Development 127, 155-166 (2000)
printed in great britain © the company of biologists limited 2000
DEV2488

Segmental expression of *Hoxb2* in r4 requires two separate sites that integrate cooperative interactions between Prep1, Pbx and Hox proteins

E. Ferretti[1], H. Marshall[2], H. Pöpperl[2], M. Maconochie[2], R. Krumlauf[2] and F. Blasi[1,*]

[1]Molecular Genetics Unit, DIBIT, Università Vita-Salute S. Raffaele, via Olgettina 58, 20132 Milan, Italy
[2]Division of Developmental Neurobiology, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK
*Author for correspondence (e-mail: blasi.francesco@hsr.it)

Accepted 13 October; published on WWW 8 December 1999

SUMMARY

Direct auto- and cross-regulatory interactions between *Hox* genes serve to establish and maintain segmentally restricted patterns in the developing hindbrain. Rhombomere r4-specific expression of both *Hoxb1* and *Hoxb2* depends upon bipartite *cis Hox* response elements for the group 1 paralogous proteins, Hoxa1 and Hoxb1. The DNA-binding ability and selectivity of these proteins depend upon the formation of specific heterodimeric complexes with members of the PBC homeodomain protein family (*Pbx* genes). The r4 enhancers from *Hoxb1* and *Hoxb2* have the same activity, but differ with respect to the number and organisation of bipartite Pbx/Hox (PH) sites required, suggesting the intervention of other components/sequences. We report here that another family of homeodomain proteins (TALE, Three-Amino acids-Loop-Extension: Prep1, Meis, HTH), capable of dimerizing with Pbx/EXD, is involved in the mechanisms of r4-restricted expression. We show that: (1) the r4-specific *Hoxb1* and *Hoxb2* enhancers are complex elements containing separate PH and Prep/Meis (PM) sites; (2) the PM site of the *Hoxb2*, but not *Hoxb1*, enhancer is essential in vivo for r4 expression and also influences other sites of expression; (3) both PM and PH sites are required for in vitro binding of Prep1-Pbx and formation and binding of a ternary Hoxb1-Pbx1a (or 1b)-Prep1 complex. (4) A similar ternary association forms in nuclear extracts from embryonal P19 cells, but only upon retinoic acid induction. This requires synthesis of Hoxb1 and also contains Pbx with either Prep1 or Meis1. Together these findings highlight the fact that PM sites are found in close proximity to bipartite PH motifs in several Hox responsive elements shown to be important in vivo and that such sites play an essential role in potentiating regulatory activity in combination with the PH motifs.

Key words: PREP1, Pbx/EXD, Hox expression, Ternary complex

INTRODUCTION

The vertebrate hindbrain is a segmentally organized structure in which the expression and function of members of the *Hox* gene family are coupled to the cascade of segmental processes that govern hindbrain patterning (Krumlauf, 1994; Lumsden and Krumlauf, 1996). Labial-like *Hoxb1* and *Hoxa1* are essential for regulating rhombomere identity and normally function in multiple steps of segmental patterning, as shown by ectopic expression or retinoic acid (RA) induction of *Hoxa1* or *Hoxb1* genes (Marshall et al., 1992; Zhang et al., 1994; Hill et al., 1995; Alexandre et al., 1996; Bell et al., 1999), or by mutations (Carpenter et al., 1993; Goddard et al., 1996; Studer et al., 1996; Gavalas et al., 1998). Regulatory analysis in transgenic mice has begun to elucidate the mechanisms that control the r4-restricted expression of *Hoxb1* necessary for its function (Morrison, 1998). Retinoid dependent enhancers at the 3′ end of the *Hoxb1* and *Hoxa1* genes are needed to activate their initial domains of expression in the CNS (Dupé et al., 1997; Frasch et al., 1995; Marshall et al., 1994; Studer et al., 1998). This early expression in turn stimulates a broad domain of segmental expression of *Hoxb1*, through direct auto- and cross-regulatory interactions mediated by a highly conserved auto-regulatory enhancer (ARE) located 5′ of the gene (Pöpperl et al., 1995; Studer et al., 1996, 1998). This pattern then becomes progressively restricted to r4 via the action of a short-range repressor located upstream of the ARE (Studer et al., 1994). Finally, by a cross-regulatory interaction, *Hoxb1* itself then directly induces the r4-specific domain of *Hoxb2* expression (Maconochie et al., 1997).

Both the *Hoxb1* and the *Hoxb2* r4 enhancers contain bipartite Pbx/Hox binding sites (PH) essential for their in vivo activity (Maconochie et al., 1997; Pöpperl et al., 1995). The PH-site selectivity of Hoxa1 and Hoxb1 depends upon co-operative interactions with Pbx members. The *Pbx* family includes three members (*Pbx1*, *Pbx2* and *Pbx3*); moreover, *Pbx1* and *Pbx3* give raise to two alternatively spliced forms (Kamps et al., 1990, 1991; Nourse et al., 1990; Monica et al., 1993). Pbx proteins undergo co-operative, sequence-specific heterodimeric interactions with subsets of the Hox proteins (Chan et al., 1994; Pöpperl et al., 1995; Rauskolb and Wieschaus, 1994; Mann, 1995; Chang et al., 1995, 1996;

Knoepfler and Kamps, 1995; Lu and Kamps, 1996; Lu et al., 1995; Peltenburg and Murre, 1996; Phelan et al., 1995; Van Dijk et al., 1995). These interactions lead to activation of promoters containing a *Pbx* responsive element (Lu et al., 1995; Mann and Chan, 1996; Phelan et al., 1995; Di Rocco et al., 1997), and modulate Hox protein binding to slightly different target sequences (Chang et al., 1996).

Pbx and its *Drosophila* equivalent, EXD, also interact with members of another homeodomain subfamily, TALE (Three-Amino acids-Loop-Extension), which includes Prep, Meis and HTH (Burglin, 1997). The interaction with TALE-family proteins modifies both transcriptional activity and subcellular localization of Pbx/EXD. In fact, nuclear localization of EXD/Pbx requires dimerization with Hth/Prep/Meis, which prevents active export from the nucleus (Mann and Abu-Shaar, 1996; Rieckhof et al., 1997; Pai et al., 1998; Berthelsen et al., 1999; Abu-Shaar et al., 1999).

TALE/Pbx complexes bind both PH and specific Prep/Meis (PM) motifs (Chang et al., 1997; Rieckhof et al., 1997; Knoepfler et al., 1997; Berthelsen et al., 1998a). TALE/Pbx and Pbx/Hox interactions are not mutually exclusive, since they utilize different dimerization surfaces, allowing the formation of ternary Prep1/Pbx/Hoxb1 complexes in vitro on bipartite PH motifs (Knoepfler et al., 1997; Berthelsen et al., 1998a). The interaction between Pbx and Hox proteins requires both homeodomains, a stretch of 20 amino acids C-terminal to the Pbx homeodomain, and the conserved pentapeptide sequence YPWMX or a similar ANW amino acid motif N-terminal to the Hox homeodomain (Chan and Mann, 1996; Chan et al., 1996; Chang et al., 1995, 1996; Knoepfler and Kamps, 1995; Lu and Kamps, 1996; Lu et al., 1995; Mann and Chan, 1996; Peltenburg and Murre, 1996; Phelan et al., 1995; Van Dijk et al., 1995). Prep1 or Meis1 interaction with Pbx, on the other hand, requires conserved amino-terminal sequences in both proteins (Berthelsen et al., 1998a; Chang et al., 1997). Therefore, by combining with Hox and Pbx, TALE proteins may also play an in vivo role in the mechanisms that serve to establish and maintain control of r4 identity.

