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Repression by HoxA7 Is Mediated by the Homeodomain and the Modulatory Action of Its N-Terminal-Arm Residues

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***Hox* genes encode homeodomain-containing proteins that are presumed to control spatial patterning during murine embryogenesis through their actions as transcriptional regulatory proteins. In this study, we have investigated the transcriptional function of a prototypic member of this family, HoxA7. We demonstrate that HoxA7 functions as a potent transcriptional repressor and that its action as such requires several domains, including both activator and repressor regions. The repressor regions are contained within the homeodomain and a C-terminal acidic region, both of which are well conserved among members of the Hox family. Accordingly, we show that two other members of this family also function as repressors, although they vary in their relative repressor potency. Finally, we explore the novel observation that the homeodomain of HoxA7 functions as a transcriptional repressor domain. We show that the homeodomain compared with two other DNA-binding domains, is unique in its ability to function as a repressor domain and that repression requires conserved residues in helix III. We further show that residues in the N-terminal arm of the homeodomain contribute to the differential repressor actions of various Hox proteins. These findings demonstrate that the transcriptional function of HoxA7 and possibly of Hox proteins in general is determined by their unique combination of conserved and nonconserved regions as well as through the complex actions of their homeodomains.**

Establishment of the embryonic body plan requires a highly intricate network of regulatory genes whose appropriate spatial and temporal expression specifies cell fate and ultimately leads to regional identity along several embryonic axes. Among these essential developmental regulators are the *Hox* genes, which are major players in the complex program that directs axial patterning during murine embryogenesis (reviewed in references 32 and 44). The murine *Hox* family consists of 38 related genes grouped on four chromosomes (referred to as *HoxA*, *HoxB*, *HoxC*, and *HoxD*). Being the evolutionary descendants of the *Drosophila* HOM-C complex, *Hox* genes share many features with HOM-C genes, including their characteristic colinearity of chromosomal organization and spatial expression in the developing embryo (2, 32, 44). This feature is manifested as the coordinate relationship of their anterior expression boundaries and their relative positions on the chromosome so that *Hox* genes located 3′ have more rostral expression boundaries whereas those located 5′ have more posterior expression boundaries (15, 17, 20, 62). This graded distribution of *Hox* gene expression along the anterior-posterior body axis presumably constitutes a combinatorial code (the so-called *Hox* code) which specifies positional information in the developing embryo (32, 44). The implication of such a *Hox* code, now well supported by numerous targeted-disruption studies, is that the roles of individual *Hox* genes are highly complex, having functions that are partially overlapping, partially combinatorial, and partially selective.

Hox genes share a conserved motif termed the homeobox, which encodes a DNA binding domain (the homeodomain), and therefore their protein products are presumed to function by regulating the transcription of specific downstream target genes (3, 18, 35, 55). However, far less is known about the actions of Hox proteins as transcriptional regulators or the identity of their downstream target genes than is known about their biological roles during murine embryogenesis. One of the main obstacles has been the pervasive issue of deciphering the mechanisms of target gene selection by homeodomain proteins (discussed in reference 3). Thus, despite the fact that their DNA binding properties are characteristically overlapping and nonselective in vitro, homeodomains are known to mediate functional specificity in vivo (9, 19, 33, 42, 65). This obvious paradox suggests that the intrinsic role of the homeodomain is relatively broad and that DNA binding activity may account for only one aspect of its actual function. Some clues as to how functional specificity is achieved have been elucidated in recent years. For instance, we and others have shown that despite their similar DNA binding specificities, Hox proteins exhibit differences in their relative affinities for DNA, suggesting that a component of specificity may include competition for binding sites (12, 48, 50). An additional aspect of target gene selectivity is likely to be derived from specific interactions of Hox proteins with other protein factors. In particular, the homeodomain protein Pbx has recently been shown to interact cooperatively with several Hox proteins both in vitro and in vivo to modulate their DNA binding specificity, and this interaction has been conserved with their *Drosophila* homologs (8, 10, 29, 39, 41, 49, 51, 59, 61). However, it is unlikely that either cooperative protein-DNA interactions or differential binding affinity are sufficient to account for the complexity of Hox protein function, particularly since some actions may be mediated primarily through protein-protein rather than protein-DNA interactions (7, 64).

To address the issue of transcriptional control by Hox proteins, we have been studying a prototypic member of this family, HoxA7 (formerly Hox 1.1 [54]). Since HoxA7 is encoded by one of the first *Hox* genes identified, a considerable volume of biological data regarding the expression of *HoxA7* during em-

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FIG. 1. Domain organization of HoxA7. A schematic representation of HoxA7 showing the positions of conserved domains (shared by Hox proteins in paralogous groups 1 to 8 or by a subset of Hox proteins [stippled and striped boxes, respectively]) and nonconserved regions (white box) is shown. Pro-Ala, region containing a high percentage of proline (11%) and alanine (14%) residues, unique to individual Hox proteins; HP, hexapeptide motif, a sequence of six amino acids that is shared among Hox proteins in paralogous groups 1 to 8; and Acidic, region that contains a stretch of glutamic acid residues. Also shown is the position of the homeodomain.

bryogenesis and its essential function in developmental control has accumulated (5, 30, 31, 40). Moreover, its primary sequence reveals several features that are characteristic of Hox proteins and which are likely to be important for their functions in transcriptional regulation (Fig. 1). In particular, HoxA7 contains several protein regions, aside from its homeodomain, that are shared among members of the Hox family (Fig. 1). These include a hexapeptide motif adjacent to the homeodomain that mediates the interaction with Pbx (10, 49), a conserved N-terminal sequence, and a stretch of glutamic acid residues in its C-terminal region that is a feature of many, but not all, Hox proteins (Fig. 1). Between the hexapeptide and the N-terminal motif, HoxA7 contains a region that is unique (Fig. 1). This region contains a high percentage of proline and alanine residues, which are frequently associated with transcriptional regulatory domains (45). These features of HoxA7, including its expression, known biological relevance, and protein organization, make it a suitable model with which to gain insight into the transcriptional regulatory properties of Hox proteins.

Here, we show that HoxA7 functions as a potent transcriptional repressor and that this activity requires the cumulative action of multiple functional domains. Since these domains are differentially conserved among the various members of the Hox family, this provides a framework for understanding how the actions of Hox proteins may be partially overlapping and partially unique. Finally, we demonstrate that a major component of repression by HoxA7 is provided by its homeodomain, which suggests a novel role for this protein domain in addition to its well-characterized function in mediating DNA-protein interactions. We further show that other Hox homeodomains function as repressor domains, although residues within the N-terminal arm modulate the potency of their repressor action. In combination, these observations implicate a broad role for the homeodomain in transcriptional regulation, as well as a molecular basis for deciphering the complexity of Hox protein function in transcriptional control.

MATERIALS AND METHODS

Construction of the expression plasmids. cDNAs containing the full-length *HoxA7* and *HoxC8* coding sequences were obtained by reverse transcriptase-PCR amplification from 9.5-day-postcoitum (p.c.) mouse embryonic RNA. The cDNA containing full-length *HoxB4* was a generous gift of Robb Krumlauf. The 5′ and 3′ oligonucleotides used for PCR contained unique *Bam*HI and *Hin*dIII restriction sites to facilitate cloning into the pM2 eukaryotic expression plasmid in frame with sequences encoding the GAL4 DNA binding domain (amino acids 1 to 147) (53). Alternatively, the full-length *Hox* sequences were cloned into the mammalian expression vector pCB6+ as detailed elsewhere (7). Truncated *HoxA7* sequences were obtained by PCR amplification of full-length *HoxA7* using oligonucleotides that contained *Bam*HI and *Hin*dIII restriction sites to facilitate cloning into either pCB6+ or pM2. The pCB6+− *HoxA7* series also contained a heterologous epitope from the myc protein as detailed previously (66) to facilitate detection of the HoxA7 polypeptides (i.e., myc-HoxA7) in mammalian cells. Mutations were introduced into *HoxA7* by overlapping PCR mutagenesis using oligonucleotides containing the appropriate nucleotide substitutions. The chimeric genes *HoxA7*/*C8* and *HoxA7*/*B4* were constructed by overlapping PCR using oligonucleotides that joined the sequences encoding HoxA7 amino acids 2 to 128 with those encoding HoxC8 amino acids 149 to 242 or HoxB4 amino acids 161 to 250. The pCG147 plasmid and the pCG147-c-*rel* CCRxC construct containing the Rel DNA binding domain were as described in reference 25. Sequences encoding the Fos DNA binding domain were subcloned from pDS56-*wbfos* (1) into pM2 by using the *Bam*HI and *Hin*dIII restriction sites. The pCMV-*Pbx-1* expression plasmid was a generous gift of M. Kamps. All plasmids were prepared by using plasmid kits (Qiagen) according to the manufacturer's instructions. All constructs were verified by dideoxy DNA sequencing with a Sequenase version 2.0 kit (U.S. Biochemicals) according to the manufacturer's instructions.

Expression of HoxA7 polypeptides. The expression of the myc-HoxA7 and GAL4-Hox polypeptides was verified by Western blot (immunoblot) analysis performed exactly as described previously (7). The antisera used were either a monoclonal antibody directed against the GAL4 DNA binding domain (Santa Cruz Biotechnologies) or a monoclonal antibody directed against the myc epitope (66). The expression of the polypeptides in mammalian cells is shown in Fig. 2. Note that expression of the various polypeptides was similar to that of myc-HoxA7 (Fig. 2A) or GAL4-HoxA7 (Fig. 2B to E) with the exception that myc-HoxA7(129-229) (Fig. 2A) and GAL4-HoxA7(2-60) and GAL4-HoxA7(31-129) (Fig. 2B) were expressed at lower levels.

