX-C complex of flies and termed colinearity (5).

38 class I human HOX genes have been isolated so far. They are clustered in four complex HOX loci, HOX 1 to 4, on chromosomes 2, 7, 12 and 17, respectively (10, 13). Most HOX genes have been shown to be expressed in midgestation embryos and fetuses in complex spatial and temporal patterns (17) and in embryonal carcinoma cells upon retinoic acid induction.

* To whom correspondence should be addressed
(Simeone et al., 1990, 9, 10, 18). More recently some HOX homeoproteins have been shown to be able to act as transcription factors in cotransfection experiments (19, 20).

Despite the similarities among their HD domains, different HD proteins, to exert their function, have to bind to different DNA sequences or to similar DNA sequences but with different affinity (21). Recent structural studies have shown that there are only few sequence-specific contacts between the HD and the DNA and led to the suggestion that less conserved parts of the HD proteins may contribute to the final specificity (22, for a review).

We have produced three complete recombinant human HOX proteins, HOX 2C, 3C and 4B, using the Baculovirus system and investigated their binding properties to DNA sequences derived from a binding site for Antp present in the Drosophila engrailed gene promoter and from the HOX 3D, 4C, 1C and 4B promoters, in comparison with the recombinant HD fragment of Antennapedia.

MATERIALS AND METHODS

Expression of HOX 2C, HOX 3C and HOX 4B recombinant proteins

The Alu-Alu HOX 3C cDNA fragment, (851bp), the entire HOX 2C cDNA (23) and the Apal-Apal (851 bp) HOX 4B cDNA fragment (24) were inserted in the BamHI site of pACYMI (Fig. 1). HOX 3C and 4B were trimmed to remove most of the non translated portions of the cDNAs to maximize subsequent expression.

Recombinant Baculoviruses were obtained by cotransfection of Sf9 cells with wild type AcNPV virus and pACYMI/HOX 3C, pACYMI/HOX 4B and pACYMI/HOX 2C and purified by limiting dilution and dot hybridization.

For production of the three HOX proteins, Sf9 cells were infected with purified recombinant viruses at 1-10 pfu (plaque forming units)/cell. At 3 days post-infection the cells were lysed by extensive dialysis against Buffer D (20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) at 4°C. The DNA binding activity of the renatured samples was measured by EMSA as described below. Recombinant homeoproteins were purified to near-homogeneity using oligonucleotide (BS2-18) affinity chromatography (27) with the exception that we used 5 μg/ml poly(dl-dC)/(dl-dC) instead of calf thymus DNA. The eluted recombinant proteins were dialyzed against Buffer D and stored at -80°C in aliquots after addition of 10% glycerol (final concentration). The concentration of the purified recombinant proteins was estimated by densitometric scanning of silver stained bands in SDS-PAGE corresponding to different amounts of each recombinant protein in comparison to known amounts of two marker proteins (Carbonic Anhydrase and Bovine Serum Albumin).

Southern-Western Blot

Nuclear lysates of infected Sf9 cells were electrophoresed on a 12% SDS-PAGE and transferred to nitrocellulose according to standard procedures. The blots were subjected to denaturation/renaturation and incubation with radioactive oligodeoxynucleotides (28) with minor modifications. Briefly, after transfer the filter was incubated in binding buffer (25mM NaCl, 1mM MgCl2, 0.5mM DTT and 25mM HEPES pH 7.9) supplemented with 6M guanidium hydrochloride, for 5' at 4°C on a rotatory shaker. Half of the solution was then replaced with binding buffer without guanidine and the filter was incubated for 5' a 4°C. This dilution step was repeated for 5 times. The filter was incubated in binding buffer supplemented with 2% nonfat dry milk for 30' a 4°C. Then BS2-18 probe (1x106 cpm/ml) was added and allowed to bind for 2 h. The filter was washed 3 times for 10' with binding buffer/1% dry milk and exposed for autoradiography.

