Functional dissection of a transcriptionally active, target-specific Hox–Pbx complex

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Hox genes control cell fates and specify regional identities in vertebrate development. Hox proteins show a relaxed DNA-binding selectivity *in vitro*, suggesting that functional specificity is achieved *in vivo* through the action of transcriptional co-factors. Pbx proteins are good candidates for such a role, on the basis of both genetic and biochemical evidence. We report that the human Pbx1 and HOXB1 proteins can cooperatively activate transcription through a genetically characterized Hox target, i.e. an autoregulatory element directing spatially restricted expression of the murine *Hoxb-1* gene (b1-ARE) in the developing hind-brain. On the b1-ARE, only a restricted subset of HOX proteins (HOXA1, HOXB1, HOXA2) are able to bind cooperatively with Pbx1 and activate transcription. Selective recognition of the b1-ARE is mediated by the N-terminal region of the HOX homeodomain. The DNA-binding and protein–protein interaction functions of HOXB1 and Pbx1 are all necessary for the assembly of a transcriptionally active complex on the b1-ARE. Functional dissection of the complex allowed the localization of the main activation domain in the HOXB1 N-terminal region, and of an additional one in the C-terminal region of Pbx1 contained in the Pbx1a but not in the alternatively spliced Pbx1b isoform. Our results indicate that Pbx1 acts as a transcriptional co-factor of Hox proteins, allowing selective recognition and cooperative activation of regulatory target sequences.

Keywords: developmental regulation/gene expression/homeobox/protein–protein interaction/transcription

Introduction

Genes encoding the Hox family of homeodomain-containing transcription factors regulate cell fates and developmental pathways in metazoans, eventually leading to the generation of morphological differences along body axes (reviewed in Krumlauf, 1994). Despite the high degree of functional specificity observed *in vivo*, a large number of Hox proteins (up to 38 in mammalian species) appear to bind *in vitro* to a restricted set of very similar sites with similar affinities. For this reason, the specificity of action of Hox genes *in vivo* has been postulated to rely not only on their DNA-binding properties, but also on the activity

of transcriptional (co-)factors (reviewed in Manak and Scott, 1993). Good candidates for such a role are the homeodomain-containing products of the *extradenticle* (*exd*)/*Pbx* gene family. *exd* was originally identified as a mutation causing patterning defects in *Drosophila* without affecting homeotic gene expression (Peifer and Wieschaus, 1990). *exd* function is not dependent on Hox gene activity (Wieschaus and Noell, 1986), while it is required for the regulation of Hox-controlled target genes (Chan *et al.*, 1994; Rauskolb and Wieschaus, 1994), consistent with the proposed role of *exd* as a Hox co-factor.

Three mammalian *Exd* homologues, termed Pbx1, 2 and 3, have been isolated independently due to the involvement of Pbx1 in a chromosomal translocation associated with pre-B cell human leukaemia (Kamps *et al.*, 1990; Nourse *et al.*, 1990). The high degree of homology (~70%) between *exd*, the mammalian *Pbx1*, 2 and 3, and the *Caenorhabditis elegans* *ceh-20* genes (Burglin and Ruvkun, 1992) parallels that observed for the Hox genes, and suggests the conservation throughout evolution of co-factor functions. In agreement with the proposed role as Hox co-factors, *exd*/Pbx proteins have been found to cooperatively stimulate sequence-specific DNA binding of several Hox proteins (Chan *et al.*, 1994; Van Dijk and Murre, 1994; Chang *et al.*, 1995; Knoepfler and Kamps, 1995; Lu *et al.*, 1995; Pöpperl *et al.*, 1995), and to select different consensus binding sites in association with different Hox proteins *in vitro* (Chan and Mann, 1996; Chang *et al.*, 1996). The formation of Hox–Pbx complexes has been shown to rely on protein–protein contacts mediated by the Hox homeodomain and a short conserved motif (consensus sequence: YPWM) located upstream of the homeodomain in a subset of Hox proteins (reviewed in Mann and Chan, 1996). The ‘YPWM’ motif appears to be required (Chang *et al.*, 1995; Knoepfler and Kamps, 1995; Phelan *et al.*, 1995), although it is not sufficient (Chan *et al.*, 1994; Johnson *et al.*, 1995), to stabilize the Hox–Pbx complex.

The function of *exd*/Pbx proteins as Hox co-factors has been studied extensively *in vivo*, in *Drosophila* and in transgenic mice, as well as *in vitro* (reviewed in Mann, 1996; Mann and Chan, 1996). However, few studies have directly addressed the role of these proteins as modulators of Hox function at the level of transcriptional regulation (Lu *et al.*, 1995; Peers *et al.*, 1995; Phelan *et al.*, 1995; Chang *et al.*, 1996; Lu and Kamps, 1996a). We have analysed the transcriptional activity of Hox–Pbx complexes on a well characterized Hox target, the *Hoxb-1* autoregulatory element (Pöpperl *et al.*, 1995). The expression of *Hoxb-1* in the developing mouse hindbrain is confined to a segmentally organized cellular compartment referred to as rhombomere 4 (reviewed in Keynes and Krumlauf, 1994), as the result of a positive autoregulatory feedback loop, mediated by a conserved 148 bp genomic

region (Pöpperl *et al.*, 1995). The activity of the *Hoxb-1* autoregulatory element (b1-ARE) was shown in transgenic flies to be dependent on the functions of both *exd* and the *Drosophila Hoxb-1* homologue *labial* (Pöpperl *et al.*, 1995). The b1-ARE contains three related sequence motifs (repeats 1, 2 and 3) which are nearly identical to the *in vitro* defined Pbx consensus binding sequence ATCAAT-CAA (Van Dijk *et al.*, 1993; Lu *et al.*, 1995). These repeats are necessary and sufficient to drive expression of a reporter gene in rhombomere 4 of transgenic mice. In particular, repeat 3 (R3) proved to be crucial for rhombomere-restricted expression, and to mediate cooperative binding *in vitro* of *hoxb-1* and *exd* proteins, indicating that the b1-ARE may indeed represent a natural target for positive regulation by a Hoxb-1–Pbx complex (Pöpperl *et al.*, 1995).

Here we show that the human HOXB1 and Pbx1 proteins cooperatively activate transcription through the b1-ARE in transfected cells. The functional specificity of various HOX–Pbx1 complexes on the b1-ARE was analysed by co-expressing with Pbx1 a set of HOX proteins, belonging to different clusters and/or paralogy groups, and assaying for transcriptional activation. We show that transcription from the b1-ARE is activated selectively by members of HOX paralogy groups 1 and 2 in combination with Pbx1. Domain-swap experiments showed that the homeodomain N-terminal region of HOXB1 is sufficient to transfer the ability to bind to the R3 site *in vitro*, and to activate transcription from the b1-ARE in transfected cells, to the otherwise inactive HOXB3 protein. The transcriptional activity of the HOXB1–Pbx1 complex on the b1-ARE is dependent essentially on the activation domain of HOXB1, located in the N-terminal portion of the protein. An additional activation domain was mapped to the C-terminus of the Pbx1 protein, within a region present in the Pbx1a but not in the alternatively spliced Pbx1b isoform.

