The *Hox-1.3* homeo box protein is a sequence-specific DNA-binding phosphoprotein

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We report that the murine *Hox-1.3* homeo domain protein is a nuclear phosphoprotein capable of binding to specific DNA sequences. DNase I protection of the *Hox-1.3* gene promoter region with the *Hox-1.3* protein identifies a binding site 144 bp upstream from the start of transcription. Both phosphorylated and nonphosphorylated forms bind DNA directly in a sequence-specific manner. Electrophoretic mobility shift assays were performed with a set of synthetic oligonucleotides representing either the DNase I-protected region of the *Hox-1.3* gene or partially homologous sequences present in promoter regions of other characterized viral, yeast, and mammalian genes. From the results, we deduce a consensus binding motif of CPyPyNATTAT/GPy. Base substitutions in the core ATTA sequence severely reduce or abolish binding. In the SV40 enhancer, the *Hox-1.3* binding motif overlaps both the octamer (Octa2) and the transactivator protein-1 (AP-1) binding sites. The *Hox-1.3* binding motif also overlaps the nuclear factor III (NF-III) octamer motif in the adenovirus-2 origin of DNA replication. Overlap among DNA-binding sites suggests that regulation imparted by certain *cis*-elements may be integrated by these different factors.

[Key Words: *Hox-1.3*; homeo domain–DNA binding; phosphoprotein]

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The molecular mechanisms that govern both DNA replication and gene expression are the instruments affecting developmental processes. Interactions between regulatory factors, their DNA-binding sites, and other components of the molecular machinery play fundamental roles in the realization of the developmental programs. Genetic analysis of these processes in the fruit fly *Drosophila melanogaster* has identified a number of genes whose correct function is required for both the elaboration and identity of its body parts (Ouweneel 1976; Nüsslein-Volhard and Wieschaus 1980). Many of these genes contain a homeo box sequence and encode homeo domain containing nuclear proteins (McGinnis et al. 1984; Scott and Weiner 1984; for review, see Gehring 1987). The presence of multiple copies of the homeo box sequence in the genomes of eukaryotes indicates that the encoded proteins represent a conserved family of related proteins with shared characteristics (McGinnis et al. 1985; Holland and Hogan 1986).

The most conserved region of the homeo domain shares amino acid sequence and structural similarities with DNA-binding, regulatory proteins known to con-

trol cell fate, such as the yeast mating-type *MATa1* and *MATa2* proteins (Laughon and Scott 1984; Shepherd et al. 1984). These observations have led to the proposal that homeo domain proteins function by binding to *cis*-acting regulatory elements and modulating the transcriptional activity of specific genes. In support of this hypothesis, DNA-binding studies employing either homeo domain fusion proteins or full-length homeo domain proteins synthesized in *Escherichia coli* have demonstrated that a structural element resides within the homeo domain, which binds DNA in a sequence-specific manner (Desplan et al. 1985; Fainsod et al. 1986; Cho et al. 1988; Hoey and Levine 1988). Additionally, Hiromi and Gehring (1987) have provided functional evidence that the *Drosophila* homeo box gene, *fushi tarazu* (*ftz*), is autoregulated; although direct DNA-binding studies have not yet been published, the presence of the *ftz* protein is required for the proper functioning of its own transcriptional enhancer.

The observation that divergent *Drosophila* homeo domain proteins recognize similar DNA sequences (Hoey and Levine 1988) suggests that these proteins represent a

family of regulatory factors that compete for similar DNA-binding sites and, once bound, perform unique or similar functions (Desplan et al. 1985; Harding et al. 1985). Sequence homology between the DNA targets of different binding proteins has been observed with non-homeo-domain proteins, as well. In yeast, both the AP-1 and GCN-4 transcription factors bind to similar DNA sites, but, nonetheless, affect expression of relevant genes differently (Harshman et al. 1988). The tissue specific and ubiquitous proteins that bind the octamer (Rosales et al. 1987) or CCAAT box (Dorn et al. 1987; Santoro et al. 1988) sequences represent other families of regulators that share DNA target sites.

Here, we report that the murine *Hox-1.3* protein (Odenwald et al. 1987; Fibi et al. 1988), prepared with the aid of a eukaryotic expression system, is a nuclear protein that undergoes multiple phosphorylation events. Multiple endogenous *Hox-1.3* forms are also present in the nuclei of cultured fibroblasts, and their differential salt elution from nuclei suggests that phosphorylated forms bind more avidly to nuclear sites. Both phosphorylated and unphosphorylated forms are sequence-specific DNA-binding proteins. The DNA target sequence of the *Hox-1.3* homeo domain protein (CPyPyNATTAT/GPy) is present, both in origins of DNA replication and in *cis*-regulatory elements that control cellular and viral gene expression. Many of these *cis*-regulatory elements contain *Hox-1.3* DNA-binding sites that either lie within, overlap with, or are adjacent to other sequence motifs recognized by non-homeo-domain transcription and/or DNA replication factors. These observations suggest that in addition to intrafamily competition, homeo domain proteins may also compete with other families of regulatory proteins for DNA-binding sites.

Results

Multiple forms of recombinant Hox-1.3 protein accumulate in the nuclei of spodoptera frugiperda cells

Previously, we reported several characteristics of the *Hox-1.3* homeo domain protein extracted from cultured fibroblasts or the central nervous system (CNS) of mid-gestation mouse embryos that suggested the protein was post-translationally modified. The proteins migrated aberrantly in SDS-polyacrylamide gels and could be resolved as several immunoreactive bands with apparent molecular weights that were larger than those predicted from the DNA sequence (Odenwald et al. 1987; M. Tani et al., unpubl.). These observations guided us in the selection of a recombinant DNA expression system for the generation of substrate amounts of the *Hox-1.3* protein. The baculovirus expression system was chosen because of its demonstrated high yield of recombinant protein and the ability of the eukaryotic host cells (*Spodoptera frugiperda*) to post-translationally modify recombinant nuclear proteins, such as *c-myc* (Miyamoto et al. 1985), in a correct manner.

Double homologous recombination between the wild-type baculovirus genome and the shuttle vector pAC YM-1-*Hox-1.3* (see Methods) yielded a polyhedrin-nega-

tive recombinant virus containing a single copy of a polyhedrin-*Hox-1.3* gene. Controlled by the polyhedrin promoter, the hybrid gene encodes an RNA that contains (in 5' → 3' order) all of the polyhedrin 5'-untranslated leader, a 14-base synthetic linker, 833 bases of *Hox-1.3* cDNA sequence, starting at the presumed initiation codon and ending 18 bases 3' of the termination codon (Odenwald et al. 1987), fused to the polyhedrin 3'-untranslated trailer. The 5'- and 3'-untranslated portions of the endogenous *Hox-1.3* cDNA contain sequences which may either reduce the translation efficiency (Bürglin et al. 1987) or message stability (Shaw and Kamen 1986), and the deletion of these sequences from the recombinant DNA construction should be noted.