Oligodeoxynucleotides and Electrophoresis Mobility Shift Assay (EMSA)

Oligodeoxynucleotides were synthesized on a Beckman SM oligosynthesizer and purified by polyacrylamide gel electrophoresis. The sequences of the filled-in, double stranded oligodeoxynucleotides are:

Purification of the recombinant proteins

200-500x10^6 Sf9 cells were infected with recombinant Baculovirus and on the fourth day after infection nuclei were lysed in 8 M urea, 1% 2-mercaptoethanol, 5 mM Tris, pH 8.0. Nuclear lysates of Sf9 cells infected by recombinant Baculovirus expressing HOX 4B, HOX 3C and HOX 2C were directly loaded onto Q Sepharose Fast Flow columns (Pharmacia-LKB) previously equilibrated in 5 mM Tris, pH 8.0. The columns were extensively washed with 8 M urea, 1% 2-mercaptoethanol, 20 mM NaCl, 5 mM Tris, pH 8.0 and then eluted with a linear gradient of 20-300 mM NaCl in 8 M urea, 1% 2-mercaptoethanol, 5 mM Tris, pH 8.0. The presence of the recombinant HOX protein was assessed by silver staining of SDS-PAGE and Southern-Western assays as described below. The fractions corresponding to 150 mM, 50 mM and 200 mM NaCl contained HOX 4B, HOX 3C and HOX 2C, respectively. Fractions containing HOX 3C were further chromatographed on a S Sepharose Fast Flow column (Pharmacia-LKB). The column was washed and eluted as described above. The fractions corresponding to 200 mM NaCl contained HOX 3C. Fractions containing the recombinant proteins were pooled and renatured by extensive dialysis against Buffer D (20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) at 4°C. The DNA binding activity of the renatured samples was measured by EMSA as described below. Recombinant homeoproteins were purified to near-homogeneity using oligonucleotide (BS2-18) affinity chromatography (27) with the exception that we used 5 μg/ml poly(dl-dC)/(dl-dC) instead of calf thymus DNA. The eluted recombinant proteins were dialyzed against Buffer D and stored at -80°C in aliquots after addition of 10% glycerol (final concentration). The concentration of the purified recombinant proteins was estimated by densitometric scanning of silver stained bands in SDS-PAGE corresponding to different amounts of each recombinant protein in comparison to known amounts of two marker proteins (Carbonic Anhydrase and Bovine Serum Albumin).

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EDTA, 2mM DTT, 4% Ficoll and incubated at 20°C for 1 hr (unless otherwise indicated). Poly (dl-dC) (dl-dC) was included in the reaction (5 μg/100 μl) when total nuclear extracts were used instead of the purified proteins. The DNA-protein complexes were resolved on 10—12% polyacrylamide gels in 0.5xTBE buffer at 4°C. Gels were dried and exposed to autoradiography films at —70°C. Slices of dried gels corresponding to radioactive bands (bound and free oligonucleotides) were cut out, incubated in Pico-Fluor 40 (Packard) and counted by liquid scintillation spectroscopy.

**Calculation of the Equilibrium Binding Constants**

The data obtained from the saturation experiments were plotted and the following equilibrium equation was used to calculate $K_a$ from the midpoint of the curves:

$$[\text{HD-DNA}] = \frac{[\text{HD}]_0 \times K_a ([\text{DNA}]_0 - [\text{HD-DNA}])}{1 + K_a ([\text{DNA}]_0 - [\text{HD-DNA}])}$$

$[\text{HD}]_0$ and $[\text{DNA}]_0$ are the total concentrations of the protein and the oligodeoxynucleotide, respectively, and $[\text{HD-DNA}]$ is the concentration of the complex. $K_a$ is the association constant. $K_q$ is $1/K_a$.

**Molecular mass determination of the DNA-HOX complex**

BS2-18 (0.2 nM), end-labelled with $^{32}$P as described above, was incubated in binding buffer with the three HOX proteins (10 nM) for 1 hr at 20°C. Molecular mass markers (albumin, ovalbumin and chymotrypsinogen) were added to the reaction mixtures (200 μl), which were then loaded onto a 600×7.5 mm TSK 2000 HPLC column (Beckman) equilibrated in binding buffer. The molecular mass markers peaks were detected by recording the absorption of the effluent at 280 nm. 0.1 ml fractions were collected and counted to identify the position of the BS2-18 complex with each of the three HOX proteins.

**RESULTS**

**Production and purification of HOX 2C, HOX 3C and HOX 4B**

The HOX 2C, 3C and 4B cDNAs (see Materials and Methods) were inserted in pACYM1 and transferred by homologous recombination to Baculovirus under the control of the polyhedrin promoter, as described in Materials and Methods. After isolation in pure form, the recombinant viruses were used to infect Sf9 cells. Three days after infection the cells were collected, lysed with NP40 and the nuclear fraction recovered by centrifugation. Both the cytoplasmatic lysate and the nuclear extract, prepared as described in Materials and Methods, were analysed in SDS-PAGE and stained with silver. In all cases a band, varying in molecular mass and absent from cells infected with a control virus, was present in the SDS-PAGE profile of the nuclear extracts, accounting for 2—5% of the total proteins (not shown).