Despite the ability of the *Hoxb1* and *Hoxb2* enhancers to mediate similar patterns of r4-restricted expression in vivo, they differ in organization and number of bipartite PH sites. *Hoxb1* enhancer has three PH motifs embedded in a highly conserved 331bp region, all participating in r4-restricted expression (Pöpperl et al., 1995). In the case of the *Hoxb2* enhancer there are no large blocks of conservation between species and only a single PH site is present (Maconochie et al., 1997). Multimerization of each of the four single sites in vivo directs r4-restricted expression, illustrating that they all have the potential to mediate segmental expression (Maconochie et al., 1997; Pöpperl et al., 1995). Possibly, other components/sequences may be important for facilitating r4 activity in a different manner in the two enhancers. Therefore we have compared and analyzed them in more detail both in vivo and in vitro. We have found that PM binding sites are located in both enhancers in close proximity to PH sites and that in the case of *Hoxb2* the PM site is essential for enhancer activity. The combination of closely positioned but distinct PH and PM sites allows the binding of binary complexes leading to a ternary Prep1-Pbx-Hoxb1 complex. The ternary complex also forms with proteins present in extracts from embryonal carcinoma P19 cells, but only upon retinoic acid induction. Furthermore we distinguish between early and late induced ternary complexes, which also appear to include, in addition to Prep1, a higher proportion of its functional homolog Meis1. Together our findings reveal that the TALE homeodomain proteins, through interactions with Hox and Pbx proteins, play a key role in the mechanisms of r4-restricted *Hox* expression.

MATERIALS AND METHODS

Generation and analysis of transgenic mice

Transgenic embryos were generated by pronuclear injection of linearized DNA inserts into fertilized mouse eggs from an intercross of F_1 hybrids (CBA × C57Bl6) followed by transfer of the injected eggs into pseudopregnant females hosts, as previously described (Whiting et al., 1991). F_0 founder embryos were harvested at the appropriate stage of development (d.p.c., days post-coitum) and stained for *LacZ* reporter activity in whole-mount preparations according to Whiting et al. (1991).

Transgenic DNA constructs

For the *Hoxb1* constructs, both wild-type and mutant versions contained a 331 bp *Stu*I-*Hin*dIII r4 enhancer fragment from the 5′ flanking region of the locus (Pöpperl et al., 1995) inserted in the antisense orientation into the *Spe*I site of the vector BGZ40, which contains the *lacZ* gene and *SV40* poly(A) signal driven by a minimal human β-globin promoter (Yee and Rigby, 1993). Mutations in conserved block 1 containing the Prep/Meis site were generated by site-directed mutagenesis (TTTGTCA to cTctgtA, in association with a change of the adjacent TAAT to CCGG). Mutations were confirmed by sequencing.

The minimal control *Hoxb2* r4 enhancer construct contained a 181 bp *Stu*I fragment; the larger *Hoxb2* construct contained a 2.1 kb *Bam*HI-*Eco*RI fragment with both the Krox20 dependent r3/r5 and the r4-restricted enhancers from the 5′ flanking region of the locus inserted by blunt end ligation in the antisense orientation into the *Spe*I site of the BGZ40 vector (constructs 9, 10; Maconochie et al., 1997). 5′ and 3′ deletion variants of the 181 bp or *Bam*HI-*Eco*RI enhancers were made by PCR plus enzymatic digestion or generated by site-directed mutagenesis in m13 (Sculptor IVM System, Amersham), respectively. The mutation placed in the *Hoxb2* Prep/Meis site converted the CTGTCA to CTcTcA and is the same change tested in the EMSA assays, which no longer binds in vitro. Following sequencing to verify the alteration, these variants were cloned back into BGZ40 in an identical manner. For microinjection, inserts were separated from vector DNA by electrophoresis and purified using a gelase method (Epicentre Technologies).

Cell culture and extracts

P19 cells were grown in DMEM supplemented with 10% newborn calf serum (Gibco-BRL), 100 U/ml of penicillin and 100 μg/ml of streptomycin. To induce differentiation, trans-retinoic acid was added to a final concentration of 10^{-5} M. The cells were collected after 6, 12, 24, 36 and 72 hours of incubation, washed with PBS, scraped and recovered by centrifugation. Nuclear extracts were prepared as described (Berthelsen et al., 1996).

Antibodies and immunoblotting

Polyclonal rabbit antibodies against PREP1 have been described (Berthelsen et al., 1998b; Ferretti et al., 1999). Antibodies against PBX proteins were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody anti-PBX1/2/3 recognizes a common C-terminal peptide in all of the 50 kDa splice variants. The antibodies against PBX1 are reactive with PBX1A and PBX1B. Antibodies against HOXB1 are affinity-purified polyclonal rabbit antibodies, raised against a GST-HOXB1 fusion protein (Babco, USA). The

nuclear proteins were separated by 8% SDS-PAGE and blotted to PVDF membrane (Millipore).

The immunological analysis was performed with PREP1 antiserum (1:8000 dilution), PBX antibodies (1:1000), Meis1 (1:5000; a kind gift from A. M. Buchberg), or HOXB1 antibodies (1:500), using the BM Chemoluminescence kit (Boehringer-Mannheim).

In vitro transcription-translation

All pSG5 derived expression vectors were translated in vitro using the coupled TNT transcription/translation system (Promega), in the presence of [^{35}S]methionine (Amersham). Prep1 and Pbx were cotranslated (plasmids in equimolar amounts). Proteins were visualysed by SDS-PAGE followed by autoradiography.

Electrophoretic mobility shift assays

For electrophoretic mobility shift assays (EMSA) 1 μl of nuclear extract (5 μg proteins) or 2 μl of reticulocyte lysate containing the desired combinations of in vitro cotranslated proteins, were mixed with binding buffer (10 mM Tris-Cl, pH 7.5, 75 mM NaCl, 1 mM EDTA, 6% glycerol, 3 mM spermidine, 1 mM DT, 0.5 mM PMSF, 1 μg poly(dIdC), 40,000 c.p.m ^{32}P-labeled oligonucleotides and, when needed, antibody or unlabeled competitors) to a total volume of 20 μl. After 30 minutes' incubation on ice the reactions were separated by 5% PAGE in 0.5× TBE. The sequences of the oligonucleotides used in this study are shown in Fig. 3. For the analysis of dissociation rate, a 100-fold excess of unlabeled competitor oligonucleotide was added after 10 minutes of incubation with the labeled probe. Samples from the same binding reactions were taken at different times and immediately loaded on native gels.

RESULTS

Identification of a PM binding site required for r4 restricted expression of *Hoxb2*

The 181 bp *Stu*I fragment from *Hoxb2* is capable of directing r4-restricted expression in transgenic mice (Fig. 1A; and Maconochie et al., 1997) and contains a bipartite PH site (AGATTGATCG) at position 97 that is essential for enhancer activity (see Fig. 3 and Maconochie et al., 1997). Deletions 3′ of this motif had no influence on reporter expression in r4 (data not shown) while a 5′ deletion, removing the first 85 bp, completely abolished reporter expression in transgenic embryos (Fig. 1B). Sequence analysis of this region revealed the presence of a PM motif (CTGTCA) 8 bp upstream of the PH site and hence with affinity for TALE proteins (Fig. 3).