Twenty hours postinfection with the plaque-purified recombinant virus, phase-dense intranuclear masses appeared in the *S. frugiperda* cells. By 40 hr, these masses occupied >50% of the nuclear volume. Immunofluorescent studies with either *Hox-1.3* peptide-specific, rabbit polyclonal, or mouse monoclonal antibodies (see Methods) identified the structures as *Hox-1.3* accumulations (Fig. 1).

The nuclear proteins extracted from *S. frugiperda* cells 36 hr post infection were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with monoclonal or polyclonal antibodies directed against the *Hox-1.3* protein. Multiple, distinct forms of the *Hox-1.3* immunoreactive proteins were identified with apparent molecular weights of 35–43 kD. No cross-reacting proteins were detected in extracts obtained from uninfected cells or cells infected with wild-type virus or unrelated recombinant virus (Fig. 2). In our previous report, we had estimated the apparent molecular weights of fibroblast *Hox-1.3* forms to be ~32–35 kD. Longer electrophoresis times and comigration of recombinant and fibroblast *Hox-1.3* proteins (Fig. 4) indicate that ~35–43 kD is a closer approximation of the apparent molecular weights.

Hox-1.3 is a nuclear phosphoprotein

We performed a series of experiments designed to determine whether the multiple forms of *Hox-1.3* were the result of post-translational modifications and what the nature of those modifications might be. Pulse-chase experiments, followed by immunoprecipitation of the labeled protein, revealed that [³⁵S]methionine first appeared in the fastest migrating form and only later in the slower migrating forms. The labeling of the slower migrating forms took place during the cold methionine chase, indicating that they were not directly labeled with free [³⁵S]methionine but were likely derived from the first labeled material. The appearance of multiple forms also suggested that *Hox-1.3* protein was undergoing multiple modifications. Additionally, the comparison of the ³⁵S-labeled immunoprecipitates with those obtained from cells incubated with [³²P]orthophosphate demonstrated that the slower migrating forms are phosphorylated, whereas no phosphate was detected in the

Figure 1. *Hox-1.3* protein accumulates in the nuclei of *S. frugiperda* cells infected with the recombinant baculovirus. Immunofluorescence and differential interference contrast microscopy (DIC) views (*a*, *b*) of host cells 20 hr postinfection with a low multiplicity of infection. Note that *Hox-1.3* protein colocalizes with intranuclear inclusions (arrowheads). Uninfected cells, immunostained with anti-*Hox-1.3* (*c*) or viewed by DIC (*d*), show that they lack the *Hox-1.3* epitope and contain no intranuclear inclusions. Cells infected with wild-type or unrelated recombinant viruses also show no immunostaining (data not shown).

faster migrating forms (Fig. 3b). Alkaline phosphatase treatment of the [35 S]methionine-labeled proteins substantiates the conclusion that phosphorylation was the principal factor in generating the multiple forms of the protein resolved by SDS–polyacrylamide gel electrophoresis. As shown in Figure 3C, treatment with alkaline phosphatase converts the slower migrating forms of the *Hox-1.3* protein into the faster migrating form. Alkaline treatment alone (100 mM NaOH for 30 min) does not alter the SDS–gel pattern (data not shown). Consequently, multiple phosphorylations, and not poly-ADP ribosylation, are responsible for the altered migration. Recently, Gay et al. (1988) reported that another homeo domain protein, the *Drosophila engrailed* protein, is also modified by phosphorylation.

Differential extraction of the Hox-1.3 proteins from nuclei

Western blot analysis of proteins extracted from NIH-3T3 nuclei with buffers containing different NaCl concentrations reveals that the multiple *Hox-1.3* forms possess different avidities for the nuclear binding sites (Fig. 4B). Higher NaCl concentrations were required to efficiently extract the slower migrating forms. The same differential extraction was also observed in cultured embryonic fibroblasts, which maintained a significantly lower steady-state level of the *Hox-1.3* protein (Fig. 4A). The comigration of the recombinant and the fibroblast *Hox-1.3* forms suggests that the different endogenous forms represent differences in the level of phosphoryla-

tion and that the differential salt extraction of *Hox-1.3* proteins from nuclei of fibroblasts is a manifestation of the increased affinity of phosphorylated forms for nuclear factors.

Hox-1.3 protein binds the promoter region of its own gene

The protein-/DNA-binding studies of others have demonstrated that fusion proteins containing homeo domains and full-length unmodified homeo domain proteins bind DNA in a sequence-specific manner (Desplan et al. 1985; Fainsod et al. 1986; Cho et al. 1988; Hoey and Levine 1988). Furthermore, genetic studies of *Drosophila* homeo box genes have provided evidence that they regulate their own expression, as well as the expression of other homeo box genes (for review, see Gehring 1987). These activities are presumed to depend on the homeo domain proteins' ability to bind to the *cis*-acting elements controlling these genes. To determine whether the *Hox-1.3* gene product also binds to the 5' region of its own gene, we performed DNase I protection analyses. Depending upon the amount of the recombinant *Hox-1.3* protein added in the assay (1.2–9.6 μ g of total protein), different regions of the 5' sequences were protected (Fig. 5). The sequence a–b was protected with the least amount of extract, whereas the adjacent sequences (c and d) required eight times more extract to obtain significant protection (under the conditions of our assay, the a–b footprint was also detected with 0.4 μ g of extract). None of these footprints was detected

Figure 2. Immunoblot analysis of nuclear extracts demonstrates that *Hox-1.3* immunoreactive proteins are unique to extracts from cells infected with the *Hox-1.3* recombinant virus. Nuclear extracts were prepared according to the Dignam et al. (1983) protocol from *Hox-1.3* recombinant virus infected cells (36 hr) (lane 1); wild-type baculovirus infected cells (36 hr, PI) (lane 2), uninfected cells (lane 3), and unrelated recombinant virus-infected cells (36 hr, PI) (lane 4). Proteins (~10 μ g) were resolved in a 12.5% polyacrylamide-SDS gel and electroblotted onto nitrocellulose. (Left half) Amido black staining; (right half) immunoblot with *Hox-1.3* peptide-specific monoclonal antibodies. The same immunostaining profile was observed with a peptide-specific polyclonal antibody (data not shown).

when equivalent amounts of extracts not containing the *Hox-1.3* protein were used (Fig. 5).