Results

HOXB1 and Pbx1 cooperatively activate transcription through the Hoxb-1 gene autoregulatory element

The ability of the HOXB1–Pbx1 complex to regulate transcription was assayed in transient co-transfection experiments, using the b1-ARE (Pöpperl *et al.*, 1995) as a responsive element. The pAdMLARE reporter construct contains a 148 bp fragment from the b1-ARE upstream of the adenovirus major late (AdML) minimal promoter in the pAdMLluc luciferase reporter gene (Figure 1A). Transfection of COS7 cells with the pAdMLARE reporter (ARE in Figure 1B), together with a construct expressing either HOXB1 or Pbx1 under the control of the SV40 promoter (pSGHOXB1 and pSGPbx1 respectively), did not significantly stimulate the reporter basal activity (Figure 1B). Conversely, co-transfection of both the Pbx1 and the HOXB1 expressors caused a 9-fold enhancement of the pAdMLARE activity (ARE in Figure 1B), but not of the enhancer-less pAdMLluc reporter transfected as a control (C in Figure 1B).

Among the three related sequence motifs present in the b1-ARE, R3 was shown to mediate most of the activity of the b1-ARE in transgenic mice (Pöpperl *et al.*, 1995).

To study the relative role of this motif in mediating transcriptional activation from the HOXB1–Pbx1 complex, we generated a knockout mutation of R3 within the b1-ARE reporter (pAdMLAREmR3, Figure 1A). Co-transfection of pSGHOXB1 and pSGPbx1 expressors with the pAdMLAREmR3 reporter (AREmR3 in Figure 1B) led to a relative stimulation of the reporter basal activity of ~40% of that observed with the wild-type pAdMLARE, indicating that R3 is required for maximal activity of the ARE element. To test whether R3 alone was sufficient for mediating transcriptional activation by HOXB1 and Pbx1, we generated a reporter construct containing a 3mer of R3 (pAdMLR3, Figure 1A). As shown in Figure 1B, HOXB1 and Pbx1 were able to activate the pAdMLR3 reporter (R3) at a level comparable with that observed with the complete b1-ARE. Transfection of either protein alone had no effect on the reporter activity (Figure 1B). Identical results were obtained with a reporter in which the R3 3mer was cloned upstream of a different minimal promoter, such as the herpes simplex virus thymidine kinase (TK) –81 promoter (Figure 1A and C). Cooperative action of HOXB1 and Pbx1 in activating transcription from the b1-ARE was also observed by co-transfection of a different cell type, i.e. the murine embryonal carcinoma P19 cell line (Figure 1D).

These data show that a Pbx1-dependent activation of the b1-ARE by the HOXB1 protein can be reproduced in cultured cells. In this context, Pbx1 behaves as a transcriptional co-factor of HOXB1, allowing cooperative activation of the b1-ARE in a promoter- and cell context-independent fashion. Within the b1-ARE, the R3 site appears to be sufficient for transactivation by HOXB1 and Pbx1.

The b1-ARE selectively mediates Pbx1-dependent binding and transcriptional activation by members of the HOX paralogy groups 1 and 2

Next we tested whether other HOX gene products could transactivate the b1-ARE in combination with Pbx1. Twelve HOX genes belonging to different clusters and/or paralogy groups were co-expressed with Pbx1 in transient transfections of COS7 cells, and tested for cooperative transcriptional activation of the pAdMLARE reporter. As shown in Figure 2A, none of the tested HOX proteins alone were able to transactivate the pAdMLARE reporter. Expression of the same set of HOX proteins in combination with Pbx1 led to significant (i.e. 4- to 6-fold) transactivation of the reporter activity only in the case of HOXA1, HOXB1 and HOXB2, whereas very weak (<2-fold) or no transactivation was observed for the other HOX proteins (Figure 2B). Identical results were obtained by co-transfecting the pAdMLR3, containing the R3 trimer (data not shown). Production of HOX proteins by the transfected plasmid was quantitatively comparable, as tested by an electrophoretic mobility shift assay (EMSA) of the transfected cell nuclear extracts with an oligonucleotide containing a Hox consensus binding site (not shown).

A representative subset of the same HOX proteins was tested for cooperative DNA binding with Pbx1 to the b1-ARE R3 site in EMSA. As shown in Figure 2C, only the HOXA1, HOXB1 (lanes 3 and 4) and HOXB2 (not shown) proteins formed a detectable retarded complex with a labelled R3 oligonucleotide in the presence of Pbx1. No

Fig. 1. HOXB1 activates transcription from the murine *Hoxb-1* autoregulatory enhancer (b1-ARE) in combination with Pbx1. **(A)** Schematic representation of the reporter constructs utilized in the transfection assay. R1, R2 and R3 indicate the evolutionarily conserved repeated sequences within the b1-ARE genomic region. Mutated bases within the R3 repeat are underlined. A grey box represents the luciferase reporter gene. **(B)** Luciferase activity, in arbitrary units, assayed from extracts of transiently transfected COS7 cells. Cells were transfected with 4 μ g of the SV40-driven HOXB1, and/or 8 μ g Pbx1 expression constructs, together with 8 μ g of alternatively pAdMLuc (C), pAdMLARE (ARE), pAdMLAREmR3 (AREmR3) or pAdMLR3 (R3) reporter constructs. **(C)** Luciferase activity, assayed from extracts of transiently transfected COS7 cells. Four μ g of the HOXB1 and/or 8 μ g of the Pbx1 expression constructs were transfected together with 8 μ g of the pTK R3 (TK-R3) reporter. **(D)** Luciferase activity, assayed from extracts of transiently transfected P19 cells. Four μ g of the HOXB1, and/or 8 μ g of the Pbx1 expression constructs were transfected together with the pAdMLuc (C), or the pAdMLARE (ARE) reporter. In all experiments, 0.2 μ g of the pCMV β -gal plasmid were co-transfected as an internal standard. Bars represent the mean $\pm$ SEM of at least four independent experiments.

binding was detected with Pbx1 alone (lane 2), or with either of the HOX proteins alone (not shown). Polyclonal antibodies against the full-length Pbx1 protein completely abolished the formation of the HOXA1-Pbx1 and HOXB1-Pbx1 complexes (Figure 2C, lanes 12 and 13).

These results show that the b1-ARE, and the R3 site within it, selectively mediate transcriptional activation by HOX-Pbx1 complexes made of members of the HOX paralogy groups 1 and 2. DNA binding *in vitro* experiments suggest that this selectivity relies on the differential

recognition of the R3 site by HOX proteins, allowing only members of paralogy groups 1 and 2 to bind this sequence cooperatively with Pbx1.