Transient-transfection assays. Transfection assays were carried out essentially as described by Catron et al. (7). NIH 3T3 cells (below passage 10) were seeded 16 to 24 h prior to transfection at 10^5 cells per 35-mm-diameter dish in Iscove's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. F9 embryonic carcinoma cells (below passage 10) were seeded 16 to 24 h prior to transfection at 10^5 cells per 35-mm-diameter dish (0.3% gelatin coated) in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum and 2 mM L-glutamine. Cells were transfected by a calcium phosphate procedure. Transfection assays included the indicated amount of the expression plasmid, 1,000 ng of the luciferase reporter plasmid per dish, and 1,000 ng of the internal-control plasmid pCMV-βgalactosidase. The GAL4-luciferase reporter plasmids containing the simian virus 40 (SV40) promoter were described in reference 7, and the WIP luciferase reporter plasmids were described in reference 24. The thymidine kinase (TK)-luciferase and the GAL4–TK-luciferase plasmids were kindly provided by P. Traber. Cells were harvested 48 h posttransfection in 1× reporter lysis buffer (Promega). Luciferase activity was measured as counts per minute in a scintillation counter by using a luciferase assay system (Promega) according to the manufacturer's instructions, and β-galactosidase activity was assayed as described previously (7). Luciferase assays were normalized to levels of β-galactosidase activity to account for differences in transfection efficiency. For all assays described, baseline transcriptional activity was defined as the luciferase activity obtained with an expression plasmid (either pM2 or pCB6+) that lacked the HoxA7 sequences. Data were expressed as fold luciferase activity relative to the baseline and represented as zerofold (rather than onefold) for the purpose of clarity in presentation. Experiments were carried out in duplicate and performed a minimum of six times or as indicated.

RESULTS

HoxA7 functions as a transcriptional repressor. To investigate the transcriptional properties of HoxA7, we performed transient-transfection assays with NIH 3T3 cells, using an expression plasmid that contained the *HoxA7* coding sequence directed by the cytomegalovirus promoter. As is the case for a majority of Hox proteins, natural target sequences for HoxA7 have not yet been identified. Therefore, we used a reporter plasmid that contained a genomic element isolated from the Wnt-1 enhancer, termed WIP, that contains a single homeodomain binding site which has previously been shown to be required for appropriate expression of a *Wnt-1* transgene in vivo (24). Thus, the WIP element provides a consensus DNA site within a biologically relevant context through which to test the transcriptional activity of HoxA7. The WIP element was situated upstream of the SV40 early promoter, which directed expression of the luciferase gene so that transcriptional activity was measured indirectly as luciferase enzymatic activity (Fig. 3A). As shown in Fig. 3, HoxA7 repressed transcription through the reporter plasmid that contained the WIP element. Specifically, the luciferase activity obtained by transfection of the reporter plasmid along with the expression plasmid encoding HoxA7 was reduced by 18-fold relative to activity obtained when the reporter plasmid was transfected with an expression plasmid lacking HoxA7 (Fig. 3A). Furthermore, repression was mediated through the WIP element, since the luciferase activity of a reporter plasmid that did not contain this site was

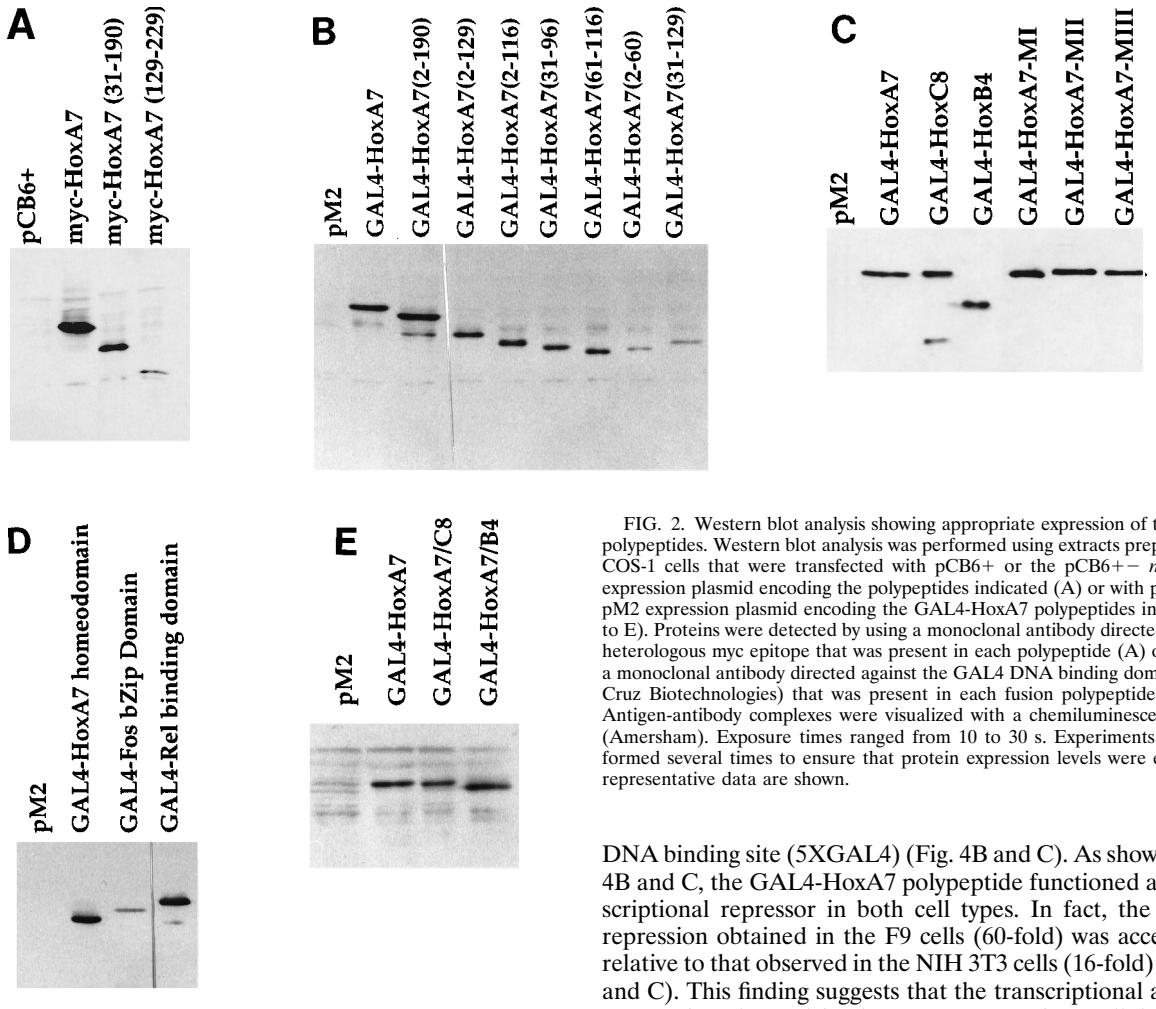

FIG. 2. Western blot analysis showing appropriate expression of the HoxA7 polypeptides. Western blot analysis was performed using extracts prepared from COS-1 cells that were transfected with pCB6+ or the pCB6+− *myc-HoxA7* expression plasmid encoding the polypeptides indicated (A) or with pM2 or the pM2 expression plasmid encoding the GAL4-HoxA7 polypeptides indicated (B to E). Proteins were detected by using a monoclonal antibody directed against a heterologous myc epitope that was present in each polypeptide (A) or by using a monoclonal antibody directed against the GAL4 DNA binding domain (Santa Cruz Biotechnologies) that was present in each fusion polypeptide (B to E). Antigen-antibody complexes were visualized with a chemiluminescent reagent (Amersham). Exposure times ranged from 10 to 30 s. Experiments were performed several times to ensure that protein expression levels were equivalent; representative data are shown.

not affected by HoxA7 (Fig. 3A). Since basal levels of the reporter plasmids with or without the WIP sites were similar (~2,000 cpm), we conclude that HoxA7 is acting to repress transcription rather than to inhibit the binding of an endogenous factor. We further investigated the transcriptional properties of HoxA7 in the presence of the Hox cofactor Pbx1. When tested on its own, Pbx1 produced a modest repression (approximately fourfold) through the WIP reporter plasmid, likely because of weak Pbx1 DNA binding sites in this element (Fig. 3A). However, Pbx1 neither potentiated nor diminished the repressor action of HoxA7 through the WIP reporter plasmid (Fig. 3A). This observation indicates that the potent repressor action of HoxA7 does not require Pbx1, although we cannot rule out the possibility that NIH 3T3 cells already contain Pbx or other HoxA7 cofactors.

To further characterize the transcriptional properties of HoxA7, we examined its activity when directed to a heterologous DNA binding element through the GAL4 DNA binding domain (Fig. 4). This approach is particularly useful for studying the transcriptional properties of proteins for which actual target elements have not yet been defined. For this purpose, we constructed an expression plasmid that encoded a fusion protein containing the GAL4 DNA binding domain and full-length HoxA7 (Fig. 4A, GAL4-HoxA7). Transient-transfection assays were performed with both NIH 3T3 cells and F9 cells, using the GAL4-HoxA7 expression plasmid and a reporter plasmid that contained five copies of the canonical GAL4 DNA binding site (5XGAL4) (Fig. 4B and C). As shown in Fig. 4B and C, the GAL4-HoxA7 polypeptide functioned as a transcriptional repressor in both cell types. In fact, the level of repression obtained in the F9 cells (60-fold) was accentuated relative to that observed in the NIH 3T3 cells (16-fold) (Fig. 4B and C). This finding suggests that the transcriptional action of HoxA7 is enhanced in the more appropriate cellular milieu provided by the F9 cells, which are of embryonic carcinoma origin (43). To investigate the transcriptional properties of GAL4-HoxA7 in another promoter context, we used a reporter plasmid that contained the GAL4 sites situated upstream of the TK promoter. Similar levels of repression by GAL4-HoxA7 were observed whether the reporter plasmid contained the SV40 or TK promoter (~20-fold), although repression of a reporter plasmid that contained the GAL4 sites positioned downstream of the TK promoter was less effective (~10-fold) (53a). In combination, these findings indicate that HoxA7 functions as a transcriptional repressor through various promoter contexts and in various cell lines.