To confirm the specificity of the *Hox-1.3* protein-DNA interaction and determine whether both phosphorylated and nonphosphorylated forms bind DNA, we carried out Southwestern analysis, using both specific and nonspecific double-stranded DNA probes (Fig. 6). The results of these experiments demonstrate that when separated by SDS-polyacrylamide gel electrophoresis and immobilized on nitrocellulose, all forms of the *Hox-1.3* protein bind DNA in a sequence-specific manner. This indicates that the *Hox-1.3* protein interacts directly with DNA by itself, and not with the aid of other nuclear factors.

The *Hox-1.3* and *Hox-2.1* (Krumlauf et al. 1987) homeo box genes are thought to have originated from a

duplication event that included adjacent regions of the *Hox-1* and *Hox-2* homeo box gene clusters, located on murine chromosomes 6 and 11, respectively (Hart et al. 1987; Odenwald et al. 1987; Verrijzer et al. 1988). The observed similarity in the pattern of gene expression, in both embryonic and adult tissues, argues that the regulatory regions of these genes may exhibit considerable homology (for review of embryonic expression, see Holland and Hogan 1988). The putative promoter regions of these two genes contain multiple blocks of homologous sequences. One of these 5' regions shares 68% homology (over a sequence of 33 bp) with the human immunoglobulin heavy chain transcription enhancer (Rabbitts et al. 1983) and corresponds to sequences contained in the c and d footprints (Fig. 7). Multiple copies of sequences found in the b footprint are also part of the immunoglobulin enhancer. The *Hox-1.3* a-b protected sequence shares homology with footprints obtained with other homeo domain fusion proteins or full-length recombinant proteins (Cho et al. 1988; Hoey and Levine 1988). Footprints c and d (Figs. 5 and 7) do not share homology with each other or with the a-b footprint.

The specificity of the *Hox-1.3* protein-DNA binding was further investigated using an electrophoretic mobility shift assay and end-labeled synthetic DNA fragments. Incubation of a 30-bp segment of DNA corresponding to the a-b footprint sequence (Table 1), with *Hox-1.3*-containing extract, resulted in the formation of multiple complexes (Fig. 8A). The addition of the monoclonal 1D-9 antibody to the binding reaction revealed (via altered complex migration) that *Hox-1.3* protein was associated with the faster migrating complexes. These complexes were not detected when extracts prepared from uninfected or wild-type baculovirus-infected cells were used (data not shown). Competition experiments with an unlabeled, unrelated control 30-bp fragment (Table 1) indicated that the other complexes, unaffected by the *Hox-1.3* antibody, were the result of nonspecific binding (Fig. 8A). Increasing the poly[d(I-C)] concentration fourfold also blocked the nonspecific binding without altering the formation of *Hox-1.3* protein-DNA complexes (Fig. 8B,C). The formation of *Hox-1.3* associated complexes was not influenced when unlabeled control DNA was included in the binding buffer but was blocked when the unlabeled specific DNA fragments were included in competition experiments (Fig. 8C). Because polyacrylamide gel percentages $\geq 8\%$ were necessary to resolve the individual *Hox-1.3* protein-DNA complexes, it is unlikely that the changes in mobilities are due to multimerization. Rather, it suggests that some secondary alteration in the protein's conformation (perhaps induced by phosphorylation) may account for the different mobilities of the protein-DNA complexes. Mobility shift assays designed to examine the effect of monovalent salt concentrations on the *Hox-1.3* protein-DNA binding revealed that although binding was detected in the presence of 800 mM KCl, the formation and/or stability of the faster migrating complexes were selectively diminished in higher salt concentrations (Fig. 9). The selective retention of the slower migrating forms

Figure 3. Multiple phosphorylation events alter the mobility of *Hox-1.3* in polyacrylamide-SDS gels. (A) Pulse-chase experiments reveal that multiple immunoreactive *Hox-1.3* proteins arise from the fastest migrating form. (B) Comparison of [^{35}S]methionine- and [^{32}P]orthophosphate-labeled *Hox-1.3* immunoprecipitated proteins demonstrates that the slower migrating forms of *Hox-1.3* are phosphoproteins. (C) Disappearance of the *Hox-1.3* phosphoproteins after treatment with alkaline phosphatase suggests that their slower mobilities are due to phosphorylation. (PO₄), *Hox-1.3* recombinant [^{35}S]methionine-labeled extract preincubated with 200 mM NaPO₄ buffer (pH 8.0) before immunoprecipitation; (A.P.) extract preincubated with *E. coli* alkaline phosphatase; (A.P. + PO₄), as A.P. but in the presence of 200 mM NaPO₄ buffer (pH 8.0) to demonstrate that the disappearance of the *Hox-1.3* phosphoproteins in A.P. is not due to proteolysis.

Figure 4. Differential salt elution of nuclear *Hox-1.3* proteins from cultured mouse embryo cells and NIH 3T3 fibroblasts. (A) Autoradiographs of [^{35}S]methionine-labeled immunoprecipitated proteins resolved in a 12.5% polyacrylamide-SDS gel. (Lane 1), *Hox-1.3* recombinant proteins (autoradiographic exposure time, 48 hr); (lane 2), *Hox-1.3* immunoreactive proteins present in a 600 mM NaCl wash of nuclei from cultured mouse primary embryonic fibroblasts (exposure, 3 weeks); (lane 3), *Hox-1.3* immunoreactive proteins present in these nuclei after the 600 mM NaCl wash, solubilized by RIPA buffer (exposure, 3 weeks). Note the comigration of the fibroblast proteins with those of the recombinant. Also note that a significant amount of the slower migrating (presumably phosphorylated) protein is not eluted from the nuclei during the 600 mM salt wash. (B) Nuclei prepared from nonconfluent 3T3 fibroblasts were aliquoted and exposed to one of four different NaCl concentrations. The proteins in these salt washes were resolved in a 12.5% SDS-polyacrylamide gel, electroblotted onto nitrocellulose, and immunostained with *Hox-1.3*-specific antibodies. The observed differential elution profile of the *Hox-1.3* proteins suggests that the slower migrating forms may have a higher affinity for nuclear binding sites.

Figure 5. DNase I protection analysis of the *Hox-1.3* promoter region. A 310-bp genomic fragment that contains 250 bp of *Hox-1.3* upstream sequences from the 5' cap site of the major transcript was employed (see Fig. 6 and Methods). (A + G) Maxam and Gilbert purine ladder. (No Ext) DNase-I digest of ~1 ng of end-labeled DNA after incubation with 1.0 µg poly[d(I-C)], in reaction to buffer containing 200 mM KCl and no nuclear extract. Amounts of 1.2, 2.4, and 9.6 µg of the *Hox-1.3* protein-containing nuclear extract were preincubated with the end-labeled fragment, as above, prior to DNase I treatment. Nuclear extracts from wild-type virus-infected (8.0 µg) and uninfected (10.0 µg) were also tested. In the flanking cartoon, boxes represent protected regions and arrows indicate DNase-I hypersensitive sites induced by the *Hox-1.3*-containing extract. Note that protected regions *a* and *b* are detected in the digests that contain 1.2 µg of extract, whereas regions *c* and *d* show significant protection only with 9.6 µg.

indicates these DNA-protein complexes were more stable. These observations and the relative resistance of the phosphorylated forms of *Hox-1.3* to salt extraction from isolated nuclei (Fig. 4) strengthen the notion that the multiple complexes may arise by phosphorylation of the *Hox-1.3* protein.