Specific target recognition by the HOXB1-Pbx1 complex is encoded in the Hox homeodomain N-terminal arm, and can be transferred to HOXB3 by domain swapping

To identify the HOX protein domain responsible for the differential activation of the R3 site *in vivo*, we carried

Fig. 2. The *Hoxb-1* ARE selectively mediates transcriptional activation by members of Hox parity groups 1 and 2. (A) COS7 cells were transiently transfected with 4 μ g of SV40-driven expression vectors producing the indicated HOX full-length proteins, and 8 μ g of the pAdMLARE reporter construct. Bars represent mean luciferase units $\pm$ SEM of at least four independent experiments. (B) COS7 cells were transfected with 4 μ g of the same HOX expressors as in (A), together with 8 μ g of the Pbx1 expression vector and 8 μ g of the pAdMLARE reporter construct. Bars represent mean fold activation over the activity obtained by transfecting the HOX proteins alone with pAdMLARE $\pm$ SEM of at least four independent experiments. (C) EMSA of a 30 bp double-stranded oligonucleotide representing the R3 site of the b1-ARE. The Pbx1 and the indicated HOX proteins were produced and labelled by coupled transcription-translation from their expression vectors in rabbit reticulocyte lysates. Lysates (1–4 μ l depending on protein content) were mixed with the radiolabelled R3 oligonucleotide in a binding reaction, and subjected to EMSA. F = free probe. Anti-Pbx1 rabbit antiserum (α -Pbx1), was added to the binding reactions containing HOXA1 and HOXB1 (lanes 12 and 13). lys = complexes arising from endogenous reticulocyte lysate binding activities.

out a domain-swap experiment between HOXB1 and a HOX protein unable to activate transcription through the b1-ARE in combination with Pbx1, i.e. HOXB3. Previous studies had indicated that the specificity of action of HOX genes *in vivo* resides within the homeodomain and its flanking regions (reviewed in Krumlauf, 1994). In particular, the flexible N-terminal arm of the homeodomain was shown to be crucial for HOX specificity both *in vivo* (Lin and McGinnis, 1992; Furukubo-Tokunaga *et al.*, 1993; Zeng *et al.*, 1993) and in transfected cells (Zappavigna *et al.*, 1994). The conserved 'YPWM' motif was identified as an important interaction surface between Hox and Pbx proteins (reviewed in Mann, 1996). Therefore, we decided to replace a region encompassing the 'YPWM' motif (FDWM in HOXB1 and FPWM in HOXB3) and the

homeodomain N-terminus in the HOXB3 protein (amino acids 130–201) with the corresponding region of HOXB1 (175–211) (Figure 3A), and to assay the resulting chimeric protein (HOXB3/B1HN in Figure 3A) for transcriptional activation of the pAdMLARE reporter in combination with Pbx1. As shown in Figure 3B, while the wild-type HOXB3 was unable to transactivate the pAdMLARE reporter, either alone or in combination with Pbx1, the HOXB3/B1HN chimera could activate the reporter together with Pbx1 to a level comparable with that obtained with HOXB1. Accordingly, the HOXB3/B1HN chimera could cooperatively bind to the R3 site in EMSA with Pbx1 (Figure 3C).

To narrow down further the region responsible for the differential activity of HOXB1 and HOXB3 on the R3

Fig. 3. The homeodomain N-terminal region of HOXB1 confers on HOXB3 the ability to bind and activate transcription from the b1-ARE. (A) Schematic representation of the HOXB1, HOXB3 and HOXB3/B1HN proteins. Numbers indicate amino acid positions, dark grey or striped boxes indicate the 'YPWM' motif and the homeodomain, horizontal lines represent the helical regions within the homeodomain. The amino acid sequences of the HOXB1 and HOXB3 homeodomains are shown aligned, vertical bars highlight conserved residues, boxed sequences represent the 'YPWM' and the homeodomain regions respectively. (B) COS7 cells were transfected with 8 μ g of the pAdMLARE reporter (ARE), together with 8 μ g of the Pbx1 expressor where indicated, and with 4 μ g of the HOXB1, HOXB3, HOXB3/B1HN and HOXB3/B1N expressors where indicated. In all experiments, 0.2 μ g of the pCMV β -gal plasmid were co-transfected as an internal standard. Bars represent the mean luciferase activity $\pm$ SEM of at least three independent experiments. (C) EMSA of the 30 bp double-stranded R3 oligonucleotide. The Pbx1, HOXB1, HOXB3, HOXB3/B1HN and HOXB3/B1N proteins were produced and labelled by coupled transcription-translation from their expression vectors in rabbit reticulocyte lysates. Lysates (1–4 μ l depending on protein content) were mixed with the radiolabelled R3 oligonucleotide in a binding reaction, and subjected to EMSA. F = free probe; lys = complexes arising from endogenous reticulocyte lysate binding activities.

site, we constructed an additional chimeric mutant (HOXB3/B1N in Figure 3A), in which the N-terminal portion of the HOXB3 homeodomain (amino acids 184–201) was replaced by the corresponding region of the HOXB1 homeodomain (195–211). As shown in Figure 3B, the substitution of the 17-most N-terminal amino acids of the HOXB3 homeodomain with the corresponding amino acids from HOXB1 is sufficient to allow the HOXB3/B1N chimera to activate transcription in combina-

tion with Pbx1 at a level comparable with that of HOXB1. Consistently, the HOXB3/B1N chimera was able to bind cooperatively with Pbx1 the R3 site in EMSA (Figure 3C)

These results indicate that the homeodomain N-terminus region of HOXB1 is sufficient to confer to HOXB3 the ability to bind cooperatively with Pbx1 at the R3 site and to activate transcription through the b1-ARE in transfected cells. These data also indicate that the absence of activation by the HOXB3 protein in combination with Pbx1 on the

A

B

C

Fig. 4. Mutational analysis of the HOXB1–Pbx1 complex transcriptional activation. (A) Schematic representation of the HOXB1 (left side) and of the Pbx1 (right side) mutants. Black boxes represent the homeodomains, grey boxes the FDWM, a white box the W177A substitution within the motif, and striped boxes the PBC-A and PBC-B domains conserved in *exd*/Pbx proteins. (B and C) COS7 cells were transfected with 8 μ g of the pAdMLR3 reporter (R3), together with 8 μ g of expressor plasmids for Pbx1 and its mutants, and with 4 μ g of expressor plasmids for HOXB1, and its mutants in the indicated combinations. Mutants are identified in (A). In all experiments, 0.2 μ g of the pCMV β -gal plasmid were co-transfected as an internal standard. Bars represent the mean luciferase activity $\pm$ SEM of at least three independent experiments.

b1-ARE element is not due to the lack of a functional activation domain on the HOXB3 protein, but rather to the inability of a HOXB3–Pbx1 complex to form on the b1-ARE R3 site.