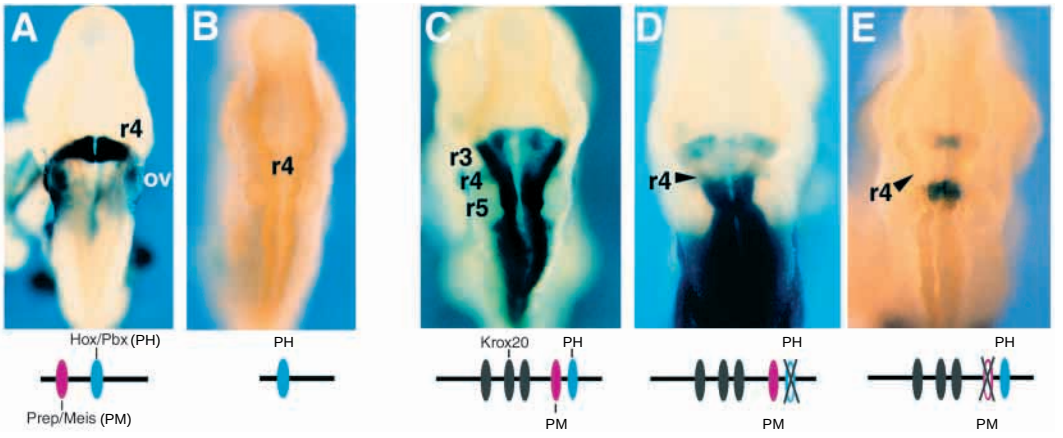

Fig. 1. PM is required in combination with PH for r4-restricted expression of *Hoxb2* enhancer. Dorsal views of *lacZ* transgene expression in the hindbrain of 9.5-9.75 d.p.c. mouse embryos. Below each panel is a diagram of the *Hoxb2* enhancer, indicating the composition of the relevant wild-type and mutant binding sites. Purple, PM; blue, the bipartite PH; gray, the three Krox20 sites. An X through a site indicates the presence of a mutation. (A) Strong r4 expression from the wild-type 181bp *Stu*I enhancer. (B) Loss of r4 expression upon deletion of the first 85bp of the 181bp *Stu*I enhancer, leaving the PH site intact. (C) Reporter staining in r3, r4, r5 and posterior regions of the neural tube and mesoderm mediated by a wild-type 2.1kb *Bam*HI-*Eco*RI *Hoxb2* enhancer. (D) Point mutations in the PH site specifically eliminate expression in r4 (arrowhead). The lower level of r3 staining in this and in (E) is due to the embryos being slightly older than in C when Krox20 dependent expression in r3 has already begun to decrease. (E) Mutations in the PM site result in a loss of transgene expression in r4 (arrowhead) and also in more posterior regions. The absence of more posterior expression, not seen with the PH site mutation, indicates that the PM site may have additional roles in *Hoxb2* expression. OV, otic vesicle.

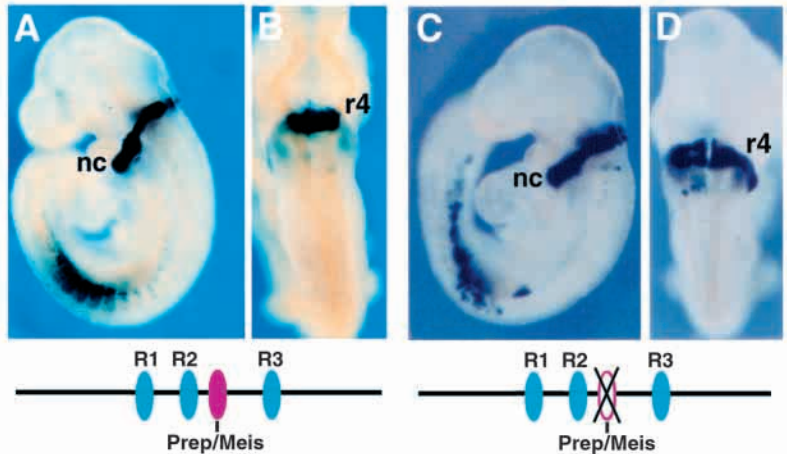

Fig. 2. A PM site is present in a *Hoxb1* enhancer but is not required for r4 expression. Lateral (A,C) and dorsal (B,D) view of a *Hoxb1 lacZ* transgene in 9.5 d.p.c. mouse embryos. Below each panel a diagram of the *Hoxb1* enhancer used indicates the composition of the relevant wild-type and mutant sites. Blue, the three bipartite PH sites (R1 to R3); purple, the PM site positioned between R2 and R3. An X indicates the presence of a mutation. (A,B) Strong r4-restricted expression is mediated by a wild-type version of a 331 bp *Stu*I enhancer from the *Hoxb1* gene. Neural crest (nc) can been seen migrating out of r4 into the second branchial arch. (C,D) Identical expression is directed by an mutated enhancer in which mutations have been introduced into the PM site. The PM site is not essential for reporter expression in r4.

To determine if this putative PM motif was required for enhancer activity in vivo, we generated a mutation in this site, converting it to CTCTTA in the context of a larger enhancer from *Hoxb2* capable of mediating a more global pattern of expression. In transgenic embryos the wild-type version of this 2.1kb *Bam*HI-*Eco*RI fragment directs segmental expression in r3, r4, r5 and more posterior regions, and mutation of the PH site specifically eliminates the r4-restricted domain of reporter staining (Fig. 1C,D; Maconochie et al., 1997). The mutation in the putative PM site, which is the same alteration tested in in vitro binding assays (Fig. 6), has an even stronger influence on transgene expression (Fig. 1E). Not only is the r4 domain abolished but many other posterior sites of reporter staining are also absent, leaving only the r3 and r5 domains dependent upon the *Krox20* enhancer in this construct (Sham et al., 1993; Vesque et al., 1996) (Fig. 1E). Thus both the bipartite PH and the PM sites are essential for r4 regulatory activity of the *Hoxb2* enhancer in vivo. Furthermore, it appears that factors interacting with the PM site exert additional influences on *Hoxb2* independent of the PH site.

A PM site located in the Hoxb1 r4 enhancer is not essential for activity

The examination of the *Hoxb1* r4 enhancer showed, in addition to three bipartite PH sites (repeats R1-R3) in the highly conserved 331 bp *Stu*I r4 enhancer (Pöpperl et al., 1995), a single putative PM motif (TTGTCA) 7 bp downstream of the R2 site and 17 bp upstream of R3. To determine its role in r4 expression we generated mutations in the site (cTctgtA) and assayed for activity in transgenic embryos. The wild-type *Stu*I enhancer from *Hoxb1* directs strong staining in r4 and neural crest, migrating from this segment into the second branchial arch, in addition to a small posterior domain in the tail (Fig. 2A,B; Pöpperl et al., 1995). An identical expression pattern is also observed with the construct with mutated PM motif (Fig. 2C,D), indicating that in contrast to *Hoxb2* this site is not essential for r4-restricted enhancer activity from *Hoxb1*. This does not preclude this site from influencing relative levels of expression, which would not be detected by our reporter assay. Nevertheless, putative PM sites are found in close proximity to PH sites and, in some cases, are essential for potentiating in vivo activity in transgenic embryos.