Multiple domains of HoxA7 contribute to its overall transcriptional function. To define the relevant domains that contribute to the transcription activity of HoxA7, we constructed a series of expression plasmids that encoded truncated HoxA7 polypeptides on their own (Fig. 3B) or as fusion polypeptides with the GAL4 DNA binding domain (Fig. 4A). The transcriptional activities of the HoxA7 polypeptides in NIH 3T3 cells were examined by using the WIP reporter plasmid (Fig. 3B), whereas the GAL4-HoxA7 series was tested in both NIH 3T3 cells and F9 cells with the GAL4 reporter plasmid (Fig. 4B and C). When tested in NIH 3T3 cells, a GAL4-HoxA7 polypeptide lacking the C-terminal acidic region [GAL4-HoxA7(2-190)] functioned as a transcriptional repressor, although it was somewhat less effective than GAL4-HoxA7 (Fig. 4B). Further C-terminal truncation greatly reduced repressor function so

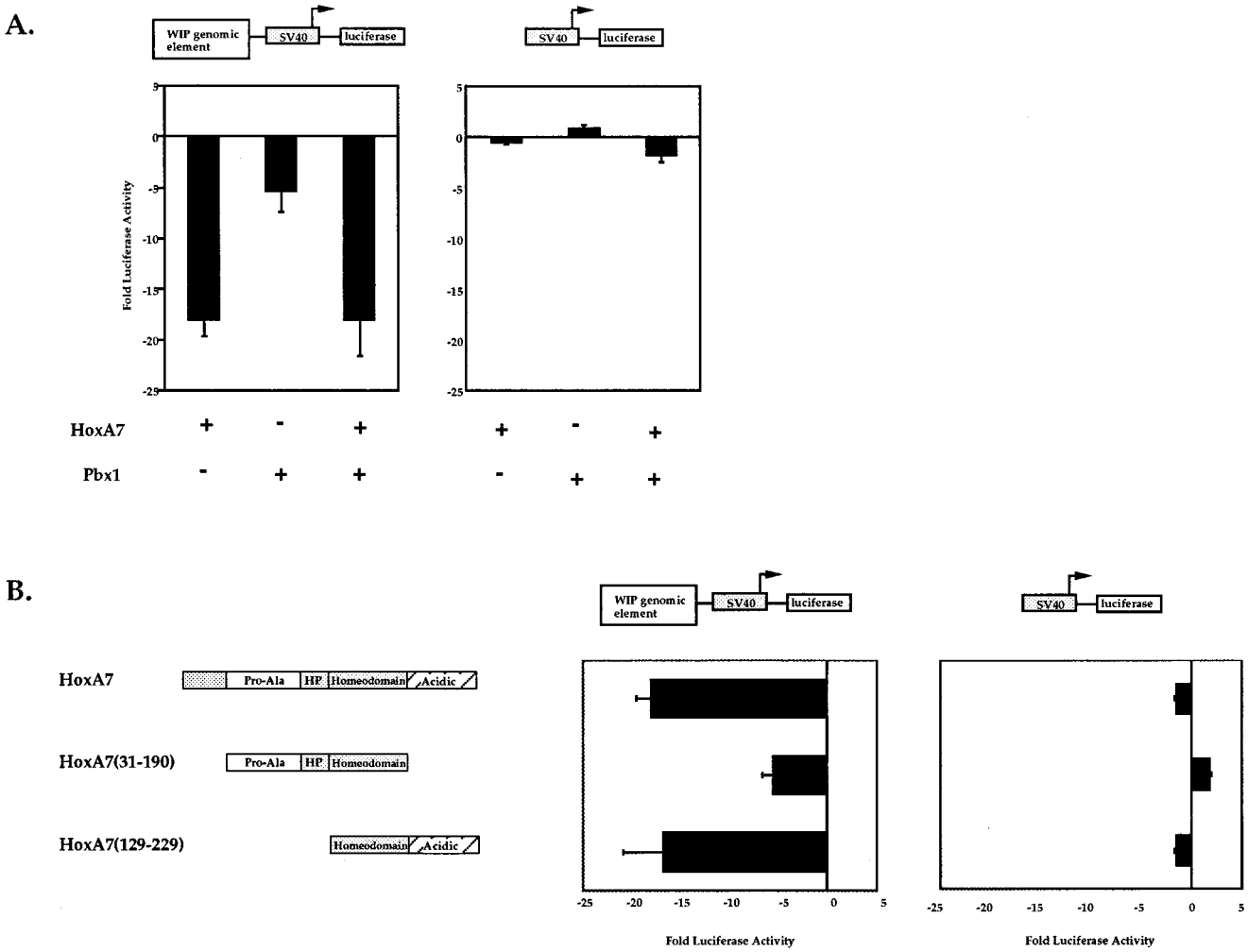

FIG. 3. HoxA7 represses transcription through a genomic binding site. (A) Transfection assays were performed with NIH 3T3 cells in the presence (+) or absence (−) of an expression plasmid encoding HoxA7 (500 ng), Pbx1 (500 ng), or both (500 ng of each), along with a reporter plasmid (1,000 ng) containing a single copy of the WIP element (left) or the parental reporter plasmid (1,000 ng) with no additional sites (right). (B) The expression plasmids encoded the full-length HoxA7 or truncated HoxA7 proteins corresponding to the amino acids indicated in parentheses. The protein domains encoded by each construct are represented by the white and stippled boxes as described in the legend to Fig. 1. Transient-transfection assays were performed with NIH 3T3 cells with the indicated HoxA7 expression plasmid (500 ng) along with a reporter plasmid (1,000 ng) that contained the WIP element (left) or the parental plasmid (1,000 ng) without additional sites (right). Data are expressed as fold difference in luciferase activity obtained with the HoxA7 expression plasmid compared with that obtained by using the parental plasmid that did not contain HoxA7 sequences. Assays were repeated a minimum of three times in duplicate; variability between assays was <15%. Shown are data from a representative assay; bars indicate errors between duplicates.

that GAL4-HoxA7(2-129), which lacked the homeodomain, did not repress transcription (Fig. 4B). Consistent with this observation, a HoxA7 polypeptide lacking the C-terminal acidic region [HoxA7(31-190)] had reduced repressor activity relative to HoxA7 through the WIP reporter plasmid, whereas a polypeptide that contained the C-terminal region of HoxA7 but lacked N-terminal regions [HoxA7(129-229)] had potent repressor activity (Fig. 3B). These findings show that the conserved sequences in the C-terminal region, including the homeodomain itself, contribute to repression by HoxA7. An alternative possibility is that the observed contribution of the homeodomain towards repression is actually due to its interaction with fortuitous homeodomain DNA sites in the SV40 promoter or in the reporter plasmid. This is unlikely, since the transcriptional activities of the HoxA7 polypeptides and the GAL4-HoxA7 fusion polypeptides were mediated through their respective cognate DNA binding sites in the reporter plasmids (Fig. 3B and 4B).

Subsequent truncation of HoxA7 unmasks a fairly potent transcriptional activation domain [Fig. 4B, GAL4-HoxA7(2-116)]. The presence of this domain suggests either that HoxA7 functions as a transcriptional activator in other cellular contexts (other than the ones tested in this study) or, alternatively, that this domain modulates the potency of the HoxA7 repressor function in the context of the full-length protein. Indeed, HoxA7 has been shown to activate transcription in *Saccharomyces cerevisiae* (22). The minimal activation domain corresponds to a 65-amino-acid segment [Fig. 4B, GAL4-HoxA7 (31-96)] which has a high content of proline (11%) and alanine (14%) residues and is located within the unique region of HoxA7 (Fig. 1). It is noteworthy that the hexapeptide motif, which has recently been shown to mediate protein-protein interactions with Pbx (10, 29, 49), appears to modulate the activity of the Pro-Ala region [Fig. 4B, compare GAL4-HoxA7(2-129) with GAL4-HoxA7(2-116)].