The transcriptional activation function of the HOXB1–Pbx1 complex resides mainly in the HOXB1 activation domain

We took advantage of the activity of HOXB1 and Pbx1 on the b1-ARE to dissect the functional properties of the HOXB1–Pbx1 complex in transcriptional activation. To identify the protein domain(s) responsible for transcriptional activation, we generated a series of deletion mutants in the HOXB1 and Pbx1 proteins (Figure 4A), and tested them for the ability to activate the pAdMLR3 reporter in transfected COS7 cells. Deletion of the N-terminal region (amino acids 1–155) of HOXB1 (HOXB1HD in Figure 4A) caused a reduction in the activity of the HOXB1–Pbx1 complex of ~70% with respect to the complex containing the wild-type HOXB1 (Figure 4B). The

HOXB1HD mutant was still able to bind cooperatively with Pbx1 on the b1-ARE R3 site (Figure 5, lane 8). Conversely, two N-terminal deletions of Pbx1 (Pbx1a Δ 1–140 and Pbx1a Δ 1–230 in Figure 4A), lacking one or both of the conserved PBC-A and PBC-B domains previously identified in *exd*/Pbx family members (Burglin and Ruvkun, 1992), led to cooperative activation of the pAdMLR3 reporter activity at levels comparable with, or higher than, those observed with the wild-type Pbx1 (Figure 4B). Interestingly, the Pbx1a Δ 1–230 mutant was able to activate the pAdMLR3 reporter even in the absence of HOXB1 (Figure 4B). These data indicate that the transcriptional activating function of the HOXB1–Pbx1 complex on the b1-ARE element resides essentially in the HOXB1 N-terminal region. This region contains a *bona fide* activation domain, since it was active also in the context of a fusion with the yeast GAL4 DNA-binding domain (amino acids 1–147) on a GAL4-responsive reporter in transfected COS7 cells (results not shown).

To test whether binding of either HOXB1 or Pbx1 to

Fig. 5. Cooperative DNA binding of HOXB1 and Pbx1 mutants to the 30 bp double-stranded oligonucleotide representing the R3 site of the b1-ARE. Pbx1, HOXB1 and their mutants were produced and labelled by coupled transcription–translation from their corresponding expression vectors in rabbit reticulocyte lysates. Lysates (1–4 μ l depending on protein content) were mixed with the radiolabelled R3 oligonucleotide in a binding reaction, and subjected to EMSA. F = free probe; lys = complexes arising from endogenous reticulocyte lysate binding activities.

DNA is necessary for activation of the pAdMLR3 reporter, we generated and tested DNA-binding-defective mutants in both proteins. A HOXB1 mutant lacking helix 3 of the homeodomain (B1 Δ 236–274 in Figure 4A) did not stimulate the pAdMLR3 reporter basal activity when expressed in combination with Pbx1 (Figure 4C). Similarly, a Pbx1 deletion mutant lacking three amino acids within helix 3 of the homeodomain (Pbx1 Δ 283–285, in Figure 4A) showed no activity when co-expressed with HOXB1 (Figure 4C). When tested in EMSA, neither B1 Δ 236–274 (not shown) nor Pbx1 Δ 283–285 (Figure 5, lane 12) were able to bind cooperatively to the R3 site in association with the wild-type Pbx1 or HOXB1 respectively. Thus, mutations affecting DNA binding of either HOXB1 or Pbx1 disrupt the formation of a complex *in vitro* and suppress transcriptional activation in transfected cells.

Cooperative DNA binding by Hox and *exd*/Pbx proteins has been shown to require the Hox 'YPWM' motif, in particular the highly conserved tryptophan residue (reviewed in Mann and Chan, 1996), and the Pbx1 C-terminal region (Chang *et al.*, 1995; Lu and Kamps, 1996a; Mann and Chan, 1996), which are supposed to mediate protein–protein contacts between the two proteins. To test the role of the 'YPWM' motif in the function of the HOXB1–Pbx1 complex, we generated two HOXB1 mutant derivatives, HOXB1 Δ WM (Figure 4A) in which the conserved tryptophan and methionine residues were deleted, and HOXB1W177A in which the single tryptophan residue was replaced by an alanine residue. As shown in Figure 4C, both mutations completely abolished the ability of HOXB1 to activate the pAdMLR3 reporter

Taken together, these results indicate that the DNA-binding domains of both HOXB1 and Pbx1, and the putative protein-protein interaction domains on both proteins, are all individually necessary for the assembly of a cooperative DNA-binding and transcriptionally active complex.

As reported above, the deletion of the N-terminal region of HOXB1 caused a significant but not complete reduction in the transcriptional activity of the HOXB1-Pbx1 complex (HOXB1HD mutant in Figure 4B). This observation led us to assume that the Pbx1 protein could contribute in part to the transcriptional activity of the HOX-Pbx complex, and therefore be responsible for the residual activity of the B1HD-Pbx1 complex on the b1-ARE element. To identify a potential activation domain within the Pbx1 protein, we focused on the C-terminal region, since deletions within the N-terminus did not reduce the activity of the HOXB1-Pbx1 complex ($\Delta 1-140$ and $\Delta 1-230$ mutants in Figure 4B). In particular, we tested the activity of the Pbx1b isoform, a naturally occurring, alternative splicing variant of the full-length Pbx1 protein (or Pbx1a), which is 83 amino acids shorter at the C-terminus (Monica *et al.*, 1991). As shown in Figure 6A, co-expression of Pbx1b and HOXB1 led to activation of the pAdMLR3 reporter at levels of ~50% of those obtained with Pbx1a, while co-expression of Pbx1b and the HOXB1 mutant carrying a deletion of the N-terminal, major activation domain (HOXB1HD) showed no residual activity. These data indicate that the C-terminal 83 amino acids of the Pbx1a isoform carry an activation domain which is not contained in Pbx1b, and which contributes to the overall transcriptional activity of the HOXB1-Pbx1 complex.

As described above, a Pbx1a deletion mutant lacking amino acids 1–230 (Pbx1a Δ 1–230) was able to activate the pAdMLR3 reporter even in the absence of HOXB1 (Figure 4B). To test whether this activity relied on the activation domain located within the C-terminal region of Pbx1a, we generated a Δ 1–230 mutant also of the Pbx1b isoform (Pbx1b Δ 1–230), and compared its activity with that of Pbx1a Δ 1–230. As shown in Figure 6A, Pbx1b Δ 1–230 alone had no significant activity on the pAdMLR3 reporter, and showed an activity consistently lower than that of Pbx1a Δ 1–230 in combination with HOXB1. The Pbx1b variant showed the same efficiency of Pbx1a in

Fig. 6. The C-terminus of Pbx1a contains an activation domain lacking in the alternatively spliced Pbx1b variant. **(A)** COS7 cells were transfected with 8 μ g of the pAdMLR3 reporter (R3), together with 8 μ g of the Pbx1a, Pbx1b, Pbx1a Δ 1–230 (1a Δ 1–230) and Pbx1b Δ 1–230 (1b Δ 1–230) expressors where indicated, and with 4 μ g of the HOXB1 or HOXB1HD (B1HD) expressors where indicated. **(B)** Cells were transfected with 8 μ g of the pTUAS reporter (UAS) together with 4 μ g of the pGAL1–147 (GAL1–147) or the pGAL1–147Pbx1aCT (GAL1–147Pbx1aCT) expression construct. In all experiments, 0.2 μ g of the pCMV β -gal plasmid were co-transfected as an internal standard. Bars represent the mean luciferase activity $\pm$ SEM of at least five independent experiments.

cooperative binding to the b1-ARE R3 site *in vitro*, in combination with either HOXB1 or its N-terminal deletion HOXB1HD (Figure 5, lanes 6 and 9).