The PM sites of the *Hoxb1* and *Hoxb2* enhancers support in vitro multimeric complex association

We next examined the in vitro binding properties of the putative PM site. In addition, since Prep/Meis/HTH proteins interact with Pbx/EXD and affect their function (Chang et al., 1997; Knoepfler et al., 1997a; Berthelsen et al., 1998a, 1999; Abu-Shaar et al., 1999), we also investigated whether PM and PH sites would synergize in in vitro binding assays.

The DNA sequences of *Hoxb1* and *Hoxb2* r4 enhancers, including their PM and PH sites, are shown in Fig. 3, together with the sequences of nucleotides used in the following experiments.

In the experiment shown in Fig. 4, in vitro translated Prep1, Pbx1a and Hoxb1 proteins were tested for binding the b1-R3 and b1-AR2 oligonucleotides containing, respectively, the PH and PH+PM binding sites shown in Fig. 3. When all three proteins were present, the b1-R3 oligonucleotide bound two distinct heterodimers corresponding to Hoxb1/Pbx1a and Prep1-Pbx1a. A weak, slower migrating band (ternary complex, see below) was observed when Prep1 homeodomain was deleted (ΔHDPrep1). The b1AR2 oligonucleotide, containing PH and PM sites, not only bound a Prep1-Pbx1a heterodimer but also a slower migrating, possibly ternary Prep1-Pbx1a/Hoxb1

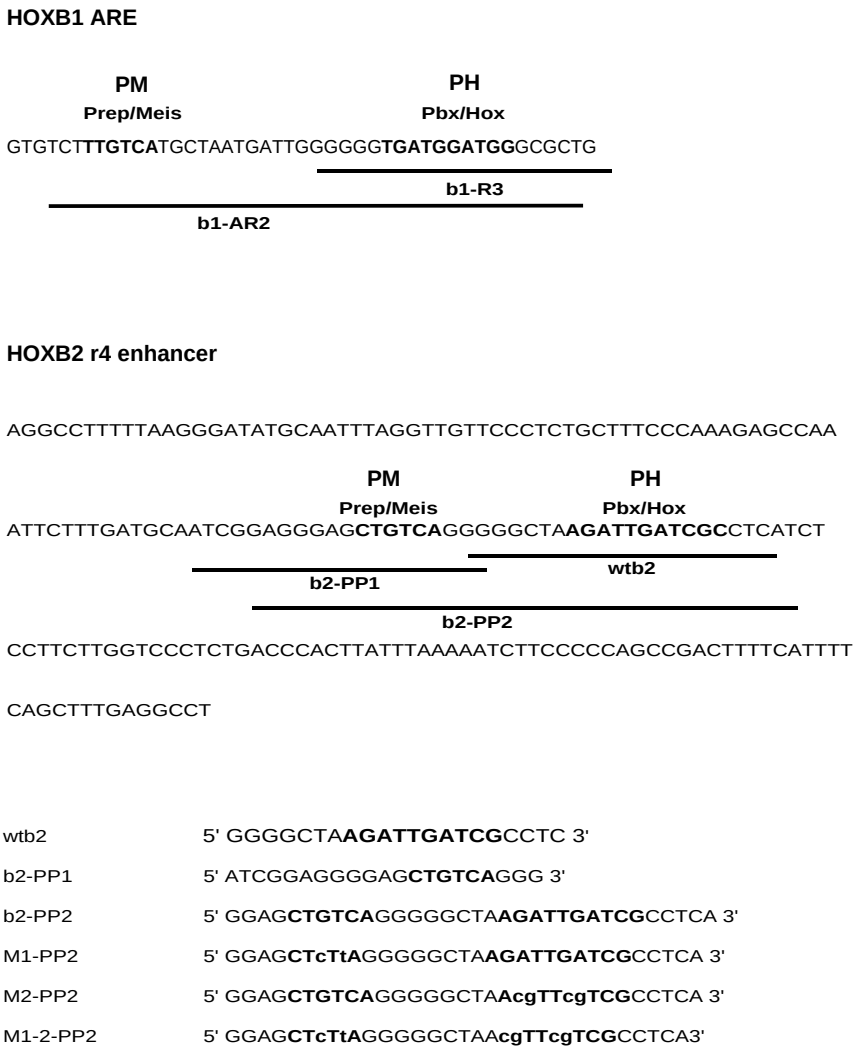

Fig. 3. The DNA sequences of *Hoxb1* and *Hoxb2* r4 enhancer contain a combined PH-PM element. (Top) The DNA sequence of the *Hoxb1* ARE, containing a PM and a PH site. The sequences underlined correpond to the oligonucleotides employed in the EMSA experiments (symbols indicated below the line). (Middle) The DNA sequence of the *Hoxb2* r4 enhancer with its PM and PH sites. The sequence of the oligonucleotides wtb2, b2-PP1 and b2-PP2 is underlined. Their sequence is repeated at the bottom, along with the mutants used for EMSA.

complex. Deletion of Prep1 homeodomain prevented ternary complex formation, as previously observed (Berthelsen et al., 1998a).

We next investigated the *Hoxb2* r4 enhancer (see Fig. 3, for sequences and oligonucleotides). The PH motif alone (wtb2), bound both Pbx1a-Hoxb1 and Prep1-Pbx1a dimers (Fig. 5A). Using all three proteins, Prep1, Pbx1A and Hoxb1, we saw no additional slower migrating band. As in b1AR2, Prep1 homeodomain deletion (ΔHDPrep1, Fig. 5A), induced an additional, weaker and slower migrating band.

The PM motif alone (b2-PP1 oligonucleotide) did not bind Prep1-Pbx1a, Pbx1a-Hoxb1 or Prep1-Pbx1a-Hoxb1 mixtures (Fig. 5B). The O1 control oligonucleotide, which comprises a PM site (Berthelsen et al., 1996, 1998b), did however bind Prep1-Pbx complexes. This result emphasizes the importance of the sequence context for these interactions. With both PM and PH motifs (b2-PP2) Prep1-Pbx1a and Pbx1a-Hoxb1 mixtures produced a shift with a distinguishable migration rate. However, with all three proteins, a major, additional slower migrating band was observed (Fig. 5B). A second minor band comigrating with the dimeric complexes (Prep1-Pbx1a and Pbx1a/Hoxb1) was still visible. The deletion of the Prep1 homeodomain (ΔHD) negatively affected slower complex formation.

The slower migrating band observed with b2-PP2 was tested with specific antibodies. Pbx1a-Hoxb1 complex was inhibited by anti-Pbx and abolished by anti-Hoxb1 antibodies. Prep1-Pbx1a complex was inhibited by anti-Pbx and prevented altogether by anti-Prep1 antibodies. With all three proteins, Pbx1a, Hoxb1 and Prep1, anti-Prep1 and anti-Hoxb1 antibodies completely inhibited formation of the slower band (Fig. 5C). The faster bands (dimeric complexes) were prevented by anti-Pbx and only partially by anti-Prep and anti-Hoxb1 antibodies, suggesting that it is composed of both Pbx1a/Prep1 and Pbx1a/Hoxb1 heterodimers. The anti-Pbx antibodies, although in general less effective in this type of experiment, confirmed the presence of Pbx1a in both the slower and faster migrating bands. In conclusion, the slower migrating band contains Pbx1a, Prep1 and Hoxb1.