To footprint regions *c* and *d*, greater amounts of extract were required when compared to the *a*-*b* footprint. However, the *c* and *d* footprints were specific for the extract containing the *Hox-1.3* protein, indicating that either *Hox-1.3* protein can bind these sequences, albeit with lower affinity, or other DNA-binding proteins in the extract can interact in a cooperative manner with *Hox-1.3* to bind adjacent sequences. The lack of shared sequence homology between these different footprints and the inability of a 30-bp fragment containing the footprint *c* sequence to compete for *Hox-1.3* binding (Fig. 8C) argue in favor of the latter. Distinction between these two possibilities and the role of phosphorylation await the purification of the individual *Hox-1.3* isoforms.

Hox-1.3 protein requires an ATTA box for efficient binding

To determine those base pairs necessary for efficient *Hox-1.3* binding, we carried out a series of mobility shift assays with different synthetic 30-bp oligonucleotides that share partial homology with the *a*-*b* *Hox-1.3* footprinted sequence (Fig. 8B,C; summarized in Table 1). Assays were also performed to test whether *Hox-1.3* protein-binding sites (predicted by sequence homology) existed in the promoter regions of other homeo box genes and in well-characterized *cis*-regulatory elements of other non-homeo-box genes (Fig. 8; Table 1). The results of these assays highlight the importance of the core 5'-ATTA-3' for efficient *Hox-1.3* binding. Single base alterations in the core ATTA box significantly reduce or abolish detectable binding under the conditions of our assays (see Methods and legend to Fig. 8). The lack of demonstrable *Hox-1.3* binding to the mHox 1.3e oligonucleotide (ATTA → AAAT; Table 1) further indicates that *Hox-1.3* protein interacts in a selective, sequence-specific manner with the ATTA sequence and does not bind indiscriminately to AT-rich sequences. Overlapping ATTA boxes in both orientations, 5'-TAATTA-3', also represent a high affinity core recognition sequence,

Figure 6. Both phosphorylated and nonphosphorylated *Hox-1.3* proteins are sequence-specific DNA-binding proteins. Nuclear extract from *Hox-1.3* recombinant virus-infected cells was fractionated electrophoretically through a 12.5% polyacrylamide-SDS gel and transferred to a sheet of nitrocellulose. This sheet was cut into individual strips, each of which contain separated proteins between apparent molecular weights of ~27–60 kd. Adjacent strips were treated in the following way: (Strip 1) The amido black staining of the immobilized proteins. Strip 2 was probed with a radiolabeled, catenated DNA fragment containing multiple *a-b* DNase-I-protected sequences (see Fig. 5 and Methods). After autoradiographic exposure (2A), immunoblot analysis of strip 2 was carried out to identify *Hox-1.3* immunoreactive proteins (2B). Strip 3 was processed the same as strip 2 but probed with a radiolabeled concatemer that lacks the *a-b* sequence (3A) (see Methods). (3B) The *Hox-1.3* immunoblot of this strip. Note that all forms of the *Hox-1.3* protein that are identified by the antibody bind DNA in a sequence-specific manner.

as detected in our mobility shift assays with the yeast α 1B-factor gene P-box *cis*-control element (Fig. 8C; Table 1). Our gel shift assays identified base pairs in the flanking sequences that enhanced or reduced binding. From these data, we compiled a consensus *Hox-1.3* binding motif (5'-CPyPyNATTAT/GPy-3') that reflects the sequences present in the 'good' binding oligonucleotides (Table 1). As shown in Figure 10, this DNA-binding motif shares overlapping sequence homology with the DNA-binding motifs of both the DNA replication nuclear factor III (NF-III) (Prujijn et al. 1987; Rosenfeld et al. 1987) and the transcription factor AP-1/c-jun (Bohmann et al. 1987; Lee et al. 1987).

Discussion

Here we have combined high level expression of a recombinant murine homeo domain protein, *Hox-1.3*, DNase I footprint analysis, Southwestern protein-DNA binding, and electrophoretic mobility shift assays to characterize the DNA-binding specificity of the *Hox-1.3*

protein. Our studies highlight several features of the interaction of this homeo domain protein with its nuclear target(s). First, the *Hox-1.3* recognition sequence is not unique to the *Hox-1.3* gene. Closely related sequences are found in other homeo box gene promoters and in many other *cis*-regulatory elements, both viral and cellular. A comparison of these sequences tested for *Hox-1.3* binding allowed us to deduce a consensus sequence, 5'-CPyPyNATTAT/GPy-3', with the core ATTA being required for efficient binding. Second, in certain *cis*-elements, this consensus sequence overlaps sequences recognized by well-studied transcription factors such as AP-1 or the proteins binding to the octamer sequence. Third, post-translational modifications give rise to multiple *Hox-1.3* forms distinguishable by their degree of phosphorylation, and these forms appear to display a characteristic binding avidity for nuclear factors. From these features, it is evident that the interaction of this homeo domain protein with its DNA-binding sites, if at all relevant in gene regulation, cannot be viewed separately from other systems of gene regulation; competition among different proteins for the same DNA target, binding of the same protein to related targets in different genes, and the regulation of the strength of binding to nuclear factors by post-translational modification all point to gene regulation, dependent upon control systems both highly integrated and well balanced. In the following text, we will discuss the points mentioned above.

Determinants of *Hox-1.3* protein-DNA binding

All *Antennapedia* (*Antp*) class homeo domain proteins studied thus far recognize sequences sharing the ATTA core sequence (see references cited above). In *Drosophila*, all homeo domain footprints reported contain the core ATTA box, with the exception of the *even-skipped* and *paired* protein footprints of the *even-skipped* promoter region (Hoey and Levine 1988). Yet, even in the *even-skipped* e4 DNase I-protected region, the sequence 5'-CCTCGTTATC-3' matches the *Hox-1.3* consensus sequence, except for 1 base, G. It is noteworthy in this context that substitution of GTTA for the core ATTA in an otherwise perfect consensus sequence still shows *Hox-1.3* binding, albeit weak (see Fig. 8; Table 1). This DNA recognition sequence conservation may reflect no more than that the proteins' DNA-binding domain (the homeo domain) has also been conserved during evolution. For example, all *Antp* class homeo domains share homologous, if not identical, amino acid residues in the region thought to contact DNA (for review, see Gehring, 1987). If distinct homeo domain proteins bind to the same sequence and yet accomplish unique functions, domains not directly involved in DNA binding are likely to play an important role in this specificity. It is conceivable that such protein domains interact in very specific ways with other components of the transcriptional machinery.