Finally, to prove the existence of an activation domain in the Pbx1a C-terminus, we fused the 83 amino acid region to the DNA-binding domain (amino acids 1–147) of the yeast GAL4 transcription factor to generate the GAL4Pb $\times$ 1aCT chimeric protein. This protein was able to activate transcription from a reporter containing five GAL4-binding sites (UAS in Figure 6B).

Discussion

The specificity of action of Hox gene products *in vivo* is probably achieved through the activity of still ill-defined co-factors modulating their transcriptional functions. Requirement for such factors *in vivo* would account for the relatively relaxed target specificity displayed by Hox proteins *in vitro*. While Hox gene products are apparently able to regulate transcription in a co-factor-independent manner (Zappavigna *et al.*, 1991; Arcioni *et al.*, 1992; Jones *et al.*, 1992, 1993; Pöpperl and Featherstone, 1992), regulation of some target genes *in vivo* was indeed shown to require the presence of additional factors (Chan *et al.*, 1994; Pöpperl *et al.*, 1995; Gross and McGinnis, 1996). The products of the *exd*/Pbx genes have been proposed as Hox co-factors on the basis of genetic analysis and of their ability to modulate DNA binding of Hox proteins *in vitro* (reviewed in Mann and Chan, 1996). A large amount of data has been gathered on site-selective and cooperative DNA binding by Pbx and Hox proteins on artificial sites *in vitro*, and a few studies on the E2A–Pbx oncogene fusion provided evidence for func-

tional cooperativity between the two families of proteins (Lu *et al.*, 1995; Peers *et al.*, 1995; Phelan *et al.*, 1995; Chang *et al.*, 1996; Lu and Kamps, 1996a). Nevertheless, the complex formed by wild-type Pbx and Hox proteins had never been functionally characterized in terms of its ability to activate transcription cooperatively from known target sequence.

We have shown here that Pbx1 can modulate Hox protein function at the level of transcription, by acting as a co-factor in the activation of a target element by a restricted class of human HOX proteins. We used as a model a genetically characterized, Pbx-dependent Hox target, the 148 bp autoregulatory element (b1-ARE) driving *Hoxb-1* gene expression in the fourth rhombomere of the mouse developing hindbrain (Pöpperl *et al.*, 1995). We show that the activity of a reporter construct containing the b1-ARE can be stimulated by the HOXB1 protein in transfected cells only upon co-expression of Pbx1. The b1-ARE contains three repeated sequences closely related to an *in vitro*-selected Pbx1 consensus binding site (Van Dijk *et al.*, 1993; Pöpperl *et al.*, 1995), one of which (R3) was shown previously to bind *in vitro* a *Hoxb-1*–*exd* complex, and to be crucial for the *in vivo* activity of the b1-ARE (Pöpperl *et al.*, 1995). Consistent with these findings, transactivation of the b1-ARE by the HOXB1–Pbx1 complex is significantly reduced by a mutation within the R3 site, while a multimerized R3 site is sufficient to mediate cooperative activation in a promoter- and cell context-independent fashion. Thus, HOXB1 requires Pbx1 as a transcriptional co-factor to regulate the activity of the b1-ARE through the R3 site.

Co-expression of Pbx1 with a representative variety of HOX proteins showed that the b1-ARE, or the multimer-

ized R3 site, is transactivated only by members of the paralogy groups 1 and 2, namely HOXA1, HOXB1 and HOXB2. The fact that the b1-ARE is activated by other proteins besides HOXB1 may reflect the existence of cross-regulation of this element *in vivo* by a subset of Hox genes. It is noteworthy, in this respect, that *Hoxa-1* and *Hoxb-2* are both expressed in rhombomere 4 during development (reviewed in Keynes and Krumlauf, 1994), and that ectopic expression of *Hoxa-1* was shown to cause activation of a *Hoxb-1-lacZ* reporter construct in the hindbrain of transgenic mice (Zhang *et al.*, 1994). This activation may, however, reflect functional redundancy among paralogous group 1 Hox proteins rather than a requirement for *Hoxa-1* in the onset and maintenance of *Hoxb-1* expression, since *Hoxb-1* is expressed normally in rhombomere 4 in *Hoxa-1* null mouse mutants (Carpenter *et al.*, 1993; Mark *et al.*, 1993). Nevertheless, expression of a b1-ARE-*lacZ* reporter in *Drosophila* embryos is not entirely dependent on the function of the *Drosophila* gene *labial* (Pöpperl *et al.*, 1995), suggesting that other Hox genes could indeed participate in the regulation the b1-ARE in a co-factor-dependent manner.

The selectivity of the b1-ARE, and particularly of the R3 site, appears to depend on its ability to allow the assembly of a Hox-Pbx1-DNA ternary complex only with group 1 and 2 Hox proteins, as indicated by DNA-binding analysis *in vitro*. This co-factor-mediated, differential site recognition appears to be specified by the extended N-terminal region of the Hox homeodomain. In fact, the ability to complex with Pbx1 on the R3 site, and to activate transcription through the b1-ARE, can be transferred to the HOXB3 protein by swapping a region containing only the homeodomain N-terminal arm. This result is in agreement with previous studies showing that the flexible homeodomain N-terminus is crucial for determining the specificity of action of Hox proteins *in vivo* (Lin and McGinnis, 1992; Furukubo-Tokunaga *et al.*, 1993; Zeng *et al.*, 1993; Chan *et al.*, 1994; Zappavigna *et al.*, 1994), and with previous models derived from *in vitro* studies on PCR-selected targets suggesting that the 'YPWM' homeodomain N-terminal region is an important determinant in the site selectivity of different Hox-Pbx complexes *in vitro* (Chan *et al.*, 1996; Chang *et al.*, 1996; Lu and Kamps, 1996b). In the context of the HOXB1-Pbx1 complex, the 'YPWM' motif therefore provides only a neutral, protein-protein interaction function, as previously suggested by yeast two-hybrid experiments (Johnson *et al.*, 1995), without necessarily contributing to the DNA-binding specificity. Indeed, mutations in the domains involved in protein-protein contacts, either in HOXB1 (deletion or mutation of the critical tryptophan in the FDWM region) or in Pbx1 [deletion of the C-terminus downstream from the homeodomain (see Chang *et al.*, 1996; Lu and Kamps, 1996b)] disrupt the formation of the ternary complex *in vitro* and abolish transcriptional activation altogether. Furthermore, we show that transcriptional activation by the HOXB1-Pbx1 complex requires intact DNA binding functions by both HOXB1 and Pbx1 proteins. Thus, unlike the MATA1-MAT α 2 homeodomain protein complex, where a MAT α 2 mutation impairing DNA binding is still able to form a functional complex with MATA1 on DNA *in vivo* (Vershon *et al.*, 1995), in the case of the HOXB1-Pbx1 complex, tethering of either

protein onto DNA through protein-protein interactions is not sufficient for activity.