When examined in F9 cells, the overall transcriptional prop-

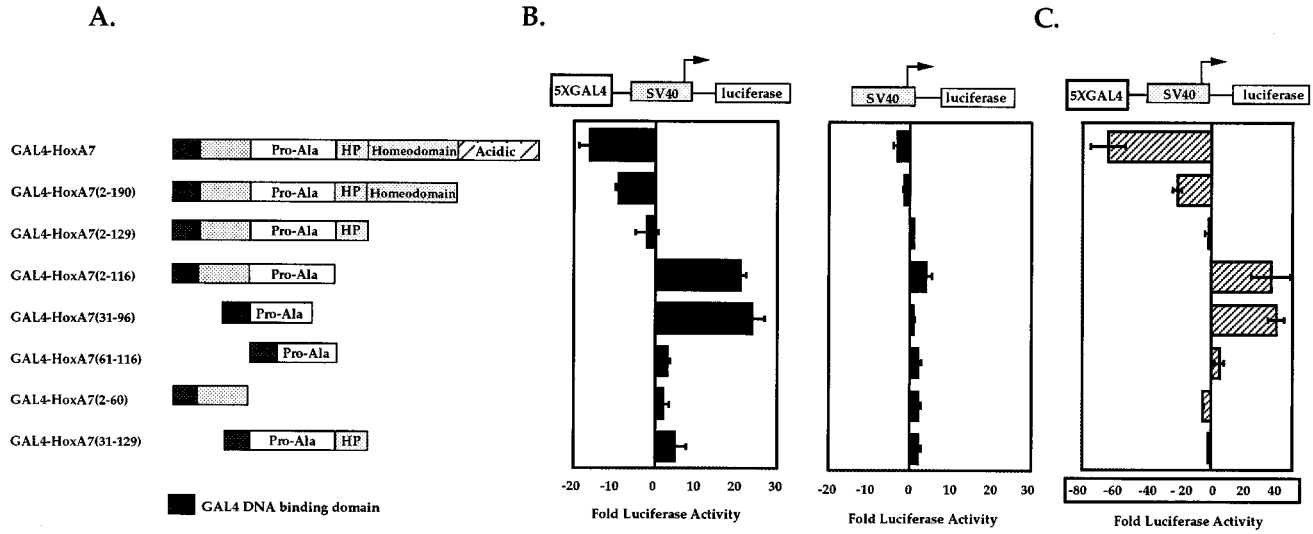

FIG. 4. Multiple domains contribute to repression and activation by HoxA7. (A) The GAL4-HoxA7 fusion polypeptides contained the GAL4 DNA binding domain and the HoxA7 protein regions corresponding to amino acids indicated in parentheses and the protein domains as shown by the white and stippled boxes. (B and C) Transfection assays were performed with NIH 3T3 cells (B) or F9 cells (C), using a reporter plasmid (1,000 ng) that contained either five tandem copies of the GAL4 binding site (5XGAL4) or no additional sites upstream of the SV40 early promoter and the luciferase gene. In panel C, the fold luciferase activity is boxed to highlight the difference in the scales compared with panel B. Assays contained GAL4-HoxA7 or the truncated GAL4-HoxA7 expression plasmid as indicated (250 ng). Data are expressed as fold difference in luciferase activity as in Fig. 3. Transfection assays were repeated a minimum of six times in duplicate; variability among assays was <15%. The data shown are from a representative assay; bars indicate errors between duplicates.

erties of the GAL4-HoxA7 polypeptides were similar to those observed in NIH 3T3 cells, although their activity was somewhat increased (Fig. 4B and C). For instance, the polypeptide that contained the Pro-Ala domain activated transcription by 40-fold in F9 cells compared with 20-fold in NIH 3T3 cells [compare GAL4-Hox(31-96) in Fig. 4B and C]. One notable difference in the activities of the GAL4-HoxA7 polypeptides in these two cell lines was the enhanced contribution of the C-terminal acidic region towards repression in the F9 cells. Thus, a GAL4-HoxA7 polypeptide that lacked the C-terminal acidic region [GAL4-HoxA7(2-190)] had significantly reduced repressor function compared with that of GAL4-HoxA7 (e.g., 21- versus 60-fold), whereas in NIH 3T3 cells, the difference between these polypeptides was less pronounced (9- versus 16-fold) (Fig. 4B and C). We attempted to confirm the repressor activity of the C-terminal acidic region directly by producing a polypeptide containing this segment; however, this sequence was not stably expressed in mammalian cells, precluding further analysis. These findings suggest that although the overall transcriptional properties of HoxA7 may be similar in various cell types, the relative contribution of particular domains is likely to vary, depending upon the specific cell type.

The transcriptional function of HoxA7 results from the composite action of its multiple domains. The preceding data demonstrate that HoxA7 is composed of at least three domains that contribute to its transcriptional properties; two of these are conserved domains that function in repression, and one is a nonconserved domain that functions as an activator region. The implication of these findings is that the overall transcriptional properties of HoxA7 reflect the cumulative action of these multiple domains. To test this idea, we examined the activity of individual domains within the full-length protein by using GAL4-HoxA7 polypeptides that contained specific amino acid substitutions in two of the relevant domains, the Pro-Ala region and the homeodomain (Fig. 5). To facilitate selection of potentially critical residues within the Pro-Ala domain, we relied on our observation that this region shares limited sequence similarity with the carboxy-terminal domain of RNA polymerase II (53a). Since the carboxy-terminal domain plays an important role in transcription initiation through its interactions with the TATA-binding protein (60), we selected for substitution residues within the Pro-Ala domain that were conserved with the carboxy-terminal domain (Table 1). A GAL4-HoxA7 polypeptide that contained such substitutions (GAL4-HoxA7-MI) exhibited an increase in repressor activity relative to that of GAL4-HoxA7 (Fig. 5). We interpret this finding as a loss of activator function within full-length HoxA7 which is manifested as an increase in its overall repressor potency. This conclusion is supported by our observation that these same substitutions within the context of the minimal activation domain abrogated its activator function (53a). Furthermore, a HoxA7 polypeptide that contained the Pro-Ala region but lacked the C-terminal acidic region [HoxA7(31-190)] also had reduced repressor activity through the WIP reporter plasmid relative to that of HoxA7 (Fig. 3B).

The homeodomain has not been previously implicated as a transcriptional repressor domain, nor does it have any obvious sequence similarity with other known transcriptional regulatory regions. Therefore, our choice of potentially critical residues for replacement was based on sequence comparisons with other homeodomains (55). Specifically, we selected residues that lie within a subsegment, i.e., helix III, that is highly conserved among many different homeodomains (Table 1, homeodomain residues 53, 55, and 57). The protein containing these substitutions (GAL4-HoxA7-MII) exhibited reduced repressor activity relative to that of GAL4-HoxA7 (Fig. 5). This finding further shows that the homeodomain and, in fact, conserved residues within this domain contribute directly to the overall repressor function of HoxA7 in the context of the full-length protein.

The HoxA7 homeodomain, but not two other DNA binding domains, functions as a transcriptional repressor region. The implication of our observation that the homeodomain of HoxA7 functions as a repressor region is that this domain may

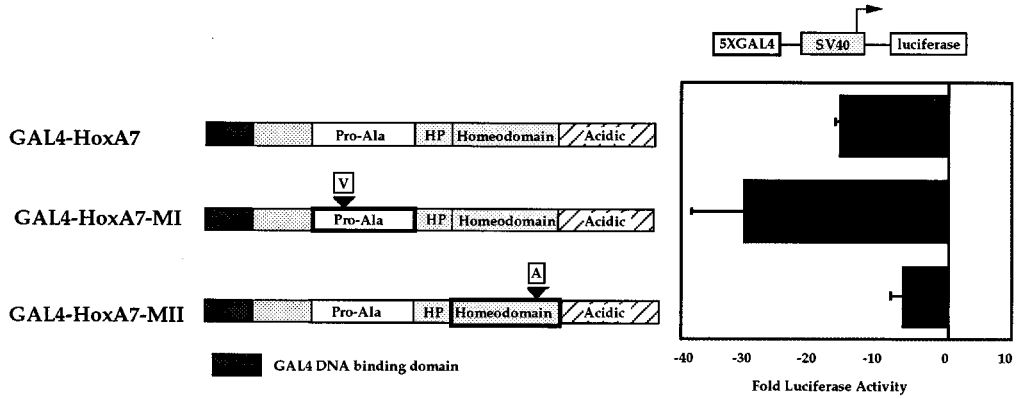

FIG. 5. Multiple domains of HoxA7 contribute to its overall transcriptional function. Transfection assays were performed with NIH 3T3 cells, using the GAL4 luciferase reporter plasmid (1,000 ng) and expression plasmids (150 ng) encoding GAL4-HoxA7 polypeptides which contained valine substitutions (V) in the Pro-Ala region (GAL4-HoxA7-MI) or alanine substitutions (A) in the homeodomain (GAL4-HoxA7-MII), as indicated. The exact amino acid substitutions are listed in Table 1. Data are expressed as fold difference in luciferase activity as in Fig. 3. Transfection assays were performed a minimum of six times in duplicate; variability among assays was <15%. Shown are results of a representative assay; bars indicate errors between duplicates.

have a broader role in transcriptional regulation than was previously thought to be the case. To further investigate this idea, we examined whether the homeodomain functioned independently as a repressor domain (Fig. 6). For this purpose, expression plasmids that encoded fusion polypeptides containing the GAL4 DNA binding domain and either the HoxA7 homeodomain or two other DNA binding domains of equivalent size (i.e., the Fos bZip domain or the Rel DNA binding region) were constructed (Fig. 6). When examined in this capacity, the HoxA7 homeodomain repressed transcription, whereas the other DNA binding domains had minimal effects on transcriptional activity (Fig. 6). In addition to the HoxA7 homeodomain, two other Hox homeodomains (i.e., HoxC8 and HoxB4) also repressed transcription in the context of the GAL4 domain (53a). As was the case for each of the GAL4-HoxA7 fusion proteins described here, the ability of the HoxA7 homeodomain to effect transcription required the presence of GAL4 DNA binding sites in the reporter plasmid (Fig. 4B) (53a). In combination with the results described above, these findings demonstrate that the HoxA7 homeodomain functions as a transcriptional repressor domain through a heterologous DNA binding site.