The comparison in mobility shift assays of DNA fragments containing different *Hox-1.3* binding sites has shown that the sequences flanking the ATTA box influ-

Figure 7. Alignment of *Hox-1.3* DNase I footprints with upstream homologies between the *Hox-1.3* and *Hox-2.1* genes. Optimal alignment of *Hox-1.3* (Odenwald et al. 1987) and *Hox-2.1* (Krumlauf et al. 1987) upstream sequences reveals blocks of noncollinear homologies. One of these regions (*Hox-1.3*, -109 to -77; *Hox-2.1*, -127 to -95) shares 68% sequence homology with a human Ig germ line heavy chain enhancer element (Rabbitts et al. 1983). Note that this region is coincident with DNase-I-protected regions *c* and *d*. Sequences homologous to protected region *b* (ATTAA) are also present in multiple copies in the heavy chain enhancer element (see Rabbitts et al. 1983). Adult lung *Hox-1.3* and *Hox-1.2* mRNA major cap sites (Murphy et al. 1988; R. Krumlauf, unpubl.) are denoted by stars; translation start codons are bracketed.

Table 1. Summary of protein-DNA binding studies

	5'	3'	Binding Efficiency
mHox 1.3	TGCCAACTCCCCATTAGTGCAGGACCT		Good
hHox 1.3	T.....		"
MFa1B (P-box)	CGA-CT-T-TA-.....C-TTC-AT-A		"
SV40 enhancer	G-ATG-AT-T-A-.....CAG-A-CCAGC		"
HSV-1 IE motif	A-GAA-.....CATG-CCCT-A		"
Ad-2 ori	A-T-C-C-T-.....TCAT-TTG-CTTC		"
BPV enhancer	GCTA-G-A-.....C-GTG-CTCT-A		"
mHox 2.1	T-TCCGAT-GT-.....T-TGTAACC-TAG		"
mHox 2.3a	CCATTT-T-A-.....C-GGA-CCT-G		"
mHox 2.3b	C-TT-C-ATT-.....T-AT-ATATT-A		"
c-fos (SRE)	A-AGG-TGT-AT-.....GA-TCT-T-G		Weak
mHox 1.3a	G.....		"
b	T.....		None detected
c	C.....		"
d	CC.....		"
e	AAT.....		"
f	C.....		"
g	G.....		"
mHox 1.3 fp.C	CTAG-GG-AT-AGGC-GATTAC-TG		"
Control oligo	ACTGCAGGTACGACC-AT-T-T-C		"

Consensus for Efficient Binding CPNATTA^T_GP

Electrophoretic mobility shift assays were performed on the above double-stranded end-labeled 30-bp oligonucleotides (sequence of one strand shown). (mHox-1.3) Sequence contains a-b footprint shown in Fig. 7 (-160 to -137 bp); (hHox-1.3) corresponding mHox-1.3 sequences present in the human *Hox-1.3* cognate promoter region [E. Tourmier et al., unpubl.]; [MF α 1B (P-box)] yeast α -factor structural gene α 1B *cis*-control region (-366 to -341 bp) contains the P-box [Bender and Sprague 1987; binding data not shown]; (SV40 enhancer) domain A, P motif [Zenke et al. 1986]; (HSV-1 IE motif) motif of α gene O promoter region (-236 to -207 bp; Mackem and Roizman 1982); (Ad-2 ori) adenovirus-2 origin of DNA replication (58-31 bp; Wides et al. 1987); (BPV enhancer) bovine papilloma virus long control region enhancer (7590-7612 bp; Mos-

ence the strength with which the *Hox-1.3* proteins bind to DNA. DNA recognition sequence comparisons of the different homeo domain protein-binding sites indicate that although the ATTA box is shared, individual proteins may require unique flanking sequences for high-affinity binding.

The degree of protein phosphorylation also appears to affect binding avidity to unknown nuclear components. At present, it is unclear whether this post-translational modification influences DNA binding, protein-protein interactions, or both. Nevertheless, when multiple DNA-binding sites with different binding avidities are available, the intranuclear concentrations of these proteins and their phosphorylation status are likely to play significant roles in modulating gene expression. Recent studies in *Drosophila* point in this direction. For instance, the *bicoid* homeo domain protein influences cell differentiation in a concentration dependent manner [Driever and Nüsslein-Volhard 1988].

kaluk and Bastia 1988); (mHox-2.1) murine *Hox-2.1* promoter region (-191 to -163 bp; Fig. 7 and Krumlauf et al. 1987); (mHox-2.3a, mHox-2.3b) murine *Hox-2.3* gene promoter region (α , 799-806 bp; β , 730-756 bp; Verrijzer et al. 1988); [c-fos (SRE)] serum response element in the *c-fos* enhancer (-319 to -269 bp; Treisman 1985); (mHox-1.3a through mHox-1.3g) variations of mHox-1.3; (mHox-1.3 fp.c) the c footprint shown in Fig. 7 (-128 to -99 bp). Dots represent identity with mHox-1.3. The relative binding efficiency represents an arbitrary assessment based on autoradiographic signal intensity comparisons between free and bound DNA (Fig. 8) (P) Pyrimidine; (N) any base.

Occurrence of the Hox-1.3 binding motif in promoter regions of other genes

As shown above, not only does *Hox-1.3* bind to sequences in its own gene and those of other homeo box genes, it also recognizes related sequences known to be to one or several host factors that bind to the IE *cis*-ele-

ment themselves [Kristie and Roizman 1987]. Because both *Hox-1.3* (Fig. 7B) and these host factors recognize similar DNA sequences and recognize them independently of Vmw65, it is tempting to speculate that they are homeo domain proteins. Another example is found regulatory elements of other genes. Some of them are worth mentioning. To stimulate expression of the im-