The transcriptional activity of the HOXB1-Pbx1 complex on the b1-ARE was exploited to characterize functional domains in both proteins. The main transcriptional activation domain within the complex was mapped to the N-terminal region of the HOXB1 protein. In fact, deletion of the 155 N-terminal amino acids of the HOXB1 protein considerably reduced the transcriptional activation strength without affecting cooperative DNA binding. The N-terminus of the HOXB1 protein activates transcription also when fused to a heterologous DNA-binding domain. However, deletion of the HOXB1 N-terminal domain did not abolish transcriptional activity completely, leading us to speculate that Pbx1 could also contribute to the activation strength of the complex. A transcriptional activation domain was indeed mapped within the 83 most C-terminal amino acids of the Pbx1 protein. This region is absent in the Pbx1b alternative splicing variant, which reproducibly displayed lower levels of activation when compared with the full-length protein (Pbx1a) in combination with HOXB1. The C-terminal region of Pbx1a is rich in Ser/Thr residues, and is able to activate transcription when fused to a heterologous DNA-binding domain. Interestingly, a Ser/Thr-rich region located within the C-terminus of the LFB1 (HNF1) homeodomain protein has been reported previously to be implicated in transcriptional activation, and to account for 50% of the LFB1 activity (Nicosia *et al.*, 1990).

The C-terminal region of Pbx1a is apparently not functional as an activation domain in the uncomplexed Pbx1a protein, which is unable to activate transcription even through a PCR-derived optimal consensus binding site (Van Dijk *et al.*, 1993; Mann and Chan, 1996). Deletion of the N-terminal 230 amino acids, containing the two conserved PBC-A and PBC-B regions (Burglin and Ruvkun, 1992), uncovers the function of the Pbx1 C-terminal activation domain, since the Pbx1 Δ 1-230 mutant is able to stimulate the b1-ARE R3 reporter activity significantly, even in the absence of HOXB1. A deletion of the N-terminal 232 amino acids of Pbx1 has been reported previously to bind to a Pbx consensus sequence with higher affinity with respect to its wild-type counterpart in the absence of Hox proteins (Lu and Kamps, 1996b), suggesting that the activity of the Δ 1-230 mutant on the R3 reporter could be due to an increased binding affinity for the R3 site. Transcriptional activation by the Pbx1a Δ 1-230 mutant is sustained by the C-terminal activation domain, since the same N-terminal deletion in the shorter Pbx1b variant, Pbx1b Δ 1-230, does not significantly activate transcription. The Pbx1b alternatively spliced variant may, therefore, represent a less active form of Pbx1, capable of forming a DNA-bound complex with HOX proteins like the longer Pbx1a variant, but leading to lower levels of transcriptional activation. Pbx1b could antagonize and/or substitute for the longer and more active Pbx1a isoform in different tissues or body regions, allowing a fine tuning of the activity of Hox-Pbx complexes on their targets.

Materials and methods

Protein expression and reporter plasmids

All expression constructs are derivatives of the SV40 promoter-driven expression vector pSG5 (Green *et al.*, 1988). Hox expression vectors

were described previously (Zappavigna *et al.*, 1991, 1994; Arcioni *et al.*, 1992; Guazzi *et al.*, 1994), with the exception of pSGA1 and pSGC6. pSGA1 was generated by ligating a *Bam*HI–*Xba*I Klenow-filled *Hoxa-1* cDNA coding sequence obtained from the pHoxa-1(HD⁺) plasmid (Phelan *et al.*, 1995) into the *Bam*HI–*Bgl*II Klenow-filled sites of pSG5. pSGC6 was obtained by cloning a PCR-amplified *Bam*HI insert containing the full-length HOXC6-coding sequence from the pCT-H3C plasmid (Arcioni *et al.*, 1992) into the *Bam*HI site of pSG5. pSGPbx1a was generated by ligating a *Hind*III-filled–*Eco*RI-filled cDNA fragment encompassing the complete coding sequence of Pbx1a (Van Dijk *et al.*, 1993) into the filled *Bam*HI site of pSG5. pSGPbx1b was reconstructed by substituting the E1A N-terminal region of E1APbx1b (kind gift of F.Biasi) at a unique *Nco*I site with the N-terminal region of Pbx1a. Pbx1a deletion mutants (pSGPbx1aΔ1–140, Δ1–230, Δ296–430 and Δ283–285) were generated by ligating *Bam*HI PCR-amplified and deleted cDNA fragments in the *Bam*HI site of pSG5. To generate pSGPbx1aΔ1–140 and Δ1–230, an ATG start codon was introduced in-frame upstream of amino acids 141 and 231, respectively. To generate pSGPbx1aΔ296–430, a TGA stop codon was added downstream of amino acid 295. pSGPbx1bΔ1–230 was generated using the same PCR primer as for Pbx1aΔ1–230 and cloning into the *Eco*RI–*Bam*HI sites of pSG5. pSGB1ΔWM and W177A were obtained by PCR mutagenesis, covering the full-length HOXB1, as *Bam*HI inserts into pSG5. In pSGB1ΔWM, amino acids W177 and M178 of the HOXB1 ‘YPWM’ motif were deleted and in pSGB1W177A amino acid W177 was replaced by an alanine. pSGB1Δ236–274 was obtained by removing the *Sac*I–*Pvu*II fragment from pSGB1 and re-ligating the vector after blunting of the *Sac*I site with T4 DNA polymerase. This generates a deletion in the HOXB1 cDNA extending from the C-terminal region of the homeo-domain (helix 3/4) to part of the adjacent C-terminus of the protein, without any amino acid substitution. pSGHOXB3/B1HN was generated by replacing the ‘YPWM’/homeodomain N-terminal region of HOXB3 (amino acids 130–201) with the analogous region of HOXB1 (amino acids 175–211). The mutated cDNA was obtained by PCR amplification and cloned as a *Bam*HI insert into pSG5. pSGHOXB3/B1N was generated by replacing the homeodomain N-terminal region of HOXB3 (amino acids 184–201) with the analogous region of HOXB1 (amino acids 195–211). The mutated cDNA was obtained by PCR amplification and cloned as a *Bam*HI insert into pSG5. pSGB1HD was generated by PCR amplification of a region comprising amino acids 155–286 and introducing a methionine residue in position 154. pSGGAL1–147 was obtained by cloning a *Bam*HI–*Bgl*II insert containing the DNA-binding domain of yeast GAL4 (amino acids 1–147) into pSG5. pGAL1–147pb×1CT was generated by PCR amplifying a region comprising amino acids 348–430 and cloning in-frame with the GAL4 1–147 protein at the *Eco*RI site of the pGAL1–147 vector. The correctness of all cloned PCR products was verified by DNA sequencing, and the expression of all proteins was tested in a rabbit reticulocyte system.