Prep1 confers higher stability to the multimeric complex

We then probed the two sites by mutational analysis (sequences in Fig. 3). Oligonucleotide M1-PP2, wild type PH and mutated PM (same mutation tested in transgenic analysis), bound both Prep1-Pbx1a and Pbx1a/Hoxb1 combinations but formed no slower migrating complex with the three proteins (Fig. 6A). Mutation of the PH site alone (M2-PP2) prevented all complex formation, as did the combination of the two (M1-2-PP2) (Fig. 6A).

Competition assays (Fig. 6B) showed that a mutated PM (M1-PP2) still competed for complex formation, although less efficiently, while mutated PH site (M2-PP2 and M1-2-PP2) no longer competed for binding. These data indicate that both sites synergize in multimeric complex formation, with the PH site representing the major binding site for the dimeric complexes.

We next compared the dissociation rate of the various complexes. Complexes were assembled by incubating labeled b2-PP2 with the various protein mixtures for 10 minutes on ice. Following addition of a 100-fold excess of the same unlabeled probe as competitor, samples were withdrawn at various times and analyzed by EMSA (Fig. 7). Prep1-Pbx1a complexes dissociated with a half-life of about 20 minutes but Pbx1-Hoxb1 and the slower migrating complex were much more stable.

The combined PH and PM sites mediate a ternary Prep1-Pbx1A-Hoxb1, and not a tetrameric, complex

The migration properties of the slower migrating complex on combined PH-PM sites are compatible with a stoichiometry of either two dimeric complexes, or of a ternary Prep-Pbx-Hox complex. To differentiate between the two possibilities (Fig. 8A), we used two alternative splicing forms of Pbx, Pbx1a and Pbx1b, with different C-terminal extensions, resulting in a 5 kDa variation in apparent molecular mass but both binding Prep1 and Hoxb1 (see below). In the presence of all four proteins, Prep1, Hoxb1, Pbx1a and Pbx1b, in the case of

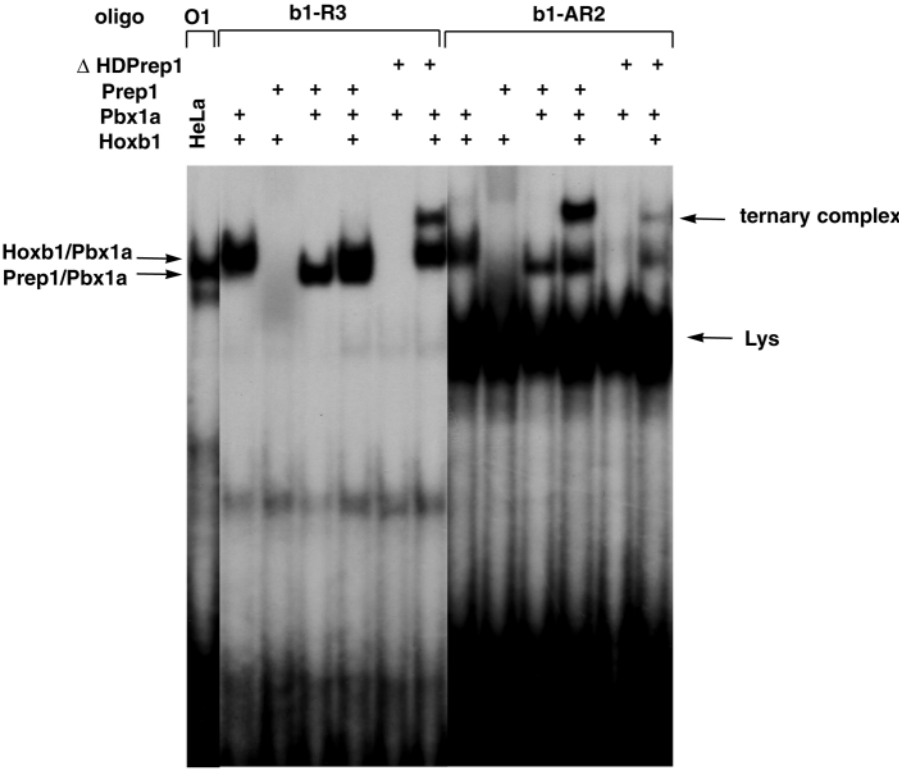

Fig. 4. Formation of a slower migrating DNA-binding complex on *Hoxb1* r4 enhancer in the presence of Prep1, Pbx1a and Hoxb1 requires both PM and PH sites. EMSA analysis of in vitro translated combinations of Prep1, Pbx1a, Hoxb1 and ΔHDPrep1, as indicated. The labeled DNA target is shown on the top (see sequences in Fig. 3). The reculocyte lysate contains a non-specific endogenous activity, marked by Lys. The first lane shows the binding of HeLa nuclear extracts to the O1 oligonucleotide containing only a PM binding site (Berthelsen et al., 1996). The arrow to the right shows a slower migrating complex produced with the Prep1, Pbx1a and Hoxb1 combination.