The C-terminal regions of other Hox proteins mediate transcriptional repression. The regions of HoxA7 required for its repression function (i.e., the C-terminal half of the protein including the homeodomain and acidic region) correspond to those conserved among members of the Hox family (Fig. 1), which suggests that repression may be a common mode of Hox protein action. To determine whether this is the case, we compared the transcriptional properties of HoxA7 with those of two other family members, HoxC8 and HoxB4 (Fig. 7). These Hox proteins were selected since their sequences and domain organization have certain features in common with HoxA7 and yet also have some noteworthy differences (Fig. 7A). For instance, both HoxC8 and HoxB4 have unique regions that contain residues prevalent among transcriptional regulatory domains, and HoxC8, but not HoxB4, has an acidic C-terminal region (Fig. 7A). To compare their transcriptional properties with those of HoxA7, we constructed expression plasmids that encoded fusion proteins containing the GAL4 DNA binding domain and the full-length sequence of either HoxC8 or HoxB4 (Fig. 7B).

When tested in NIH 3T3 cells, both GAL4-HoxC8 and GAL4-HoxB4 functioned as transcriptional repressors, as was observed for GAL4-HoxA7 (Fig. 7B). However, these Hox proteins differed in their relative levels of repressor action (Fig. 7B). HoxC8 was a more potent repressor than HoxA7, and HoxB4 was a less potent repressor (Fig. 7B). Since the results shown in Fig. 7B (and also in Fig. 7C and 8B) are averages of several independent experiments, and since Western blot analysis demonstrated comparable levels of protein expression (Fig. 2C), we conclude that these differences were due to actual variations in the repressor actions of these three Hox proteins. Subsequent analyses revealed that the serine-rich region of HoxC8 functioned as a repressor domain, which is likely to contribute to its enhanced repressor potency relative to HoxA7 (53a). Moreover, we have also observed that the reduced repressor potency of HoxB4 relative to those of the other Hox proteins, HoxA7 and HoxC8, is further accentuated in F9 cells since HoxB4 lacks a C-terminal acidic region which,

TABLE 1. GAL4-HoxA7 polypeptides

Polypeptide	Composition[a]
GAL4-HoxA7-MI	pM2 GAL4(1–147) HoxA7(2–229) (Y40V, P42V, F47V, T50V, P52V)
GAL4-HoxA7-MII	pM2 GAL4(1–147) HoxA7(2–229) (R181A, K183A, K185A)[b]
GAL4-HoxA7-MIII	pM2 GAL4(1–147) HoxA7(2–229) (G132S, Q134T, T135A)[c]
GAL4-HoxA7/C8	pM2 GAL4(1–147) HoxA7(2–128) HoxC8(149–242)
GAL4-HoxA7/B4	pM2 GAL4(1–147) HoxA7(2–128) HoxB4(161–250)

[a] Shown are the amino acid sequences and substitutions contained within each of the modified GAL4-HoxA7 polypeptides (refer to Fig. 5, 7C, and 8B). The amino acid numbers correspond to the full length sequence of HoxA7.
[b] Substitutions in HoxA7 correspond to homeodomain positions 53, 55, and 57.
[c] Substitutions in HoxA7 correspond to homeodomain positions 4, 6, and 7.

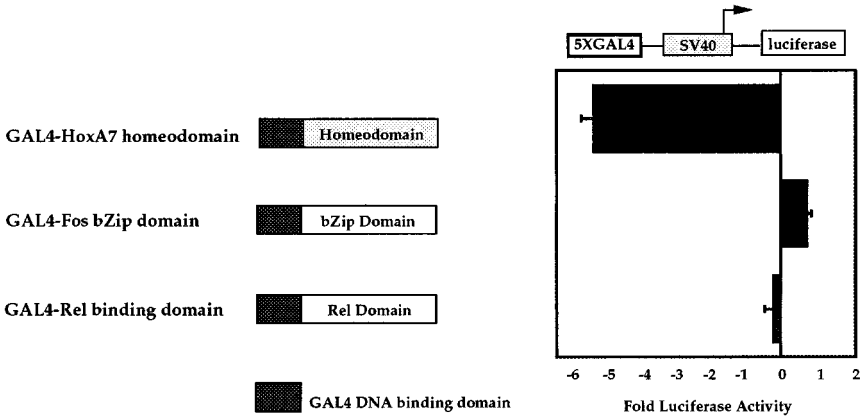

FIG. 6. The HoxA7 homeodomain, but not two other DNA binding domains, functions as a repressor region. Transfection assays were performed with NIH 3T3 cells, using the GAL4 luciferase reporter plasmid (1,000 ng) and expression plasmids (250 ng) encoding the GAL4-HoxA7 homeodomain (homeodomain), the GAL4-Fos DNA binding domain (bZip Domain), and the GAL4-Rel binding domain (Rel Domain). Data are expressed as fold difference in luciferase activity as in Fig. 3. Transfection assays were performed three times in duplicate; variability among assays was <15%. Shown are results of a representative assay; bars indicate errors between duplicates.

as we showed in Fig. 3, preferentially functions in F9 cells (53a). Thus, although repression may be a common mode of Hox protein action, the activity of individual Hox proteins is likely to vary, depending upon their unique combination of functional domains and the specific cell type in which they are expressed.

An additional implication of these data is that the C-terminal regions of HoxC8 and HoxB4, including their homeodomains, may also contribute to repression. To test whether these regions could substitute for the C-terminal sequences of HoxA7, we constructed chimeric GAL4-Hox polypeptides that contained the N-terminal region of HoxA7 and the C-terminal region (including the homeodomain) of HoxC8 or HoxB4 (Fig. 7C). As shown by transfection assays performed with both NIH 3T3 cells and F9 cells, the chimeric proteins corresponding to GAL4-HoxA7/C8 and GAL4-HoxA7/B4 also repressed transcription, as observed for GAL4-HoxA7 (Fig. 7C). We conclude from these data that repression by Hox proteins is mediated in part by conserved regions, including the homeodomain. It is noteworthy, however, that GAL4-HoxA7/B4 (compared with GAL4-HoxA7, GAL4-HoxC8, and GAL4-HoxA7/C8) exhibited reduced repressor potency in NIH 3T3 cells (Fig. 7C). Moreover, this difference was accentuated in F9 cells, presumably because of the lack of a C-terminal acidic region in HoxB4 which is present in the other two proteins (Fig. 7C).

Residues in the N-terminal arm of the homeodomain modulate the repressor potency of HoxA7 and HoxB4. The observation that GAL4-HoxA7/B4 reproducibly repressed transcription in NIH 3T3 cells to a lesser extent than the other GAL4-Hox proteins (i.e., GAL4-HoxA7, GAL4-HoxC8, and GAL4-HoxA7/C8) (Fig. 7C) suggested that its reduced repressor potency might also be attributed to differences in the homeodomain. Although these three Hox homeodomains show extensive sequence similarity overall, residues in the N-terminal arm of HoxB4 differ from those in HoxC8 and HoxA7, whereas the latter proteins have several residues in common (Fig. 8A). To determine whether the N-terminal-arm residues that differ between HoxA7 and HoxB4 contribute to the observed differences in repressor potency, we constructed a GAL4-HoxA7 expression plasmid containing substitutions of these residues for the corresponding ones in HoxB4 (Table 1). When tested in NIH 3T3 cells, a GAL4-HoxA7 polypeptide containing these substitutions in the N-terminal arm (GAL4-HoxA7-MIII) had levels of repressor activity similar to that of GAL4-HoxB4 and exhibited reduced repressor activity compared with that of GAL4-HoxA7 (Fig. 8B). Western blot analysis confirmed that these proteins were expressed at comparable levels (Fig. 2C); thus, we conclude that the transcriptional activities observed reflect actual differences in repressor potency between the polypeptides. This finding suggests that the enhanced repressor potency of HoxA7 in comparison with that of HoxB4 is due, in part, to residues in the N-terminal arm and to the presence of a C-terminal acidic region. Moreover, these data further demonstrate the contribution of the homeodomain as a repressor domain and suggest a crucial modulatory role for residues in the N-terminal arm.

DISCUSSION

Although Hox proteins are presumed to function as transcriptional regulators, few studies have actually investigated their properties as such; most have instead focused on the expression and biological functions of *Hox* genes during embryogenesis. The present study provides a comprehensive analysis of the transcriptional properties of HoxA7, as well as a general framework for understanding the functions of Hox proteins in transcriptional control. We provide evidence that HoxA7 acts as a potent transcriptional repressor, and this activity reflects the integrative action of multiple functional domains that include both repressor and activator regions. We show that some of these domains, in particular the C-terminal regions that mediate repression, are partially conserved among other Hox proteins. Accordingly, at least two other Hox proteins, HoxC8 and HoxB4, also function as repressors, although with various degrees of efficacy. Finally, we demonstrate that a major component of the repressor action of HoxA7 is its homeodomain, suggesting a novel role for this domain in transcriptional control. Our findings further indicate that repression may be an important feature of Hox homeodomains and is modulated by nonconserved residues in their N-terminal-arm sequences. Therefore, the function of HoxA7 in transcriptional control is derived from its particular combination of modular domains and from the complex action of the homeodomain itself.