The luciferase reporter construct pML is described elsewhere (Pöpperl and Featherstone, 1992). pAdMLARE (generous gift of Mark S. Featherstone) contains the *Aval*–*Hae*II R4 enhancer of *Hoxb-1* (Pöpperl *et al.*, 1995) cloned as a PCR-amplified *Hind*III–*Xho*I fragment into pML. In pAdMLAREmR3, the Pbx consensus site of R3 in the R4 enhancer TGATGGAT was changed to TGTCGACT. pAdMLR3 contains a trimer of R3 of the b1-ARE enhancer cloned as a *Bam*HI–*Hind*III fragment into pML. The same trimer of R3 was cloned into the *Bam*HI site of the pT81luc luciferase reporter vector. The sequence of the 30 bp oligo used to generate the trimer is 5′-GATCCGGGGGGTGTATGGATGGCGCTGGGA-3′. The pTUAS luc GAL-4 reporter construct was described in Zappavigna *et al.* (1996).

Protein production and DNA-binding assays

Pbx and HOX proteins were produced *in vitro* from the corresponding pSG5-derived expression vectors using a T7 polymerase-based coupled transcription–translation reticulocyte lysate system (Promega, Madison, WI) according to recommended conditions. HOX and Pbx proteins were translated separately in the presence of [³⁵S]methionine and quantitated after SDS–PAGE using a PhosphorImager (Molecular Dynamics). The amounts of proteins were normalized for the methionine content of each protein.

Gel retardation analysis was performed by pre-incubating the *in vitro* synthesized proteins for 30 min on ice in 15 μl of binding buffer (75 mM NaCl, 6% glycerol, 10 mM Tris–HCl pH 7.6, 1 mM EDTA), together with 2 μl (0.5 ng, 5×10⁴ c.p.m.) of ³²P-labelled oligonucleotide R3 probe 5′-GATCCGGGGGGTGTATGGATGGCGCTGGGA-3′. The incubation mixture was resolved by electrophoresis on a 6% polyacrylamide gel in 0.25× TBE at 10 V/cm. Gels were dried and exposed to

a Kodak X-AR film at –70°C. The amount of reticulocyte lysate added to each incubation mixture was adjusted in order to normalize for the translated protein content.

Cell culture and transfection

COS7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (Gibco), 100 IU/ml of penicillin and 100 μg/ml streptomycin. Cells were transfected by calcium phosphate precipitation (Di Nocera and Dawid, 1983). In a typical transfection experiment, 8 μg of reporter plasmid, 4–8 μg of expression construct and 0.2 μg of pCMV-β-gal as an internal control were used per 9 cm dish. Cells were harvested 48–60 h after transfection, lysed and assayed for luciferase and β-galactosidase expression as described previously (Zappavigna *et al.*, 1994).

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References

- Arcioni, L., Simeone, A., Guazzi, S., Zappavigna, V., Boncinelli, E. and Mavilio, F. (1992) The upstream region of the human homeobox gene HOXD3D is a target for regulation by retinoic acid and HOX homeoproteins. *EMBO J.*, **11**, 265–277.
- Burglin, T.R. and Ruvkun, G. (1992) New motif in PBX genes. *Nature Genet.*, **1**, 319–320.
- Carpenter, E.M., Goddard, J.M., Chisaka, O., Manley, N.R. and Capecchi, M.R. (1993) Loss of Hoxa-1 (Hox-1.6) function results in the reorganization of the murine hindbrain. *Development*, **118**, 1063–1075.
- Chan, S.-K. and Mann, R.S. (1996) A structural model for a homeotic protein–extradenticle–DNA complex accounts for the choice of HOX protein in the heterodimer. *Proc. Natl Acad. Sci. USA*, **93**, 5223–5228.
- Chan, S.-K., Jaffe, L., Capovilla, M., Botas, J. and Mann, R.S. (1994) The DNA binding specificity of ultrabithorax is modulated by cooperative interactions with extradenticle, another homeoprotein. *Cell*, **78**, 603–615.
- Chan, S.-K., Popperl, H., Krumlauf, R. and Mann, R.S. (1996) An extradenticle-induced conformational change in a HOX protein overcomes an inhibitory function of the conserved hexapeptide motif. *EMBO J.*, **15**, 2476–2487.
- Chang, C.-P., Shen, W.-F., Rozenfeld, S., Lawrence, H.J., Largman, C. and Cleary, M. (1995) Pbx proteins display hexapeptide-dependent cooperative DNA binding with a subset of Hox proteins. *Genes Dev.*, **9**, 663–674.
- Chang, C.-P., Brocchieri, L., Shen, W.-F., Largman, C. and Cleary, M. (1996) Pbx modulation of Hox homeodomain amino-terminal arms establishes different DNA-binding specificities across the Hox locus. *Mol. Cell. Biol.*, **16**, 1734–1745.
- Di Nocera, P.P. and Dawid, I.B. (1983) Transient expression of genes introduced into cultured cells of *Drosophila*. *Proc. Natl Acad. Sci. USA*, **80**, 7095–7098.
- Furukubo-Tokunaga, K., Flister, S. and Gehring, W.J. (1993) Functional specificity of the Antennapedia homeodomain. *Proc. Natl Acad. Sci. USA*, **90**, 6360–6364.
- Green, S., Issemann, I. and Sheer, E. (1988) A versatile eucaryotic expression vector for protein engineering. *Nucleic Acids Res.*, **16**, 369–373.
- Gross, C.T. and McGinnis, W. (1996) DEAF-1, a novel protein that binds an essential region in a Deformed response element. *EMBO J.*, **15**, 1961–1970.
- Guazzi, S., Lonigro, R., Pintonello, L., Boncinelli, E., Di Lauro, R. and Mavilio, F. (1994) The thyroid transcription factor-1 gene is a candidate target for regulation by Hox proteins. *EMBO J.*, **13**, 3339–3347.
- Johnson, F.B., Parker, E. and Krasnow, M. (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.