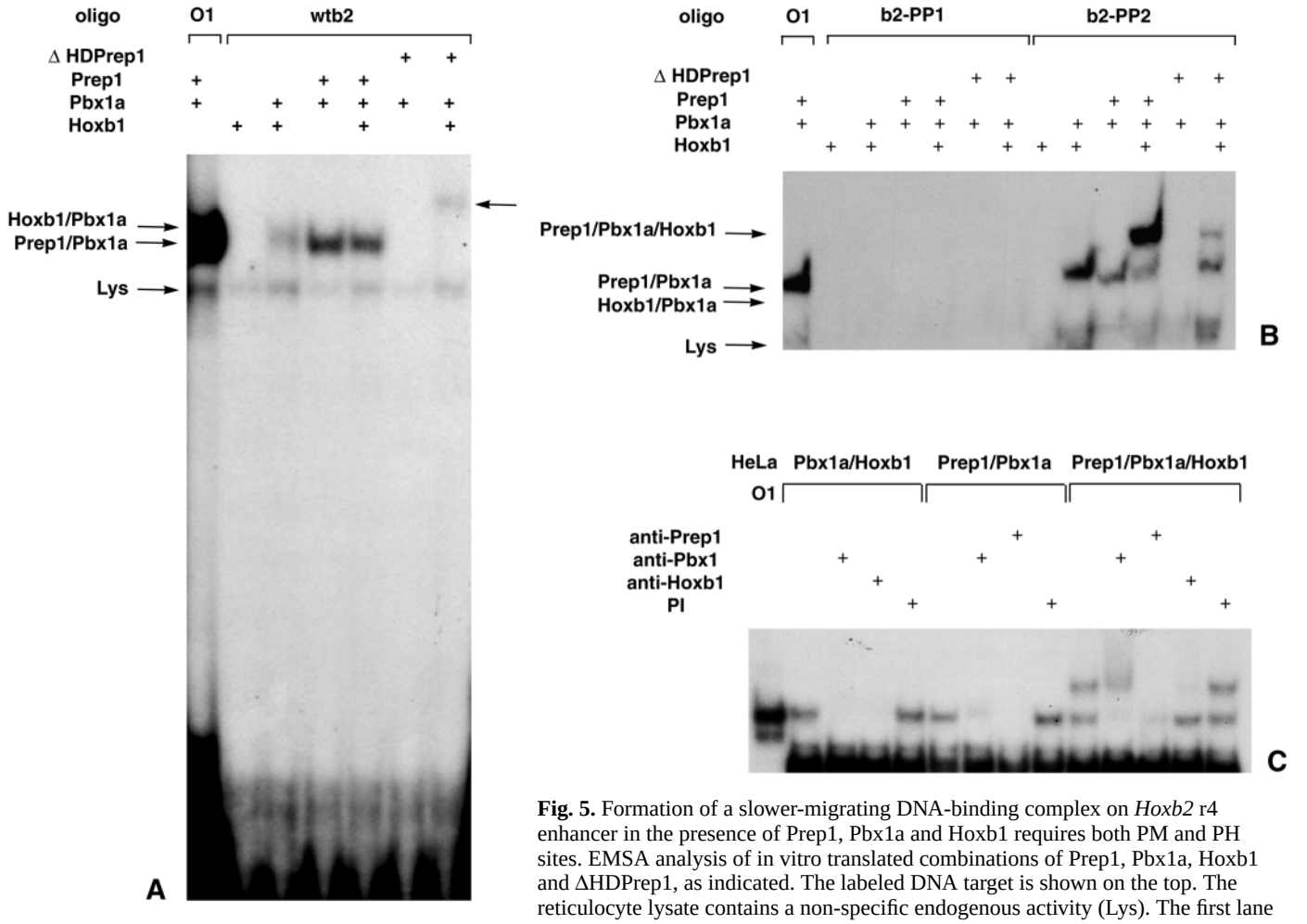

Fig. 5. Formation of a slower-migrating DNA-binding complex on *Hoxb2* r4 enhancer in the presence of Prep1, Pbx1a and Hoxb1 requires both PM and PH sites. EMSA analysis of in vitro translated combinations of Prep1, Pbx1a, Hoxb1 and ΔHDPrep1, as indicated. The labeled DNA target is shown on the top. The reticulocyte lysate contains a non-specific endogenous activity (Lys). The first lane of all panels shows the binding of in vitro cotranslated Prep1-Pbx1a (indicated to the left) to the O1 oligonucleotide. Oligonucleotide sequences are shown in Fig. 3. (A) Oligonucleotide wtb2, containing the PH site of the r4 *Hoxb2* enhancer, binds both Pbx1a-Hoxb1 and Prep1-Pbx1a heterodimers. The arrow to the right shows a weak, slower migrating complex produced only with the ΔHDPrep1, Pbx1a and Hoxb1 combination. (B) Right: b2-PP2 oligonucleotide, containing both PH and PM binding sites of the r4 *Hoxb2* enhancer. Prep1 interacts with Hoxb1-Pbx1a complex, forming a multimeric complex. Left: no DNA-binding activity is detected with the b2-PP1 oligonucleotide, containing only the PM binding site. (C) Effect of specific antibodies on the DNA-binding activity of the indicated protein mixtures: the labeled DNA target was b2-PP2 containing both PM and PH sites. The slower migrating DNA-binding activity is a multimeric complex containing Prep1, Pbx1a and Hoxb1.

tetrameric complexes we should observe three bands, while a ternary complex would form only two bands. Indeed, we observed only two slower migrating bands, showing it to be a ternary Prep1-Pbx1-Hoxb1 complex (Fig. 8B). Other controls showed that Prep1 did not bind Hoxb1 (data not shown), and that the homeodomain of Prep1 was required for ternary complex formation.

Nuclear extracts of P19 cells contain constitutive Pbx-Prep1 and are induced by retinoic-acid to form Prep1-Pbx-Hoxb1 complexes

In vivo expression of *Hoxb2* in r4 is induced by retinoic acid (RA) (Marshall et al., 1992); likewise, in cultured embryonic carcinoma cells RA induces *Hox* (Simeone et al., 1990) and Pbx expression (Knoepfler and Kamps, 1997b). The PH-PM (b2-PP2) oligonucleotide formed one retarded band with extracts from untreated cells (Fig. 9A, lane C) comigrating with that of HeLa extracts with O1 oligonucleotide (Prep1-Pbx1a heterodimers). Thus Prep1-Pbx heterodimers are present in uninduced P19 cells. Upon induction with 10 μM all-trans retinoic acid, Prep1-Pbx dimers decreased and a very strong, broad, slower migrating band(s), became visible with the b2-PP2 target. Slower migrating complexes were visible already after 6 hours induction.

We also characterized the putative ternary Prep1-Pbx1a/b-Hoxb1 complexes by immunoblotting (Fig. 9B). Prep1 was present in uninduced extracts and slightly increased upon induction. Pbx was present in uninduced extracts and increased upon RA induction. Pbx antibodies were specific only for the 50 kDa forms, and therefore detect one band. Meis1 antibodies detected two very weak bands in uninduced extracts, which strongly increased after 12 hours' induction (most likely Meis1a and 1b; Moskow et al., 1995; Jacobs et al., 1999) as Prep1 antibodies do not cross-react with Meis. Hoxb1 was absent in uninduced cells, but was induced at 6 hours and strongly at 12 hours. Thus the slower migrating DNA-binding