Figure 8. *Cis*-regulatory elements that control both transcription and DNA replication contain *Hox-1.3* protein-binding sites. (A) An end-labeled 30-bp synthetic DNA fragment (*mHox-1.3*; see Table 1) that contains the putative *Hox-1.3* protein-binding site was employed to both confirm *Hox-1.3* protein binding (via specific antibody interaction altering the mobility of the protein–DNA complexes) and to determine conditions that favor only sequence-specific binding. In a native 9.5% polyacrylamide gel, multiple-bound complexes are resolved from a binding reaction with 4 µg (total protein) of extract-containing recombinant *Hox-1.3* protein, 0.5–1.0 ng of [α - 32 P]end-labeled *mHox-1.3* fragment (2×10^4 cpm) and 1 µg of poly[d(I-C)] (lane 4 µg Ext). An additional incubation (after complex formation) with ascites fluid containing *Hox-1.3*-specific monoclonal IgG antibodies (final ascites dilution 1–100) reveals that the *Hox-1.3* protein associates with the faster-migrating complexes (lane Hox-1.3 MAb). Incubation with an unrelated monoclonal IgG did not alter complex migration (lane NS MAb). Competition with an unlabeled 30-bp control

oligonucleotide (Table 1) revealed that the slower-migrating complexes are the result of nonspecific interactions (lanes +2×, +10×, and +100×). Increasing the poly[d(I-C)] to 4 µg/reaction also diminished these nonspecific complexes (see Fig. 8B,C). (B) End-labeled synthetic 30-bp fragments shown in Table 1 were employed to determine the sequence specificity of *Hox-1.3* binding. Complex formation was performed in the presence of 4 µg (total protein) of *Hox-1.3*-containing extract, 4 µg of poly[d(I-C)], 0.5–1 ng of end-labeled oligonucleotide (2×10^4 cpm), and 200 mM KCl in 20 µl of binding buffer. (C) Competition analysis comparing *Hox-1.3* protein–DNA binding between individual unlabeled oligonucleotides (Table 1) and labeled *mHox-1.3* fragment. One-hundred-fold excesses (as determined by optical density measurements) of the unlabeled fragments were mixed with the labeled *mHox-1.3* oligonucleotide in binding buffer prior to the addition of the *Hox-1.3*-protein-containing extract.

Figure 9. Differential *Hox-1.3* protein-DNA complex affinities. Protein-DNA complexes between *Hox-1.3* proteins (4 μ g of total extract) and the end-labeled m*Hox-1.3* double stranded oligonucleotide (shown in Table 1) were formed at 4°C for 1 hr in binding buffer containing 4 μ g poly[d(I-C)] and either 0.1, 0.2, 0.4, 0.6, or 0.8 M KCl. Complexes were resolved by electrophoresis through a 9.5% polyacrylamide gel. Note that in addition to causing an overall reduced electrophoretic mobility, the higher salt concentrations selectively reduced the formation and/or stability of the faster migrating complexes.

mediate early (IE) genes of herpes simplex virus (HSV), a viral protein, Vmw65 (also known as VP16) (Preston et al. 1988; Triezenberg et al. 1988), is required. Interestingly, this protein does not bind DNA directly but binds in yeast: The α -specific upstream activating sequence P-box consensus (Bender and Sprague 1987) shares perfect homology with the *Hox-1.3* motif. Activation of the α -specific genes depends on the cooperative interaction of two factors, the pheromone response transcription factor (PRTF), which footprints the consensus motif (Bender and Sprague 1987), and the *MAT α 1* factor. Because PRTF protects sequences that match the *Hox-1.3* consensus binding motif, it is possible that this factor's DNA-binding element, as well, shares homology with the *Hox-1.3* DNA-binding domain.

Potential homeo domain binding sites also occur within origins of DNA replication. DNA sequences that function as autonomously replicating sequences (ARS) within yeast contain potential homeo domain binding sites. For example, elements within the yeast ARS-120 (Eisenberg et al. 1988) and ARS-C2G1 (Palzkill and Newlon 1988) and the human ARS-2 (Montiel et al. 1984) all contain multiple sites that share homology with the *Hox-1.3* binding motif. In addition, the origins of DNA replication of adenovirus (Wides et al. 1987),

Optimal Alignment of Transcription Factor Binding Motifs

NF-III											A	T	T	A	T	C	A	T
											.	.	.	.	.	.	.	.
Hox 1.3											C	P	P	N	A	T	T	A
											.	.	.	.	.	.	.	.
AP-1/c-JUN											T	T	A	G	T	C	A	G
											G							P

Figure 10. Overlap among transcription factor DNA-binding motifs. Alignment of NF III, an octamer binding protein (Prujñ et al. 1987; Rosenfeld et al. 1987); *Hox-1.3* (this paper); and AP-1/c-jun (Bohmann et al. 1987; Lee et al. 1987) DNA-binding motifs in 5' $\rightarrow$ 3' alignment. (P) Pyrimidine; (N) any base.

HSV (Weller et al. 1985; Elias and Lehmann 1988), and Epstein-Barr virus (Rawlins et al. 1985; Reisman et al. 1985) contain multiple potential homeo domain binding sites. Many of these recognition sequences are contained within or overlap footprints by cellular or viral factors (see the above references for footprinting data). Although the functional significance of this observation is unknown, other transcription factors such as NF-1 (Jones et al. 1987; Santoro et al. 1988) and NF-III (Prujñ et al. 1987; Rosenfeld et al. 1987) have been shown to modulate in vitro DNA replication.

Overlap of the *Hox-1.3* motif with binding sites for other transcription factors

Analysis of eukaryotic *cis*-regulatory elements reveals that the *Hox-1.3* binding site can overlap with either AP-1 or octamer binding sites. Our DNase-I protection studies and electrophoretic mobility shift assays show that *Hox-1.3* proteins bind efficiently in the absence of these transcription factor binding motifs, demonstrating the uniqueness of the *Hox-1.3* motif. In the SV40 enhancer, our mobility shift assays identify a high-affinity *Hox-1.3* protein-binding site within the *SphI*/P motifs of domain A (Zenke et al. 1986). This binding site (shown in Table 1) matches the *Hox-1.3* consensus motif and overlaps both the Octa-2 (OBP100 protein-binding site, Sturm et al. 1987) and the AP-1 binding motif (Angel et al. 1987; Lee et al. 1987). Potential homeo domain binding sites also overlap with other AP-1 motifs in the SV40 genome (Lee et al. 1987). Many of the octamer binding sites in the 5'-flanking regions of immunoglobulin genes overlap or are adjacent to potential *Hox-1.3* target sequences. Of the 20 octamer-containing Ig promoter regions shown by Parslow et al. (1984), 12 also harbor potential homeo domain binding sites of which 9 overlap with octamer motifs. The observed overlap of binding motifs for different classes of binding factors (highlighted in Fig. 10) raises the possibility that overlap in binding motifs may result in binding competition or cooperativity among different transcription factors, with the consequence that the activity of a particular transcription factor is not only determined by its own binding characteristics but also by those of others. Such mutual control among transcription factors may represent an important mechanism, by which a high number

of different genes may be coordinately regulated by a lower number of transcription factors.

In conclusion, we have shown that a mammalian homeo domain protein (*Hox-1.3*) binds DNA in a sequence-specific manner and recognizes regulatory sequences in defined *cis*-elements. It is likely, then, that this homeo domain protein can play an important role in the regulation of other genes, both cellular and viral.