Hox A7 is composed of multiple transcriptional domains: a simple explanation for functional redundancy. The observation that the transcriptional properties of HoxA7 result from

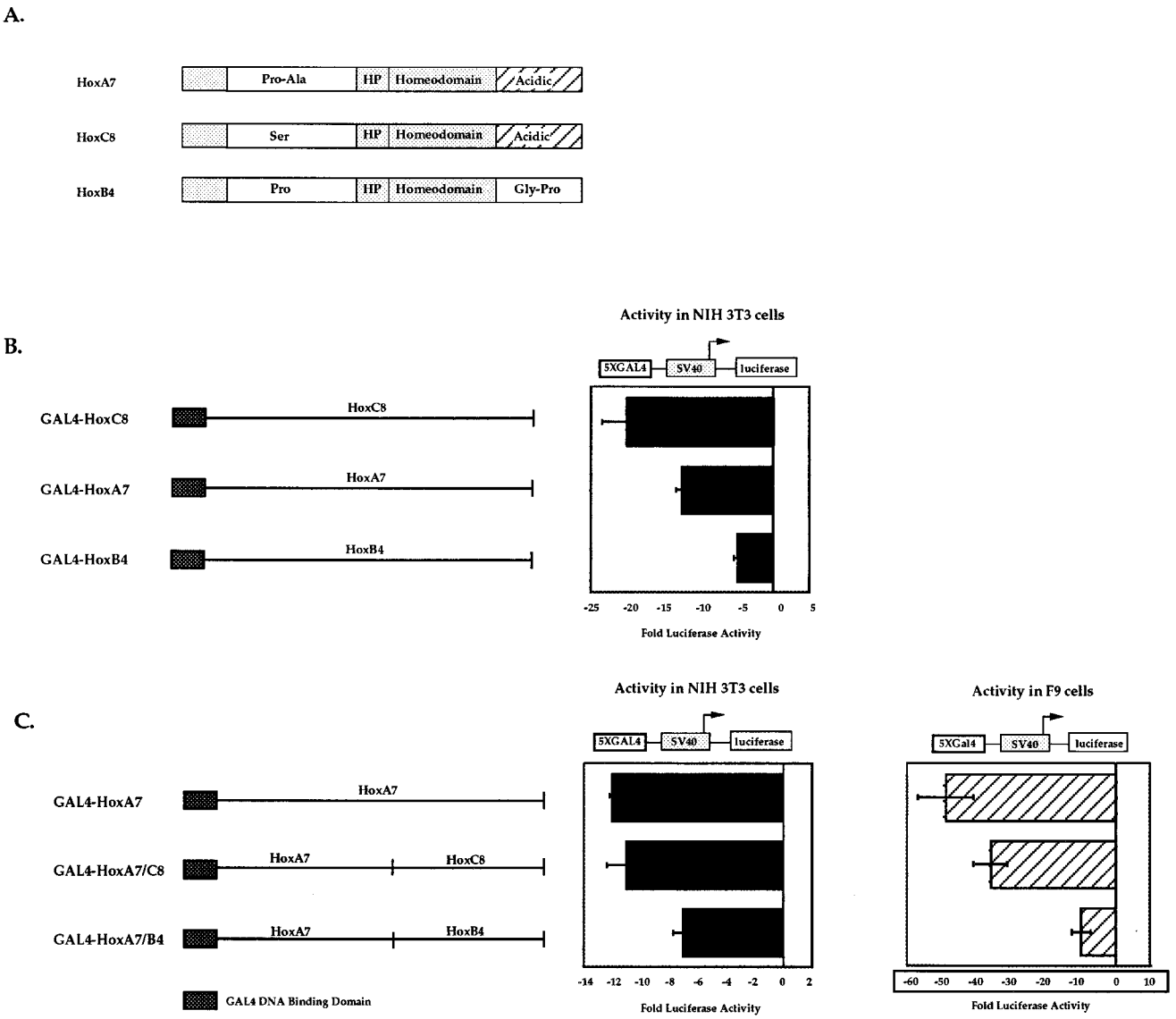

FIG. 7. Comparison of the repressor potential of Hox proteins. (A) Schematic representation comparing the domain organizations of HoxA7, HoxC8, and HoxB4. The conserved regions (stippled and striped boxes) and nonconserved regions (white boxes) are shown as for Fig. 1. Ser, region containing a high percentage of serine residues; Pro, region containing a high percentage of proline residues; Gly-Pro, region containing a high percentage of glycine and proline residues. (B and C) Transfection assays were performed in NIH 3T3 cells, using the GAL4 luciferase reporter plasmid (1,000 ng). The expression plasmids (150 ng) encoded GAL4-HoxC8, GAL4-HoxA7, or GAL4-HoxB4 fusion polypeptides (B) or GAL4-HoxA7, GAL4-HoxA7/C8, and GAL4-HoxA7/B4 chimeric proteins (C), as indicated. Table 1 describes the amino acid regions contained in each chimeric protein. Data are expressed as fold difference in luciferase activity as in Fig. 3. Assays were repeated six times in duplicate. Shown are averages of these experiments; the standard deviations are represented by the error bars.

the cumulative action of multiple domains is a prevalent theme among transcriptional regulatory proteins, which typically contain several domains that are interactive and interdependent (e.g., see references 7, 23, 37, and 46). This modular design allows for enhanced complexity of function, since the various domains may be differentially utilized depending upon the precise cellular environment and the promoter context (e.g., see references 16, 26, 27, and 58). Given the many embryonic regions in which HoxA7 is expressed (40), its discretionary use of these domains is likely to facilitate its ability to selectively regulate gene transcription in diverse cellular contexts. Indeed, we have shown that at least one of these domains, the C-terminal acidic region, functions in a cell-type-specific manner, and other domains are likely to do so as well. Presumably, an additional level of complexity is provided by the activation domain of HoxA7, the presence of which suggests that in certain situations HoxA7 may activate rather than repress transcription. This too is a recurring theme among transcriptional regulators, many of which have dual functions as activators or repressors, depending upon the promoter context (52). Together, these features of HoxA7, which epitomize many general properties of transcriptional regulatory proteins, suggest that its complex actions in transcriptional control result from the particular combination of context and composition.

Another theme that emerges from our analysis is that the transcriptional domains of HoxA7 are partially conserved among members of the Hox family. In fact, even their most conserved regions (e.g., their homeodomains) are not identical and exhibit some differences that influence the potency of their transcriptional action. These observations suggest a framework

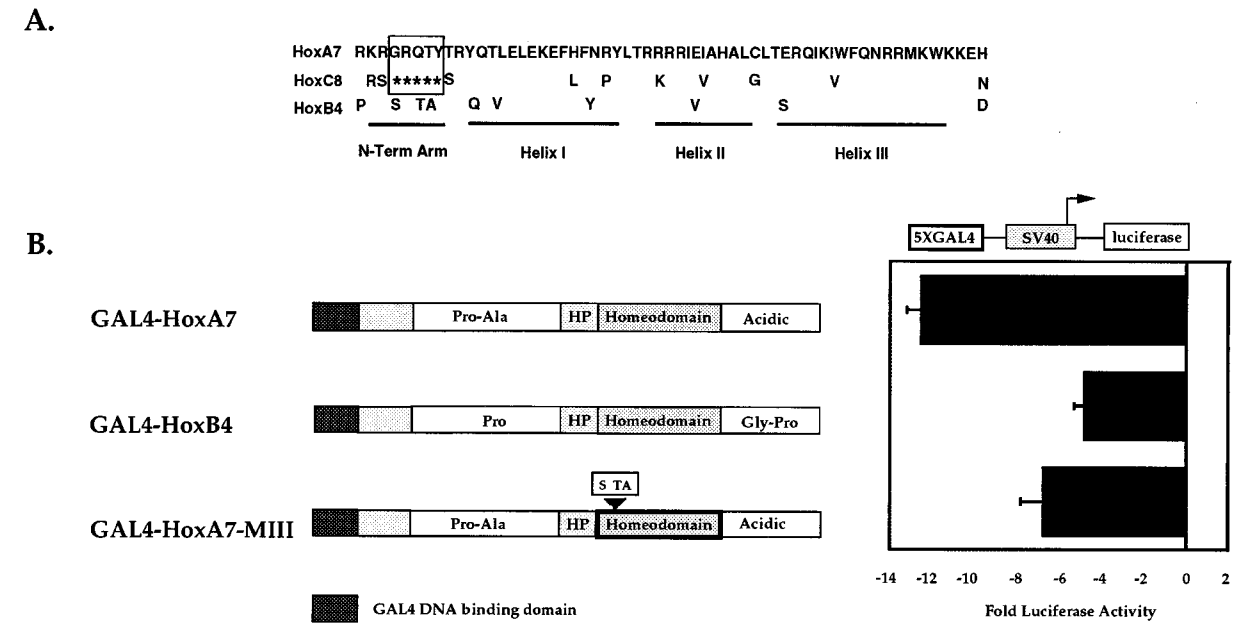

FIG. 8. The N-terminal region modulates the extent of repressor function among the Hox homeodomains. (A) Alignment of the homeodomain regions of HoxA7, HoxC8, and HoxB4. Shown is the sequence of the HoxA7 homeodomain; the residues shown for HoxC8 and HoxB4 are those that differ from the HoxA7 sequence. A sequence in the N-terminal arm shared between HoxA7 and HoxC8 (boxed amino acids and asterisks) and the positions of the homeodomain subregions, N-terminal arm (N-Term arm), and helices I, II, and III are shown. (B) Transfection assays were performed with NIH 3T3 cells, using the GAL4 luciferase reporter plasmid (1,000 ng) and expression plasmids (150 ng) which encode GAL4-HoxA7, GAL4-HoxB4, or GAL4-HoxA7-MIII containing the indicated amino acid substitutions in the N-terminal arm (S TA). Data are expressed as fold difference in luciferase activity as in Fig. 3. Assays were repeated six times in duplicate. The data shown are the averages of these experiments; standard deviations are represented by the error bars.

for understanding the complex biological functions of Hox proteins that accounts for their apparent redundancy as well as their exclusivity. Namely, inherent in their structural design are features that are unique to particular Hox proteins, features that are shared among all Hox proteins, and features that are similar, but nonidentical, among several Hox proteins. Thus, the specific domain composition combined with the particular expression patterns in a given embryonic segment may partially explain the compensatory, combinatorial, and unique functions of Hox proteins.