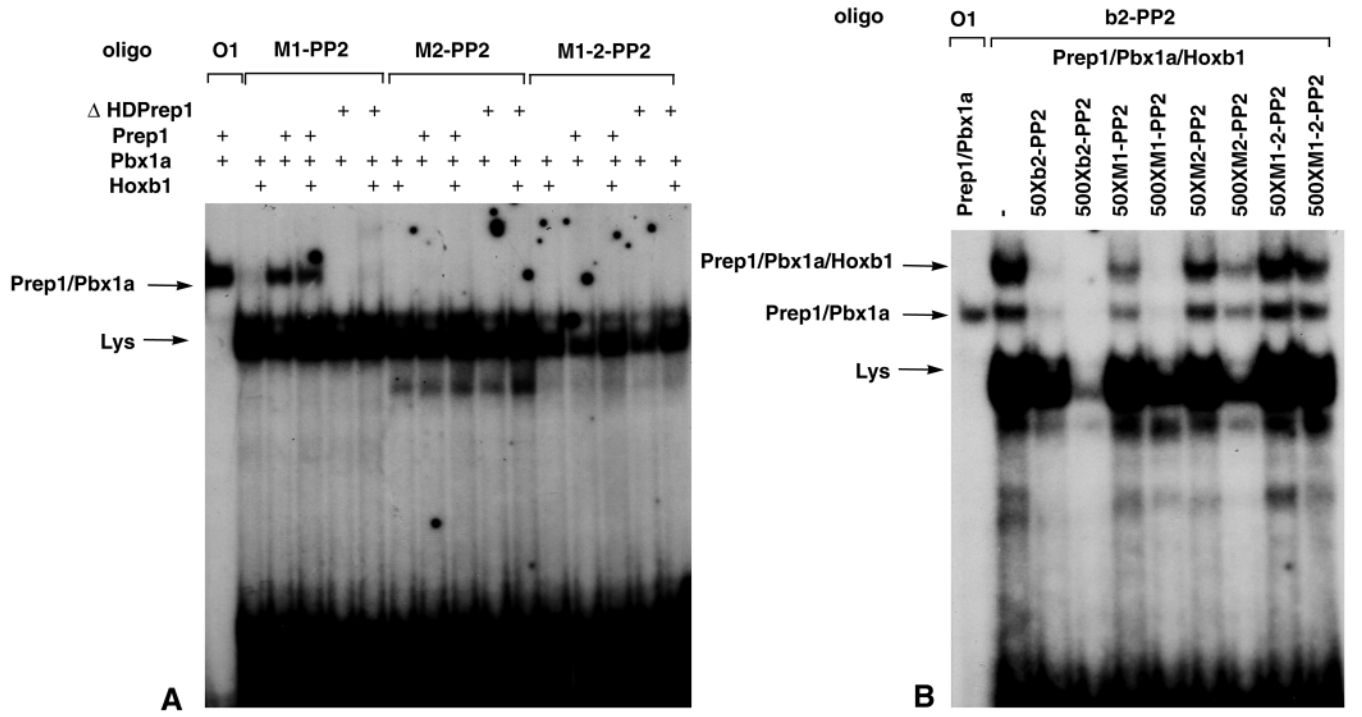

Fig. 6. Both PM and PH sites of the r4 *Hoxb2* enhancer are essential for multimeric complex formation. Prep1, Pbx1a, Hoxb1 and ΔHDPrep1 were mixed in various combinations with labeled wild-type or mutated b2-PP2 oligonucleotide (sequences in Fig. 3). Lys indicates an endogenous binding activity present in the reticulocyte lysate. The first lane is a control migration of Prep1-Pbx1a bound to oligonucleotide O1. (A) EMSA using labeled mutated oligonucleotides as probes (indicated on top). (B) EMSA with a Prep1, Pbx1a, Hoxb1 mixture using labeled b2-PP2 oligonucleotide as a probe and excess unlabeled wild-type or mutant oligonucleotides as indicated at 50- or 500-fold molar excess – indicates absence of competitor.

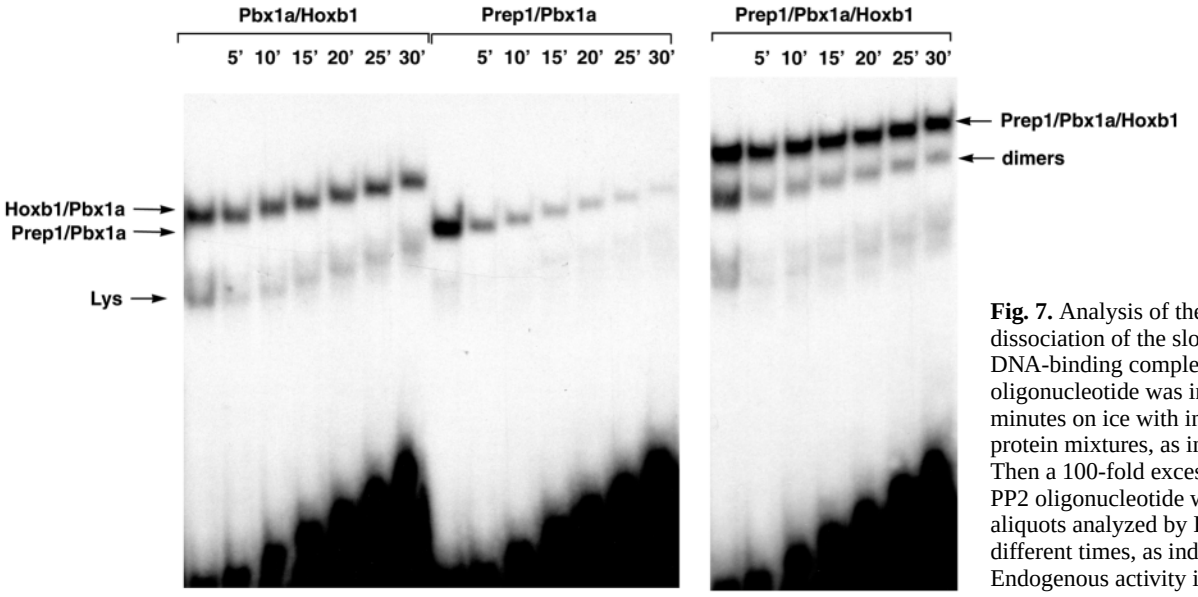

Fig. 7. Analysis of the rate of dissociation of the slower migrating DNA-binding complex. Labeled b2-PP2 oligonucleotide was incubated for 10 minutes on ice with in vitro-translated protein mixtures, as indicated on top. Then a 100-fold excess of unlabeled b2-PP2 oligonucleotide was added and aliquots analyzed by EMSA after different times, as indicated. Endogenous activity is indicated (Lys).

complex appears when Hoxb1 is induced. Moreover, at 6 hours the ternary complex contains Pbx1a, Hoxb1 and Prep1; at later times Meis1 may substitute or supplement Prep1.

Binding specificity of the 6 hour-induced P19 extracts with labeled b2-PP2 was studied by competition (Fig. 9C). Wild-type b2-PP2 totally competed at a 50-fold excess; mutant PM (M1-PP2) competed well for Prep1-Pbx in a 50× excess but only weakly for the slower migrating complexes at a 500-fold excess. Mutant PH (M2-PP2) weakly competed for both dimeric and multimeric forms but only at a 500-fold excess, while double mutant (M12-PP2) did not compete. Thus, the induced P19 cell complex reproduced the sequence specificity of in vitro-translated proteins (compare Figs 6B and 9C).

Both bands in uninduced extracts were inhibited by Prep-1

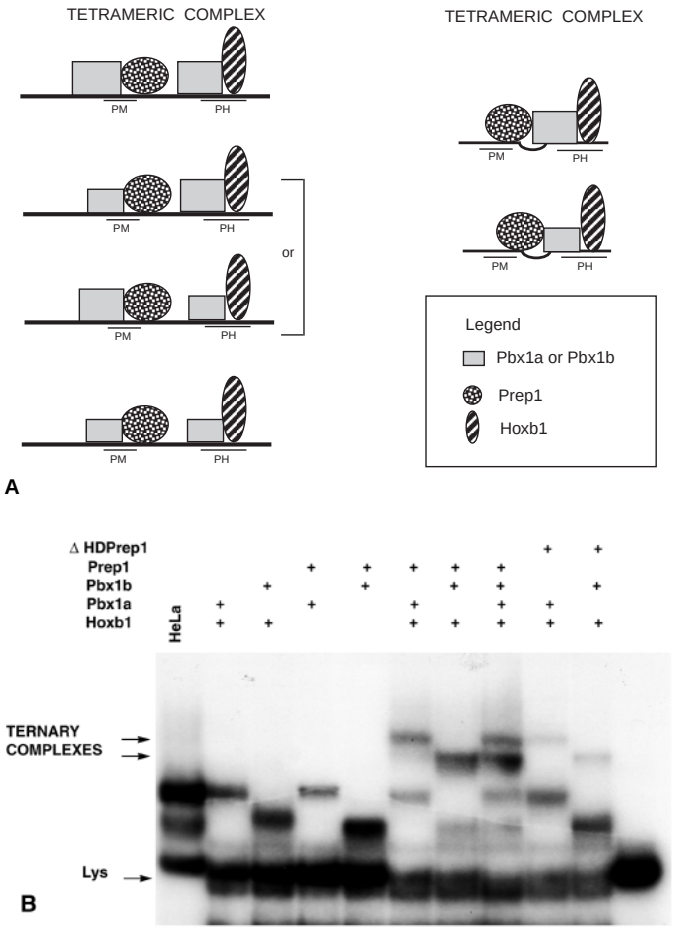

Fig. 8. The slower migrating DNA-binding activity is a ternary Prep1-Pbx1-Hoxb1 complex. (A) The scheme of the experiment and the possible results. The slower migrating multimeric complex can be either a Prep-Pbx-Hox ternary complex (right) or two heterodimers, Prep1-Pbx and Pbx-Hoxb1 bound to the same DNA molecule (left). Pbx1a and Pbx1b can both bind Prep1 and Hoxb1, but differ in their molecular mass. A binding experiment with a mixture of in vitro-translated Prep1, Hoxb1, Pbx1a and Pbx1b will distinguish between ternary and tetrameric complexes (see text). (B) EMSA with different combinations of in vitro-translated Hoxb1, Prep1, ΔHDPrep1, Pbx1a and Pbx1b and labeled b2-PP2 oligonucleotide. HeLa nuclear extract and labeled O1 oligonucleotide (lane 1) mark Prep1-Pbx1a and Prep1-Pbx1b complexes (Berthelsen et al., 1998b). The protein composition of each binding reaction is shown on top. The migration of the two ternary complexes is indicated by arrows.