Methods

Production of the recombinant Hox-1.3 baculovirus

Baculovirus shuttle vector constructions were produced by using the pUC-Xho-rhomo plasmid (obtained from S. Murphy and E. Linney, Duke University) and the *Autographa californica* nuclear polyhedrosis virus (AcNPV) shuttle vector pAcYM-1 (Matsuura et al. 1986). An 847-bp *XhoI*–*ScaI* fragment from pUC-Xho-rhomo that contains a 14-bp synthetic linker, followed by the *Hox-1.3* translation initiation codon and the entire *Hox-1.3* protein-encoding sequence plus 18-bp of 3'-untranslated sequence (Odenwald et al. 1987) was cloned into the *Bam*HI site of pAcYM-1 by filling in 5' protruding ends with the Klenow enzyme and dNTPs; blunt-end ligations followed, according to the procedures outlined by Maniatis et al. (1982).

The protocols of Summers and Smith (1987) were used for the care and propagation of both the AcNPV and its host, the *S. frugiperda* cells. After calcium phosphate cotransfection of the AcNPV genome and pAcYM-1–*Hox-1.3* DNAs into *S. frugiperda* cells, transfer of the hybrid polyhedrin–*Hox-1.3* gene into the viral genome was accomplished by double homologous recombination. The transfection protocol of Smith et al. (1983) was used. The procedures used for selecting, purification, and titering of the recombinant virus were carried out as described by Summers and Smith (1987).

Cultured cells and nuclear extracts

Mouse embryo cells were prepared according to Arnheiter and Staeheli (1983) from 16-day gestation BALB/c embryos. Experiments were performed on culture passages 4–6. These embryo cultures and the NIH-3T3 mouse fibroblast cell line were maintained in DMEM containing 10% FCS and antibiotics. *S. frugiperda* cells were maintained in Grace's medium supplemented with lactalbumin hydrolysate (3.3 g/liter), Yeastolate (DIFCO Labs) (3.3 g/liter), 10% FCS, and antibiotics. Care and maintenance of the *S. frugiperda* cells was according to Summers and Smith (1987).

Nuclear extracts were prepared according to the procedure described by Dignam et al. (1983) from subconfluent monolayers of uninfected cells and infected *S. frugiperda* cells 36 hr after virus absorption. Unless otherwise stated (see figure legends), nuclei were extracted in buffer D containing 100 mM Tris-HCl (pH 7.9), 600 mM NaCl, 5% glycerol, 5 mM MgCl₂, 1 mM DTT and 1 mM EDTA. Final extract protein concentrations varied from 1 to 2 mg/ml (as determined by a Bio-Rad protein assay kit with BSA as a standard).

Peptide synthesis

A 20-amino-acid-long peptide, corresponding to *Hox-1.3* protein residues 56–75 (Odenwald et al. 1987), was synthesized by the Merrifield solid-phase technique (Merrifield and Barany 1980) with an Applied Biosystems 430-A peptide synthesizer. The 20-mer was purified to a single peak by high pressure liquid

chromatography, and the molecular weight and purity confirmed by mass spectrophotometry. Hydrolysis of the peptide gave amino acid compositions consistent with the expected ratios. The peptide was coupled to keyhole limpet hemocyanin (KLH) by procedures described previously (Odenwald et al. 1987).

Monoclonal IgG production, Western immunoblot analysis, and immunofluorescence

The mouse monoclonal 1D-9 IgG antibody that binds the 20-mer synthetic peptide described above was produced following standard procedures (Arnheiter et al. 1981). Supernatant fluids of cell cultures containing hybridomas were screened by the enzyme-linked immunoadsorption assay (ELISA), according to the protocol described by Richardson et al. (1985). The thrice-cloned (by limiting dilution) 1D-9 hybridoma was injected into the peritoneal cavity of female BALB/c mice for the production of ascitic fluid. Immunoblot analysis of both recombinant *Hox-1.3* baculovirus infected *S. frugiperda* cell nuclei or cultured mouse fibroblast nuclei extracts using the 1D-9 monoclonal or rabbit polyclonal anti-peptide A antisera were done as described (Odenwald et al. 1987) and gave similar results. Likewise, the immunofluorescent labeling, also performed as described (Odenwald et al. 1987), with either antibody was similar. As control antibody, the monoclonal anti-peptide IgG III/21 (Arnheiter et al. 1981) was used.

Radiolabeling, alkaline phosphatase digestion, and immunoprecipitations

Radiolabeling of recombinant infected *S. frugiperda* cells (36 hr post virus adsorption) with either [³⁵S]methionine or carrier-free [³²P]orthophosphate was accomplished according to Miyamoto et al. (1985). To lower endogenous pools of methionine or free phosphate, cells were washed and incubated for 30 min in either methionine-free or phosphate-free modified Grace's medium. Nuclear extracts from the labeled cells were prepared as above, with buffer C containing 0.6 M NaCl. Pulse-chase experiments were carried out as follows. After a 30-min incubation in methionine-free medium, cells were incubated with 100 μCi of [³⁵S]methionine (1106 Ci/mM, New England Nuclear) per milliliter for 5 min, followed by three washes and incubation in complete Grace's medium for the designated times.

Alkaline phosphatase digestion of the *Hox-1.3* protein was carried out by mixing 20 μl of the *Hox-1.3* protein containing extract (~40 μg total protein) with an equal volume of *E. coli* alkaline phosphatase (~4 units, type 111-N, Sigma) in 50% glycerol, 0.5 mM MgCl₂, 0.5 mM ZnCl₂, and 5 mM Tris-HCl (pH 8.5). After 1 hr at 37°C, 400 μl of RIPA buffer containing 0.5 M NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 0.5 mM PMSF, and 50 mM Tris-HCl (pH 7.5) was added to the mixture and *Hox-1.3* proteins were immunoprecipitated as described below.

Immunoprecipitations of nuclear extracts containing radiolabeled proteins were performed using the following protocol. Twenty microliters of extract (~40 mg total protein) were added to 400 μl of RIPA buffer, mixed and added to 50 ml of a 1 : 1 (vol/vol) mixture of protein A–Sepharose 4B (Pharmacia) and RIPA buffer. This mixture was incubated on ice with occasional mixing for 30 min. The protein A–Sepharose was removed by a 30-sec centrifugation in an Eppendorf centrifuge and ascites fluid containing the 1D-9 antibody (final dilution 1 : 500) was added to the supernatant. After 3 hr of gentle rocking at 4°C, 50 μl of fresh 1 : 1 mixture of protein A–Sepharose/RIPA buffer was added and incubated for an additional 45

min at 4°C. The Sepharose beads were washed two times by centrifugation, followed by resuspension in 400 μ l of RIPA buffer, transferred to a new Eppendorf tube, and washed two additional times as above. After one final wash in 400 μ l of PBS (pH 7.5), the beads were heated in an equal volume of SDS-gel electrophoresis sample buffer (40% glycerol, 4% SDS, 3% DTT, 0.005% bromophenol blue, and 60 mM Tris-HCl at pH 6.8) at 100°C for 10 min. After removal of the beads by centrifugation, the proteins in the supernatant were resolved by electrophoresis through 12.5% SDS-polyacrylamide gels according to the method of Laemmli (1970). The electrophoresed gels were soaked in autoradiographic enhancer solution (Enlightening, DuPont) for 30 min, dried, and exposed to X-ray film at -70°C with an intensifying screen.