Repression is an important mode of Hox protein action. One of the major conclusions of the present study is that repression may be an important mode of action for HoxA7 and perhaps for Hox proteins in general. It has become increasingly evident that repression plays a significant role in directing developmental processes, particularly in the control of spatial regulation and patterning during embryogenesis (4, 21, 28, 36). Indeed, *Hox* genes play an important role in controlling such patterning events in mice (32, 44). How might the biochemical properties of Hox proteins as transcriptional repressors be linked to their biological roles in regulating pattern formation? A hint is provided by recent reports which suggest that *Hox* gene products are required for cellular proliferation. This is based on analysis of targeted disruptions of certain *Hox* genes which result in a loss of particular embryonic structures because of insufficient amounts of precursor cells (11, 13, 14, 56). Since it is well established that transcriptional repression is crucial for maintaining cells in an undifferentiated state during periods of rapid proliferation, a feasible interpretation is that disruptions of *Hox* genes, and thereby loss of Hox protein repressor function, result in premature differentiation of precursor cells rather than normal cellular proliferation. Ultimately, the identification of target genes will ascertain the precise functions of Hox proteins in transcriptional control.

A broad role for the homeodomain in transcriptional control: the HoxA7 homeodomain functions as a repressor region. A major novel finding of the present study is that the homeodomain of HoxA7 functions directly as a transcriptional repressor domain, a property that is shared by other Hox homeodomains but not other DNA binding domains in general. This observation extends the emerging notion that the homeodomain serves a broader role in transcriptional regulation which is unlikely to be attributed exclusively to its action as a DNA binding domain. Indeed, some homeodomains, including those of the Hox family, interact directly with other protein factors to influence gene transcription (7, 10, 29, 57, 63, 64). Moreover, the actions of certain homeodomain-containing proteins do not require their cognate DNA binding sites although they require their homeodomain regions to influence transcription, suggesting that in these cases the homeodomain is providing some alternative role other than DNA binding (7, 47). Even the recent studies that describe the interactions of Pbx and Hox proteins have shown that the Hox DNA binding sites (in contrast to the Pbx sites) are not absolutely required for the cooperative actions of these proteins (10, 39). These observations and those of the present study do not negate the contribution of DNA binding by the HoxA7 homeodomain for transcriptional regulation; rather, they demonstrate that homeodomains may have an additional role(s) in transcriptional control besides their function in DNA binding. Furthermore, the notion that the homeodomain functions directly as a repressor domain resonates the now-prevailing view that DNA binding domains may serve a variety of diverse functions in transcriptional control that are not restricted to DNA-protein recognition. For instance, the DNA binding region of the glucocorticoid receptor serves many other functions besides DNA-protein recognition, including nuclear localization, DNA-induced dimerization, transcriptional activation, and protein-protein interactions (refer-

ence 34 and references therein). Similarly, in certain promoter and cellular contexts, the DNA binding regions of Fos and Jun influence gene expression through direct protein-protein interactions rather than via DNA binding activity (e.g., see reference 26). Therefore, it is reasonable to suppose that, like these other transcription factors, context rather than composition dictates the action of the HoxA7 homeodomain in transcriptional control.

How might the homeodomain function as a repressor domain? One likely scenario is that it mediates interactions with other protein factors. Indeed, Hox homeodomains have been shown to interact with other homeodomain proteins, including other members of the Hox family (64) and members of the Pbx family (10, 39, 51). Alternatively, Hox homeodomains may interact with components of the core transcription complex, as has previously been shown to be the case for another homeodomain protein, Msx-1 (7). In fact, more-recent studies with Msx-1 have revealed that its interaction with the TATA-binding protein is mediated directly via residues in the N-terminal arm and that the same residues are required for repression by Msx-1 (66). It is noteworthy that the N-terminal-arm residues also play a role in repression by the HoxA7 homeodomain, since substitution of these residues modulates the potency of HoxA7 repressor action. These observations are intriguing in light of studies which have demonstrated a crucial role for the N-terminal arm in distinguishing the functional actions of homeodomain proteins in vivo (38, 65). In fact, Zeng and colleagues noted in their study that the predicted orientation of these N-terminal-arm residues renders them capable of mediating interactions with other protein factors (65). The findings presented here further support this idea and show that the modulatory action of the N-terminal-arm residues in vivo is also evident by their biochemical actions in vitro.

In summary, it remains a mystery as to how *Hox* genes function in concert to specify axial patterning events. Clearly, a careful analysis of the biochemical properties of Hox proteins will likely provide important insight into how individual Hox proteins function and, ultimately, how the actions of the *Hox* network are coordinated.

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REFERENCES

1. **Abate, C., D. Luk, and T. Curran.** 1991. Transcriptional regulation by Fos and Jun in vitro: interaction among multiple activator and regulatory domains. Mol. Cell. Biol. **11:**3624–3632.
2. **Akam, M.** 1989. *Hox* and HOM: homologous gene clusters in insects and vertebrates. Cell **57:**347–349.
3. **Andrew, D. J., and M. P. Scott.** 1992. Downstream of the homeotic genes. New Biol. **4:**5–15.
4. **Appel, B., and S. Sakonju.** 1993. Cell-type-specific mechanisms of transcriptional repression by the homeotic gene products UBX and ABD-A in *Drosophila* embryos. EMBO J. **12:**1099–1109.
5. **Balling, R., G. Mutter, P. Gruss, and M. Kessel.** 1989. Craniofacial abnormalities induced by ectopic expression of the homeobox gene *Hox-1.1* in transgenic mice. Cell **58:**337–347.
6. **Catron, K. M., N. Iler, and C. Abate.** 1993. Nucleotides flanking a conserved TAAT core dictate the DNA binding specificity of three murine homeodomain proteins. Mol. Cell. Biol. **13:**2354–2365.
7. **Catron, K. M., H. Zhang, S. C. Marshall, J. A. Inostroza, J. M. Wilson, and C. Abate.** 1995. Transcriptional repression by Msx-1 does not require homeodomain DNA-binding sites. Mol. Cell. Biol. **15:**861–871.
8. **Chan, S., L. Jaffe, M. Capovilla, J. Botas, and R. S. Mann.** 1994. The DNA binding specificity of ultrabithorax is modulated by cooperative interactions with extradenticle, another homeoprotein. Cell **78:**603–615.
9. **Chan, S.-K., and R. S. Mann.** 1993. The segment identity functions of ultrabithorax are contained within its homeo domain and carboxy-terminal sequences. Genes Dev. **7:**796–811.
10. **Chang, C.-P., W.-F. Shen, S. Rozenfeld, H. J. Lawrence, C. Largman, and M. L. Cleary.** 1995. Pbx proteins display hexapeptide-dependent cooperative DNA binding with a subset of Hox proteins. Genes Dev. **9:**663–674.
11. **Condie, B. G., and M. R. Capecchi.** 1994. Mice with targeted disruptions in the paralogous genes *Hoxa-3* and *Hoxd-3* reveal synergistic interactions. Nature (London) **370:**304–307.
12. **Corsetti, M., P. Briata, L. Sanseverino, A. Daga, I. Airoldi, A. Simeone, G. Palmisano, C. Angelini, E. Boncinelli, and G. Corte.** 1992. Differential DNA binding properties of three human homeodomain proteins. Nucleic Acids Res. **20:**4465–4472.
13. **Davis, A. P., D. P. Witte, H. M. Heieh-Li, S. S. Potter, and M. R. Capecchi.** 1995. Absence of radius and ulna in mice lacking *hoxa-11* and *hoxd-11*. Nature (London) **375:**791–795.
14. **Dolle, P., A. Dierich, M. LeMeur, T. Schimmang, B. Schuhbaur, P. Chambon, and D. Duboule.** 1993. Disruption of the *Hoxd-13* gene induces localized heterochrony leading to mice with neotenic limbs. Cell **75:**431–441.
15. **Dolle, P., J. Izpisua-Belmonte, H. Falkenstein, and A. Renucci.** 1989. Coordinate expression of the murine *Hox-5* complex homeobox-containing genes during limb pattern formation. Nature (London) **342:**767–772.
16. **Du, W., D. Thanos, and T. Maniatis.** 1993. Mechanisms of transcriptional synergism between distinct virus-inducible enhancer elements. Cell **74:**887–898.
17. **Gaunt, S. J., P. T. Sharpe, and D. Duboule.** 1988. Spatially restricted domains of homeo-gene transcripts in mouse embryos: relation to a segmented body plan. Development **104**(Suppl.)**:**169–179.
18. **Gehring, W. J., Y. Q. Qian, M. Billeter, K. Furukubo-Tokunaga, A. F. Schier, D. Resendez-Perez, M. Affolter, G. Otting, and K. Wuthrich.** 1994. Homeodomain-DNA recognition. Cell **78:**211–223.
19. **Gibson, G., A. Schier, P. LeMotte, and W. J. Gehring.** 1990. The specificities of sex combs reduced and antennapedia are defined by a distinct portion of each protein that includes the homeodomain. Cell **62:**1087–1103.
20. **Graham, A., N. Papalopulu, and R. Krumlauf.** 1989. The murine and Drosophila homeobox gene complexes have common features of organization and expression. Cell **57:**367–378.
21. **Gray, S., P. Szymanski, and M. Levine.** 1994. Short-range repression permits multiple enhancers to function autonomously within a complex promoter. Genes Dev. **8:**1829–1838.
22. **Gross, M. K., and P. Gruss.** 1994. Functional analysis of mouse Hoxa-7 in *Saccharomyces cerevisiae*: sequences outside the homeodomain base contact zone influence binding and activation. Mol. Cell. Biol. **14:**238–254.
23. **Han, K., and J. L. Manley.** 1993. Functional domains of the *Drosophila* engrailed protein. EMBO J. **12:**2723–2733.
24. **Iler, N., D. H. Rowitch, Y. Echelard, A. P. McMahon, and C. Abate-Shen.** 1995. A single homeodomain binding site restricts spatial expression of *Wnt-1* in the developing brain. Mech. Dev. **53:**87–96.
25. **Ishikawa, H., M. Asano, T. Kanda, S. Kumar, C. Gelinas, and Y. Ito.** 1993. Two novel functions associated with the Rel oncoproteins: DNA replication and cell-specific transcriptional activation. Oncogene **8:**2889–2896.
26. **Jaln, J., P. G. McCaffrey, Z. Miner, T. K. Kerppola, J. N. Lambert, G. L. Verdine, T. Curran, and A. Rao.** 1993. The T-cell transcription factor $NFAT_p$ is a substrate for calcineurin and interacts with Fos and Jun. Nature (London) **365:**352–355.
27. **Jiang, J., H. Cai, Q. Zhou, and M. Levine.** 1993. Conversion of a *dorsal*-dependent silencer into an enhancer: evidence for dorsal corepressors. EMBO J. **12:**3201–3209.
28. **Johnson, A.** 1995. The price of repression. Cell **81:**655–658.
29. **Johnson, B., E. Parker, and M. A. Krasnow.** 1995. Extradenticle protein is a selective cofactor for the *Drosophila* homeotics: role of the homeodomain and YPWM amino acid motif in the interaction. Proc. Natl. Acad. Sci. USA **92:**739–743.
30. **Kessel, M., R. Balling, and P. Gruss.** 1990. Variations of cervical vertebrae after expression of a *Hox-1.1* transgene in mice. Cell **61:**301–308.
31. **Kessel, M., F. Schulze, M. Fibi, and P. Gruss.** 1987. Primary structure and nuclear localization of a murine homeodomain protein. Proc. Natl. Acad. Sci. USA **84:**5306–5310.