Sf9 cells were then infected in large spinner cultures and the three homeoproteins purified by ion exchange and affinity chromatography, as described in Materials and Methods, to near homogeneity (Fig. 1). After purification, the three HOX proteins were able to bind BS2-18, an oligodeoxynucleotide derived from a binding site for Antp present ~2 kilobases (kb) upstream of the *Drosophila engrailed* gene promoter, as judged in a mobility shift assay (Fig. 2). The three HOX proteins showed an apparent molecular mass higher (HOX 2C 29 kDa; HOX 3C 32 kDa; HOX 4B 33 kDa) than predicted by their aminoacid sequence (24 kDa, 27 kDa and 30 kDa, respectively). In principle, this could be due to posttranslational modifications of the polypeptide chains. As the three proteins possess potential glycosylation sites for N-linked sugars, Sf9 cells were infected with the recombinant viruses, and two days after infection tunicamycin was added to the cultures. Twenty four hours later the cells were lysed and nuclear extracts run in SDS-PAGE. After transfer to a nitrocellulose sheet the electrophoresed material was fully denaturated with guanidium HCl and renaturated. The molecular mass of the HOX proteins present in the nuclear extracts was then assessed by overlaying the nitrocellulose with $^{32}$P labelled BS2-18. As shown in Fig. 3, the molecular mass of HOX 2C, 3C and 4B capable of binding the oligodeoxynucleotide remained unchanged, indicating that the DNA binding form of the HOX protein is not glycosylated. The possible phosphorylation of the three HOX protein in insect cells was investigated by incubating...
infected cells with $^{32}$P for 18 hr at 48 hr after infection. Nuclear extracts were then analysed in SDS-PAGE. A phosphoprotein with a molecular mass corresponding to HOX 3C and 4B was present in the nuclear extracts of cells infected with recombinant viruses carrying HOX 3C and 4B cDNAs, respectively (Fig. 4). No phosphoprotein with a molecular mass corresponding to HOX 2C was detected in nuclear extracts of cells infected with a recombinant virus carrying HOX 2C cDNA. The meaning of this difference is not clear, as we do not know whether the HOX 3C and 4B proteins are phosphorylated in vivo.

Determination of the equilibrium binding constant to BS2-18

The DNA binding properties of HOX 2C, 3C and 4B were assessed by determining their respective equilibrium dissociation constants ($K_d$) using the oligodeoxynucleotide BS2-18, in comparison with that of Antp HD, a 117-aminoacid peptide containing the Antennapedia homeodomain (kindly provided by Dr. M. Affolter). This sequence was chosen instead of the Antennapedia consensus sequence (30) for a better comparison with the DNA binding properties of Antp HD which were extensively studied by Affolter et al. (31) using BS2-18. However, the ability of the three HOX proteins to bind the Antennapedia consensus sequence, contained in the oligodeoxynucleotide ACS-25, was checked in similar experiments and their binding properties found very similar to those reported below for BS2-18 (data not shown).

The $K_d$s were calculated from saturation experiments, in which a fixed limiting amount of BS2-18 was mixed with increasing concentrations of the proteins. HOX-DNA complexes were separated from the free reactants in a non denaturing polyacrylamide gel. Fig. 5A represents one such experiment. Fig. 6 is a plot of the data obtained from titrations performed as in the example of Fig. 5A for HOX 2C, 3C, 4B and Antp HD. The radioactive bands corresponding to the bound and free oligonucleotide were cut out of the gel and counted. The $K_d$, found for Antp HD is $3.2 \times 10^{-9}$ M at 20°C, very close to the value reported by Affolter et al. (1-2$\times 10^{-9}$ M). The $K_d$ calculated for the three human homeoproteins was slightly lower, 1.9$\times 10^{-9}$ M (HOX 2C), 1.2$\times 10^{-9}$ M (HOX 3C), and 1.5$\times 10^{-9}$ M (HOX4B) than that of Antp HD. In another set of experiments, the $K_d$s were estimated by varying the concentration of BS2-18 mixed to a constant amount of the four proteins (as exemplified in Fig.5B). These experiments allow also the determination of the actual concentration of active HOX molecules. The total concentration of bound HOX 2C, 3C and 4B was 10.2, 12.0 and 11.9 nM, respectively. These values are about equal to the input concentration of 10 nM, indicating that, despite the denaturation step, essentially all of the purified HOX proteins are active and bind DNA with the same affinity.