DNase I footprinting

DNase I footprint analysis was carried out according to the protocol described by Lichtsteiner et al. (1987). Approximately 1 ng of the 310-bp end-labeled *XhoI-SacI* Hox-1.3 promoter region fragment (2×10^4 cpm) was added to a reaction mixture containing 25 mM HEPES (pH 7.6), and 100 mM KCl, 7.5% (vol/vol) glycerol, 0.1 mM EDTA, 0.75 mM DTT, 5 mM MgCl₂, and 1 μ g double-stranded poly[d(I-C)] DNA. Nuclear extracts were mixed in last by gentle pipetting (final volume of reactions was 50 μ l). The binding reaction continued for 90 min on ice. One microliter of DNase I (50–100 μ g/ml, Boehringer-Mannheim), prepared according to Lichtsteiner et al. (1987), was then added to the reaction mixture by gentle pipetting. After 90 sec on ice, the digestions were stopped by the addition of 100 μ l of solution containing 100 μ g/ml proteinase K, 200 mM KCl, and 100 mM Tris-HCl (pH 7.5). After 45 min at 45°C, the nucleic acids were extracted twice with phenol, followed by one chloroform extraction, and precipitated with 2.5 volumes of ethanol. The pellets were resuspended in sample buffer and electrophoresed through a 6% polyacrylamide-7M urea sequencing gel. The gels were dried and autoradiographed at -70°C with an intensifying screen.

Preparation, annealing, and end-labeling of oligonucleotides

Using cyanoethyl-phosphoramidite chemistry, 26- and 27-base-long complementary oligonucleotides (overlapping to create 5'-protruding ends) were synthesized on an Applied Biosystems 380-A DNA synthesizer. After deblocking (65°C for $\geq$ 6 hr) and lyophilization, the oligonucleotides were resuspended in deionized water, phenol extracted twice, followed by one chloroform extraction and ethanol precipitated. The pellets were resuspended in 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA. The complementary pairs were annealed by mixing 1 mg/ml of each (final concentration) in 50 μ l of 100 mM NaCl, TE (pH 8.0), heating (85°C for 10 min), followed by slow cooling. End labeling by filling in 5'-protruding ends with the Klenow enzyme to create 30-bp fragments, each containing three [α -³²P]dCTPs, was performed according to Maniatis et al. (1982). Upper strand sequences of the annealed oligonucleotides are shown in Table 1.

Southwestern analyses

The protocol developed by Miskimins et al. (1985) for the detection of DNA-binding proteins in crude nuclear extracts fractionated by SDS-polyacrylamide gel electrophoresis was employed with the following modifications. Hox-1.3 protein-containing extract was fractionated by electrophoresis through a 12.5% polyacrylamide-SDS gel and transferred onto nitrocellulose according to Towbin et al. (1979). After blocking in

binding buffer [120 mM NaCl, 5 mM MgCl₂, 0.5 mM DTT, and 25 mM HEPES (pH 7.9)] supplemented with 5% nonfat dry milk at 4°C for 1 hr, nitrocellulose strips were transferred to the above buffer containing 0.25% nonfat dry milk, 1 μ g poly-[d(I-C)]/ml, and 10⁶ cpm/ml of radiolabeled probe DNA. Synthetic, catenated DNA probes (mean length of ~120 bp) that contain either multimers of the a-b footprinted sequence or a nonspecific sequence shown below were prepared and nick translated—using [α -³²P]dCTP—according to Vinson et al. 1988.

5'-TACCCCATTTAGTGCG-3' a-b monomer
3'-GGTAATCACGCATGG-5'

5'-TAGCCACGACTTGCG-3' nonspecific monomer
3'-GTGCTGAACGCATCG-5'

After gentle shaking at 4°C for 6 hr, the strips were washed over a 30-min period with three changes of binding buffer containing 0.25% dry milk, air-dried, and exposed to X-ray film overnight with an intensifying screen at -70°C. Immunoblot analyses of the same strips were carried out using the 1D-9 monoclonal, as described above.

Electrophoretic mobility shift assays

Protein-DNA complexes were resolved in low ionic strength 9.5% polyacrylamide gels as described initially by Carthew et al. (1985), with the following modifications. Two microliters of nuclear extracts (~4 μ g total protein) were added to binding buffer (final volume, 20 μ l) containing 1.0 or 4.0 μ g of poly-[d(I-C)] (Pharmacia), 100–800 mM KCl (Fig. 8), 12% glycerol, 12 mM HEPES/NaOH (pH 7.9), 4 mM Tris-HCl (pH 7.9), 1 mM EDTA, 1 mM DTT, 300 μ g/ml BSA, 5 mM MgCl₂, and 2×10^4 cpm of ³²P-end-labeled oligonucleotide (0.2–0.8 ng). After 90 min on ice, 5 μ l of 10% Ficoll, 0.1% bromophenol blue, 0.1% xylene cyanol FF, 1 mM EDTA, 3.3 mM sodium acetate, and 6.7 mM Tris-HCl (pH 7.9) was added. For antibody incubations, 1 μ l of ascites fluid (diluted 1–10 in binding buffer) was added to the binding reaction 30 min prior to the addition of the sample buffer. Six microliters of the above mixture were carefully layered onto a pre-electrophoresed (30 min, 20 mA, at 4°C) low ionic strength 9.5% polyacrylamide gel (acrylamide/bisacrylamide 30 : 0.8; 1 mM EDTA, 3.3 mM sodium acetate; 5% (vol/vol) glycerol, and 6.7 mM Tris-HCl (pH 7.5). Electrophoresis was performed for at least 3 hr (20 mA at 4°C), with recirculation of the running buffer (1 mM EDTA, 3.3 mM sodium acetate, and 6.7 mM Tris-HCl at pH 7.5). Gels were dried and exposed to X-ray film overnight at room temperature.

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Note added in proof

After submission of this manuscript, several reports describing divergent homeo domains in known transcription factors have

been published in this journal and others. Their DNA binding sequences are strikingly similar to those which we have found for the *Hox-1.3* protein. We refer the reader to Miranda Robertson's News and Views communication (*Nature* 336: 522–524, 1988) which cites many of these contributions.

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