32. **Krumlauf, R.** 1994. *Hox* genes in vertebrate development. Cell **78:**191–201.
33. **Kuziora, M. A., and W. McGinnis.** 1989. A homeodomain substitution changes the regulatory specificity of the *Deformed* protein in *Drosophila* embryos. Cell **59:**563–571.
34. **Lefstin, J. A., J. R. Thomas, and K. R. Yamamoto.** 1994. Influence of a steroid receptor DNA-binding domain on transcriptional regulatory functions. Genes Dev. **8:**2842–2856.
35. **Levine, M., and T. Hoey.** 1988. Homeobox proteins as sequence-specific transcription factors. Cell **55:**537–540.
36. **Levine, M., and J. L. Manley.** 1989. Transcriptional repression of eukaryotic promoters. Cell **59:**405–408.
37. **Licht, J. D., W. Hanna-Rose, J. C. Reddy, M. A. English, M. Ro, M. Grossel, R. Shanknovich, and U. Hansen.** 1994. Mapping and mutagenesis of the amino-terminal transcriptional repression domain of the *Drosophila* Kruppel protein. Mol. Cell. Biol. **14:**4057–4066.
38. **Lin, L., and W. McGinnis.** 1992. Mapping functional specificity in the Dfd and Ubx homeo domains. Genes Dev. **6:**1071–1081.
39. **Lu, Q., P. Knoepfler, J. Scheele, D. D. Wright, and M. Kamps.** 1995. Both Pbx1 and E2A-Pbx1 bind the DNA motif ATCAATCAA cooperatively with the products of multiple murine Hox genes, some of which are themselves oncogenes. Mol. Cell. Biol. **15:**3786–3795.
40. **Mahon, K. A., H. Westphal, and P. Gruss.** 1988. Expression of homeobox gene *hox1.1* during murine embryogenesis. Development **104:**187–195.
41. **Manak, J. R., L. D. Mathies, and M. P. Scott.** 1995. Regulation of a *decapentaplegic* midgut enhancer by homeotic proteins. Development **120:**3605–3619.
42. **Mann, R. S., and D. S. Hogness.** 1990. Functional dissection of ultrabithorax proteins in *D. melanogaster*. Cell **60:**597–610.
43. **Martin, G. R.** 1980. Teratocarcinomas and mammalian embryogenesis. Science **209:**768–776.
44. **McGinnis, W., and R. Krumlauf.** 1992. Homeobox genes and axial patterning. Cell **68:**283–302.
45. **Mitchell, P. J., and R. Tjian.** 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science **245:**371–378.
46. **Monuki, E. S., R. Kuhn, and G. Lemke.** 1993. Cell-specific action and mutable structure of a transcription factor effector domain. Proc. Natl. Acad. Sci. USA **90:**9978–9982.
47. **Monuki, E. S., R. Kuhn, and G. Lemke.** 1993. Repression of the myelin P_0 gene by the POU transcription factor SCIP. Mech. Dev. **42:**15–32.
48. **Pellerin, I., C. Schnabel, K. M. Catron, and C. Abate.** 1994. Hox proteins have different affinities for a consensus DNA site that correlate with the positions of their genes on the *hox* cluster. Mol. Cell. Biol. **14:**4532–4545.
49. **Phelan, M., I. Rambaldi, and M. Featherstone.** 1995. Cooperative interactions between HOX and PBX proteins are mediated by a conserved peptide motif. Mol. Cell. Biol. **15:**3989–3997.
50. **Phelan, M. L., R. Sadoul, and M. S. Featherstone.** 1994. Functional differences between HOX proteins conferred by two residues in the homeodomain N-terminal Arm. Mol. Cell. Biol. **14:**5066–5075.
51. **Popperl, H., M. Bienz, M. Studer, S.-K. Chan, S. Aparicio, S. Brenner, R. S. Mann, and R. Krumlauf.** 1995. Segmental expression of *Hoxb-1* is controlled by a highly conserved autoregulatory loop dependent upon *exd*/*pbx*. Cell **81:**1031–1042.
52. **Roberts, S. G. E., and M. R. Green.** 1995. Dichotomous regulators. Nature (London) **375:**105–106.
53. **Sadowski, I., B. Bell, P. Broad, and M. Hollis.** 1992. GAL4 fusion vectors for expression in yeast or mammalian cells. Gene **118:**137–141.

53a. **Schnabel, C. A., and C. Abate-Shen.** Unpublished data.

54. **Scott, M. P.** 1992. Vertebrate homeobox gene nomenclature. Cell **71:**551–553.
55. **Scott, M. P., J. W. Tamkun, and G. W. Hartzell III.** 1989. The structure and function of the homeodomain. Biochim. Biophys. Acta **989:**25–46.
56. **Sordino, P., F. van der Hoeven, and D. Duboule.** 1995. Hox gene expression in teleost fins and the origin of vertebrate digits. Nature (London) **375:**678–681.
57. **Stark, M. R., and A. D. Johnson.** 1994. Interaction between two homeodomain proteins is specified by a short C-terminal tail. Nature (London) **371:**429–432.
58. **Sukovich, D. A., R. Mukherjee, and P. A. Benfield.** 1994. A novel, cell-type-specific mechanism for estrogen receptor-mediated gene activation in the absence of an estrogen-responsive element. Mol. Cell. Biol. **14:**7134–7143.
59. **Sun, B., D. A. Hursh, D. Jackson, and P. A. Beachy.** 1995. Ultrabithorax protein is necessary but not sufficient for full activation of *decapentaplegic* expression in the visceral mesoderm. EMBO J. **14:**520–535.
60. **Usheva, A., E. Maldonado, A. Goldring, H. Lu, C. Houbavi, D. Reinberg, and Y. Aloni.** 1992. Specific interaction between the nonphosphorylated form of RNA polymerase II and the TATA-binding protein. Cell **69:**871–881.
61. **van Dijk, M. A., and C. Murre.** 1994. Extradenticle raises the DNA binding specificity of homeotic selector gene products. Cell **78:**617–624.
62. **Wilkinson, D. G., S. Bhatt, M. Cook, E. Boncinelli, and R. Krumlauf.** 1989. Segmental expression of *Hox*-2 homeobox-containing genes in the developing mouse hindbrain. Nature (London) **341:**405–409.
63. **Xue, D., Y. Tu, and M. Chalfie.** 1993. Cooperative interactions between the *Caenorhabditis elegans* homeoproteins UNC-86 and MEC-3. Science **261:**1324–1328.
64. **Zappavigna, V., D. Sartori, and F. Mavilio.** 1994. Specificity of HOX protein function depends on DNA-protein and protein-protein interactions, both mediated by the homeodomain. Genes Dev. **8:**732–744.
65. **Zeng, W., D. J. Andrew, L. D. Mathies, M. A. Horner, and M. P. Scott.** 1993. Ectopic expression and function of the Antp and Scr homeotic genes: the N-terminus of the homeodomain is critical to functional specificity. Development **118:**339–352.
66. **Zhang, H., K. M. Catron, and C. Abate-Shen.** 1996. A role for the Msx-1 homeodomain in transcriptional regulation: residues in the N-terminal arm mediate TATA binding protein interaction and transcriptional repression. Proc. Natl. Acad. Sci. USA **93:**1764–1769.