Non specific DNA binding

To assess the $K_d$ of the interaction between HOX 2C, 3C and 4B with nonspecific DNA sequences, limiting amounts of the nonspecific oligonucleotide HAH-23, derived from the promoter of the human gene for apoferritin, were incubated with increasing amounts of the three proteins. All four proteins showed a very high $K_d$, 2-4$\times 10^{-7}$ M. This value is again in good agreement with the value reported for Antp HD (31) (between 1 and 4$\times 10^{-7}$ M with two different nonspecific oligonucleotides).

Stoichiometry of the DNA-HOX complexes

After incubation of BS2-18 (0.2 nM) with a saturating excess of the three HOX proteins (10 nM), the complexes were analysed in HPLC on a TSK 2000 column equilibrated in binding buffer. In all cases the molecular mass of the HOX-DNA complex was between 40 and 50,000 kDa (data not shown), consistent with a complex formed by a single HOX molecule bound to a single BS2-18 (predicted m.w. 40-50,000 kDa).
Figure 5. Representative saturation experiments. A. A fixed limiting amount of BS2-18 (0.2 nM) was mixed with increasing concentrations (1-40 nM) of HOX-4B, incubated 4 hr at 20°C and then run in a 12% acrylamide gel. B. A similar experiment, in which the protein concentration was kept constant at 10 nM while the concentration of BS2-18 was increased from 0.2 nM to 40 nM. After visualization by autoradiography, the bands corresponding to free (F) and bound (B) oligodeoxynucleotide were cut out and counted.

Determination of the equilibrium binding constant to four human HOX promoter sequences

Four oligodeoxynucleotides corresponding to four sequences present in the promoter of HOX 3D (20), HOX 4C (19), HOX 1C (32), and HOX 4B (33) (see Fig. 7 and Materials and Methods), named cpl 1-25, HCR-19, 1C-30, and 4B-26, respectively, were used to determine the equilibrium binding constant of the three HOX proteins. Two of these sequences, namely cpl 1-25 (20) and HCR-19 (19) have been recently shown to be target for HOX proteins. Again, Antp HD was used as a reference. The results of the saturation experiments performed with a fixed limiting amount of the four oligonucleotides and increasing concentrations of the three proteins are reported in Fig. 8.

For an easier comparison, in Table I are reported the $K_d$ values of the four proteins with the four oligodeoxynucleotides. Although Antp HD, and HOX 2C, 3C and 4B have very similar binding properties to BS2-18 and to the nonspecific oligodeoxynucleotide HAH-23, they differ considerably in their affinity for cpl11-25 and 1C-30, HOX 2C and 3C having a $K_d$ similar to that obtained with BS2-18, while HOX 4B and Antp HD showed a much lower affinity. All four proteins bind to the oligodeoxynucleotide HCR-19 with a rather low affinity. On the contrary, HOX 2C and HOX 3C bind to oligonucleotide 4B-26 with high affinity. Interestingly, 4B-26, present in the promoter of HOX 4B, is bound with high affinity also by the HOX 4B protein.

DISCUSSION

Using the Baculovirus system we have expressed three human HOX proteins, HOX 2C, 3C and 4B in a eukaryotic system. Insect cells are able to recognize the targeting signals and to transport proteins to the various intracellular compartments accordingly. All the three HOX molecules produced in S9 cells accumulate rapidly in the nuclei with very little protein left in the cytoplasm, indicating that they are actively transported and
Figure 8. Saturation experiments performed with HOX 2C, 3C and 4B and Antp HD with the oligodeoxynucleotides cpl-25, HCR-19, IC-30 and 4B-26. Experimental conditions were identical to those of Fig. 5A. A. cpl 1-25. The $K_d$ values obtained were Antp HD = (0.027 ± 0.008) $\times 10^9$ M$^{-1}$; HOX 2C = (0.25 ± 0.024) $\times 10^9$ M$^{-1}$; HOX 3C = (0.27 ± 0.040) $\times 10^9$ M$^{-1}$; HOX 4B = (0.080 ± 0.0062) $\times 10^9$ M$^{-1}$. B. HCR-19. The $K_d$ values obtained were: Antp HD = (0.033 ± 0.0012) $\times 10^9$ M$^{-1}$. C. IC-30. The $K_d$ values obtained were: Antp HD = (0.034 ± 0.001) $\times 10^9$ M$^{-1}$; HOX 2C = (0.40 ± 0.018) $\times 10^9$ M$^{-1}$; HOX 3C = (0.49 ± 0.128) $\times 10^9$ M$^{-1}$; HOX 4B = (0.040 ± 0.004) $\times 10^9$ M$^{-1}$. D. 4B-26. The $K_d$ values obtained were: Antp HD = (0.049 ± 0.0027) $\times 10^9$ M$^{-1}$; HOX 2C = (0.334 ± 0.011) $\times 10^9$ M$^{-1}$; HOX 3C = (0.293 ± 0.010) $\times 10^9$ M$^{-1}$; HOX 4B = (0.281 ± 0.006) $\times 10^9$ M$^{-1}$.