- Jones, F.S., Chalepakis, G., Gruss, P. and Edelman, G.M. (1992) Activation of the cytotoxin promoter by the homeobox-containing gene *Evx-1*. *Proc. Natl Acad. Sci. USA*, **89**, 2091–2095.
- Jones, F.S., Holst, B.D., Minowa, O., De Robertis, E.M. and Edelman, G.M. (1993) Binding and transcriptional activation of the promoter for the neural cell adhesion molecule by *HoxC6* (*Hox-3.3*). *Proc. Natl Acad. Sci. USA*, **90**, 6557–6561.
- Kamps, M.P., Murre, C., Sun, X.-H. and Baltimore, D. (1990) A new homeobox gene contributes to the DNA binding domain of the t(1;19) translocation protein in Pre-B-ALL. *Cell*, **60**, 547–555.
- Keynes, R. and Krumlauf, R. (1994) HOX genes and the regionalization of the nervous system. *Annu. Rev. Neurosci.*, **17**, 109–132.
- Knoepfler, P. and Kamps, M. (1995) The pentapeptide motif of Hox proteins is required for cooperative DNA binding with Pbx1, physically contacts Pbx1, and enhances DNA binding by Pbx1. *Mol. Cell. Biol.*, **15**, 5811–5819.
- Krumlauf, R. (1994) *Hox* genes in vertebrate development. *Cell*, **78**, 191–201.
- Lin, L. and McGinnis, W. (1992) Mapping functional specificity in the Dfd and Ubx homeo domains. *Genes Dev.*, **6**, 1071–1081.
- Lu, Q. and Kamps, M.P. (1996a) Selective repression of transcriptional activators by Pbx1 does not require the homeodomain. *Proc. Natl Acad. Sci. USA*, **93**, 470–474.
- Lu, Q. and Kamps, M.P. (1996b) Structural determinants within Pbx1 that mediate cooperative DNA binding with pentapeptide-containing Hox proteins: proposal for a model of a Pbx1–Hox–DNA complex. *Mol. Cell. Biol.*, **16**, 1632–1640.
- Lu, Q., Knoepfler, P., Scheele, J., Wright, D. and Kamps, M. (1995) 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.
- Manak, J.R. and Scott, M.P. (1993) Able assistants for homeodomain proteins. *Curr. Biol.*, **3**, 318–320.
- Mann, R. (1996) The specificity of homeotic gene function. *BioEssays*, **17**, 855–863.
- Mann, R.S. and Chan, S.-K. (1996) Extra specificity from *extradenticle*: the partnership between HOX and PBX/EXD homeodomain proteins. *Trends Genet.*, **12**, 258–262.
- Mark, M., Lufkin, T., Vonesch, J.-L., Ruberte, E., Oliver, J.-C., Dolle, P., Gorry, P., Lumsden, A. and Chambon, P. (1993) Two rhombomeres are altered in Hoxa-1 mutant mice. *Development*, **119**, 319–338.
- Monica, K., Galili, N., Nourse, J., Saltman, D. and Cleary, M. L. (1991) *PBX2* and *PBX3*, new homeobox genes with extensive homology to the human proto-oncogene *PBX1*. *Mol. Cell. Biol.*, **11**, 6149–6157.
- Nicosia, A., Monaci, P., Tomei, L., DeFrancesco, R., Nuzzo, M., Stunnenberg, H. and Cortese, R. (1990) A myosin-like dimerization helix and an extra-large homeodomain are essential elements of the tripartite DNA-binding structure of LFB-1. *Cell*, **61**, 1225–1236.
- Nourse, J., Mellett, J., Galili, N., Wilkinson, J., Stanbridge, E., Smith, S. and Cleary, M. (1990) Chromosomal translocation t(1;19) results in synthesis of a homeobox fusion mRNA that codes for a potential chimeric transcription factor. *Cell*, **60**, 535–545.
- Peers, B., Sharma, S., Johnson, T., Kamps, M. and Montminy, M. (1995) The pancreatic islet factor STF-1 binds cooperatively with Pbx to a regulatory element in the somatostatin promoter: importance of the FPWMK motif and of the homeodomain. *Mol. Cell. Biol.*, **15**, 7091–7097.
- Peifer, M. and Wieschaus, E. (1990) Mutation in the *Drosophila* gene *extradenticle* affect the way specific homeo domain proteins regulate segmental identity. *Genes Dev.*, **4**, 1209–1223.
- Phelan, M.L., Rambaldi, I. and Featherstone, M.S. (1995) Cooperative interactions between HOX and PBX proteins mediated by a conserved peptide motif. *Mol. Cell. Biol.*, **15**, 3989–3997.
- Pöpperl, H. and Featherstone, M.S. (1992) An autoregulatory element of the murine *Hox-4.2* gene. *EMBO J.*, **11**, 3673–3680.
- Pöpperl, H., Bienz, M., Studer, M., Chang, S.-K., Aparicio, S., Brenner, S., Mann, R. and Krumlauf, R. (1995) Segmental expression of *Hoxb-1* is controlled by a highly conserved autoregulatory loop dependent upon *exd/Pbx*. *Cell*, **81**, 1031–1042.
- Rauskolb, C. and Wieschaus, E. (1994) Coordinate regulation of downstream genes by *extradenticle* and the homeotic selector proteins. *EMBO J.*, **13**, 3561–3569.
- Van Dijk, M.A. and Murre, C. (1994) *extradenticle* raises the DNA binding specificity of homeotic selector gene products. *Cell*, **78**, 617–624.
- Van Dijk, M.A., Voorhoeve, P.M. and Murre, C. (1993) Pbx1 is converted into a transcriptional activator upon acquiring the N-terminal region of E2A in pre-B-cell acute lymphoblastoid leukemia. *Proc. Natl Acad. Sci. USA*, **90**, 6061–6065.
- Vershon, A.K., Jin, Y.S. and Johnson, A.D. (1995) A homeodomain protein lacking specific side chains of helix 3 can still bind DNA and direct transcriptional repression. *Genes Dev.*, **9**, 182–192.
- Wieschaus, E. and Noell, E.F. (1986) Specificity of embryonic lethal mutations in *Drosophila* analysed in germ line clones. *Wilhelm Roux's Arch. Dev. Biol.*, **195**, 63–73.
- Zappavigna, V., Renucci, A., Izpisua-Belmonte, J.C., Urier, G., Peschle, C. and Duboule, D. (1991) HOX4 genes encode transcription factors with potential auto- and cross-regulatory capacities. *EMBO J.*, **10**, 4177–4187.
- Zappavigna, V., Sartori, D. and Mavilio, F. (1994) Specificity of HOX protein function depends on DNA–protein and protein–protein interactions, both mediated by the homeo domain. *Genes Dev.*, **8**, 732–744.
- Zappavigna, V., Falciola, L., Helmer, Citterich, M., Mavilio, F. and Bianchi, E.M. (1996) HMG1 interacts with HOX proteins and enhances their DNA binding and transcriptional activation. *EMBO J.*, **15**, 4981–4991.
- Zeng, W., Andrew, D.J., Mathies, L.D., Horner, M.A. and Scott, M.P. (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.
- Zhang, M. et al. (1994) Ectopic Hoxa-1 induces rhombomere transformation in mouse hindbrain. *Development*, **120**, 2431–2442.

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