Table 1. $K_d$ values of the interaction between the three HOX proteins and Antp HD with the five oligonucleotides

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Antp HD</th>
<th>HOX 2C</th>
<th>HOX 3C</th>
<th>HOX 4B</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS2-18</td>
<td>3.2 $\times 10^{-9}$ M</td>
<td>1.9 $\times 10^{-9}$ M</td>
<td>1.2 $\times 10^{-9}$ M</td>
<td>1.5 $\times 10^{-9}$ M</td>
</tr>
<tr>
<td>cpl-25</td>
<td>40.6 $\times 10^{-9}$ M</td>
<td>4.0 $\times 10^{-9}$ M</td>
<td>3.7 $\times 10^{-9}$ M</td>
<td>14.3 $\times 10^{-9}$ M</td>
</tr>
<tr>
<td>HCR-19</td>
<td>14.5 $\times 10^{-9}$ M</td>
<td>11.6 $\times 10^{-9}$ M</td>
<td>12.5 $\times 10^{-9}$ M</td>
<td>30.0 $\times 10^{-9}$ M</td>
</tr>
<tr>
<td>IC-30</td>
<td>29.6 $\times 10^{-9}$ M</td>
<td>2.5 $\times 10^{-9}$ M</td>
<td>2.0 $\times 10^{-9}$ M</td>
<td>24.8 $\times 10^{-9}$ M</td>
</tr>
<tr>
<td>4B-26</td>
<td>20.2 $\times 10^{-9}$ M</td>
<td>3.0 $\times 10^{-9}$ M</td>
<td>3.4 $\times 10^{-9}$ M</td>
<td>3.2 $\times 10^{-9}$ M</td>
</tr>
</tbody>
</table>

$K_d$ were calculated from the saturation experiments as described in Materials and Methods. $K_d$ and their errors are reported in the legend of Fig. 6 and 8.

are thus likely to possess a nuclear localization signal peptide. Although the three HOX proteins have putative sites for N-linked oligosaccharides and Sf9 cells carry out all the glycosylation steps except the addition of sialic acid, their apparent molecular mass in SDS-PAGE remained unchanged even when the cells were incubated in the presence of tunicamycin, indicating that they do not possess sugar moieties of this type. Perhaps, this should not be surprising, as they are synthesised in the cytoplasm while the oligosaccharide transferase is located in the luminal side of the ER. The significance of the phosphorylation of two of the HOX proteins, HOX 3C and 4B in this system is unknown. Phosphorylation is a widely used mechanism to modulate protein function, but we do not know whether these proteins are phosphorylated in human embryos. Odenwald et al. (32) showed that the murine HOX-1.3 protein similarly produced using the Baculovirus system is also phosphorylated and can bind a specific sequence in both phosphorylated and non phosphorylated form. Although the $K_d$ of the interactions was not directly measured, as higher NaCl concentrations were required to extract the phosphorylated forms of HOX-1.3 from nuclear extracts of fibroblast they suggested that phosphorylated forms may have an increased affinity for nuclear factors.

The three HOX proteins show binding properties to BS2-18, an oligodeoxynucleotide whose sequence contains the canonical ATTA sequence and is specifically recognized by Antp, and to HAH-23, a nonspecific oligonucleotide, very similar to Antp HD. Their affinity to BS2-18 is indeed higher ($K_d$ = 1.9 $\times 10^{-9}$ M) than that of Antp HD ($K_d$ = 3.2 $\times 10^{-9}$ M). This latter value is slightly higher than that reported by Affolter et al. (31) ($K_d$ = 2 $\times 10^{-9}$ M), probably due to minor differences in the conditions of the saturation experiments. As found by Affolter et al. (31) for Antp HD, the three HOX proteins bind to DNA in monomeric form forming stable complexes with a very long half life (31). All the four proteins have a relatively high affinity for nonspecific DNA ($K_d$ = 10$^{-6} - 10^{-7}$ M) and, as suggested by Affolter et al. (31), nonspecific interactions might increase...
the stability of the complex. Purified HOX 2C, 3C and 4B seem then to show DNA binding properties extremely similar to Antp HD, indicating that after purification, which involves a denaturation-renaturation step, the homeoproteins, or at the very least the homeodomains, are in their native conformation. These results would also suggest a rather broad specificity of HOX proteins, as the BS2-18 sequence can hardly be considered their specific target site.

However, when we measured the affinity of HOX 2C, 3C and 4B, and Antp HD using four oligodeoxynucleotides, cp11-25, HCR-19, IC-30, and 4B-26 whose sequences are present in the promoter of HOX 3D, 4C, 1C and 4B respectively, they behaved quite differently from one another. Thus, HOX 2C and 3C bind to cp11-25, 1C-30 and 4B-26 with relatively high affinity and to HCR-19 with low affinity, while HOX 4C binds poorly to all sequences except 4B-26, which is present in its own promoter.

Auto- and cross-regulatory interactions between homeoproteins and homeobox gene promoters have been reported for a number of Drosophila loci, both in embryos and in cell culture systems (reviewed in 22). These interactions are likely to play a significant role in the establishment and/or maintenance of well defined expression pattern in the embryo, even if some of these genetically defined interactions might not have an actual functional significance (34). The expression domains of vertebrate homeobox genes in developing embryos are often overlapping, particularly in posterior regions, and the very existence of four homologous HOX clusters considerably increases the complexity of the control network, if not its redundancy.

With this in mind, we recently addressed the issue of cross-regulation of human HOX genes by analysing the activity of some homeoproteins on the HOX3D promoters by cotransfection experiments in cultured cells (20). We showed that the products of HOX3C, HOX4C, HOX4D and HOX4E are able to transactivate the HOX3D promoter in both HeLa and NIH3T3 cells. These four HOX genes belong to upstream, or more 'anterior' groups with respect to HOX3D. Conversely, the HOX3D protein and those encoded by HOX3E and HOX4B, belonging to downstream, or more 'anterior' groups are inactive on the HOX3D promoter.

It was previously shown (19) that the products of HOX4C and HOX4D are able to transactivate the HOX4D promoter whereas the murine Hox-4.3 gene product is not. Hox-4.3 is the murine homologue of the human HOX4E gene. The complex of these data suggest that cross-regulation of HOX genes, at least as assayed in cell transfection experiments, has a directional component: 'posterior' genes appear to activate at least two more 'anterior' ones (20).

In the present study, we have assayed the affinity of three HOX homeoproteins for cis-regulatory sites upstream of four HOX genes, namely HOX3D, HOX4C, HOX1C, and HOX4B. It is difficult to draw a firm conclusion from these observations but the data suggest that HOX homeoproteins have a low affinity for regulatory sequences of HOX genes located upstream from their own gene in the HOX clusters and a higher affinity for regulatory sequences of their own gene and downstream HOX genes. The low affinity for the HOX 4C regulatory site may also reflect the suggested different regulation mode of the Abd-B-like genes (upstream of HOX-4C).

It has recently been proposed (35) that the equation (3'-anterior)-early/(5'-posterior)-late, originally observed in embryonal carcinoma cells treated with retinoic acid (10, 18) and in developing limbs (36), may also be valid in general for the expression of Hox genes along the A/P axis in embryogenesis. This phenomenon whereby cells seem not to be able to express upstream members of a HOX cluster without first expressing downstream members has been called 'temporal colinearity' (35, 37). On the other hand, once expressed, 5' genes are able to override the action of more 3' genes expressed in the same region (34, 37). It is tempting to speculate that the molecular basis of these phenomena resides in differential affinities of HOX homeoproteins for HOX regulatory sequences. Downstream homeoproteins have a lower affinity than upstream homeoproteins. Early in embryogenesis, only 3' homeoproteins are expressed. Even with a low affinity they can regulate HOX gene expression. As soon as homeoproteins encoded by more upstream genes begin to be expressed these might outcompete the products of downstream genes. It is worth noting that in our experiments we assayed the affinity of the whole homeoprotein and not of the HD alone. This should give reasonably reliable information even if it is conceivable that in vivo the action of these homeoproteins could in turn be modulated through protein-protein interaction with other nuclear factors. A systematic analysis of HOX genes and gene products in different cellular contexts is clearly required to address this issue.

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