and Pbx antibodies, indicating the presence of both Prep1 and a Pbx family member in the two complexes (Fig. 10A). With induced cell extracts (Fig. 10B), the band comigrating with Prep1-Pbx1a was inhibited by anti-Prep1 and anti-Pbx antibodies. The slower migrating band was specifically, but partially, inhibited by anti-Prep1, anti-Pbx and anti-Hoxb1 antibodies. The shifts were totally inhibited in the presence of all three antibodies. The different quantitative effect of the antibodies on dimeric versus trimeric complexes suggests differential exposure of surface epitopes. The data also positively identify Prep1 as one of the components of the DNA-binding ternary complex.

DISCUSSION

In this study we have identified *cis* Prep/Meis (PM) elements near bipartite PH sites in *Hoxb1* and *Hoxb2* r4 enhancers and find that TALE homeodomain proteins increase selectivity of Pbx-Hoxb1 complexes at those sites. Co-operative interactions between TALE, Pbx and Hox proteins are important in orchestrating binding of multimeric complexes to the combined PH-PM sites both in vitro and in cell culture. This is relevant and essential for r4-restricted expression of *Hoxb2* in the developing hindbrain and might also contribute to aspects of *Hoxb1* expression in r4. The identification and analysis of other ternary complex responsive sequences will allow the identification of other in vivo Hox targets. This raises a number of important questions with respect to *Hox* regulation and specificity.

Differences between the *Hoxb2* and *Hoxb1* r4 enhancers

The presence of both PH and PM sites in r4 enhancers from two *Hox* genes suggests that it might be a common feature of many *Hox* response elements. We were able to demonstrate an essential in vivo requirement only for the PM site in *Hoxb2*. Similar data has also just been reported indicating a role for Meis1-Pbx-Hox complexes in segmental expression of *Hoxb2* (Jacobs et al., 1999). This does not exclude a role for the PM site in r4 expression of the *Hoxb1* enhancer. *Hoxb1* has three bipartite PH sites (R1-R3), and reporter expression in r4 was still detected when any one or two of the sites were mutated, although the levels were often much lower in double mutant combinations (Pöpperl et al., 1995). This demonstrated that all three PH sites in *Hoxb1* contributed to restricted expression, which was in agreement with the finding that multimerized versions of each site also mediated r4-restricted expression (Pöpperl et al., 1995; one might have to consider whether multimerized sites reproduce a PM-PH situation). Hence, in the case of *Hoxb1*, a PM site mutation might not eliminate r4 expression due to synergy between the three PH motifs. However, the PM site might still facilitate ternary complex formation with one of the three PH sites and influence relative levels of expression important in vivo for function. In contrast, the *Hoxb2* enhancer has only a single PH-PM site and altering either component abolishes activity. Thus, *Hox* target sites may vary in both number and arrangement of PH and PM sites and additional copies of either of these might serve in a redundant manner to ensure or reinforce levels of expression and spatial restrictions.

It is interesting that in the present study the PM site of the *Hoxb2* enhancer (b2-PP1) on its own was not capable of binding any of Prep1, Pbx (Fig. 5B). This is at variance with other PM sites, for example that of the human *urokinase* enhancer, which is capable of binding Prep1-Pbx heterodimers as determined by methylation interference analysis (Berthelsen et al., 1996). Since the minimum DNA sequence responsible for Prep-Pbx binding in *urokinase* and *Hoxb2* sequences is identical (TGACAG), the binding must be facilitated by adjoining bases present in the *urokinase* sequence and missing in the *Hoxb2* sequence. This point deserves deeper attention and the role of the PM motifs present in the genome in the absence of PH sites, like that in the enhancer of the *urokinase, IL-3* and *Str-1* genes (Berthelsen et

al., 1996), may have to be re-interpreted. Furthermore, it is intriguing that a mutation in the *Hoxb2* PM site has a more global effect (Fig. 1E), as it results in the loss of expression not only in r4 but also in posterior domains, an effect that is not seen with mutations in the PH site. This suggests that the PM site, either alone or in combination with other sequences in the 2.1 kb *Hoxb2* *Bam*HI-*Eco*RI, may contribute to expression independent of the PH sites. It would be useful to identify these sequences to understand the properties and target sites of TALE activity.

Formation of a ternary complex on Hox target sites

We have previously shown that one of the PH motifs (R3) in the *Hoxb1* autoregulatory enhancer bound a ternary Prep1-Pbx1-Hoxb1 complex (Berthelsen et al., 1998a). However, in this case deletion of Prep1 homeodomain increased in vitro binding of the multimeric complex as well transactivation in transfection assays. How can active ternary complexes form on such sites in vivo with full-length proteins? This study has expanded our information on the nature of *Hoxb1*-responsive sites by showing the requirement of combined PH-PM motifs in ternary complex formation. The spatial arrangement of these separate sites in close proximity allows interactions of all three homeodomains with DNA, leading to the formation of active ternary complexes (Fig. 11D). In fact, the PH motif alone is able to bind both Pbx-Hoxb1 and Prep1-Pbx complexes, while formation of a ternary complex requires a separate PM motif. The effect of Prep1 homeodomain deletion distinguishes between separated (PH+PM) and overlapping (PH only, like in R3 ARE) sites. In the former, deletion is deleterious; in the latter it is advantageous. In fact, on the single PH site, Pbx-Prep1 might even decrease Hoxb1 binding to DNA as the Pbx-Hoxb1 complex requires DNA (Fig. 11A).

The combined PM-PH sites of the *Hoxb2* and *Hoxb1* enhancers are spaced by 8 and 17 bp, respectively (Fig. 3). Thus the Prep1 homeodomain of the ternary complexes, firmly bound to the PH site, can contact sequences which differ by almost one complete turn of the helix, viewing the DNA always from the same side. The permissivity of the different spacing is best explained by looping-out or bending of the intermediate DNA (Fig. 8A) or by a flexible Prep1 homeodomain differentially extending in the two cases. We hypothesize that the ternary complex forms from a pre-existing Pbx-Prep dimer bound to the PH site (Fig. 11A,C). Recruitment of Hoxb1 by Pbx might displace Prep1 homeodomain, inducing it to contact the adjacent PM (Fig. 11D). The specific spacing requirements will have to be addressed in greater detail in the future.

Why is a ternary complex necessary?

It is not clear why a ternary complex is required for *Hoxb2* expression. Prep1 does not appear to have a transactivation domain, yet its presence in the ternary complex increases transcription (Berthelsen et al., 1998a). Dimerization of Pbx

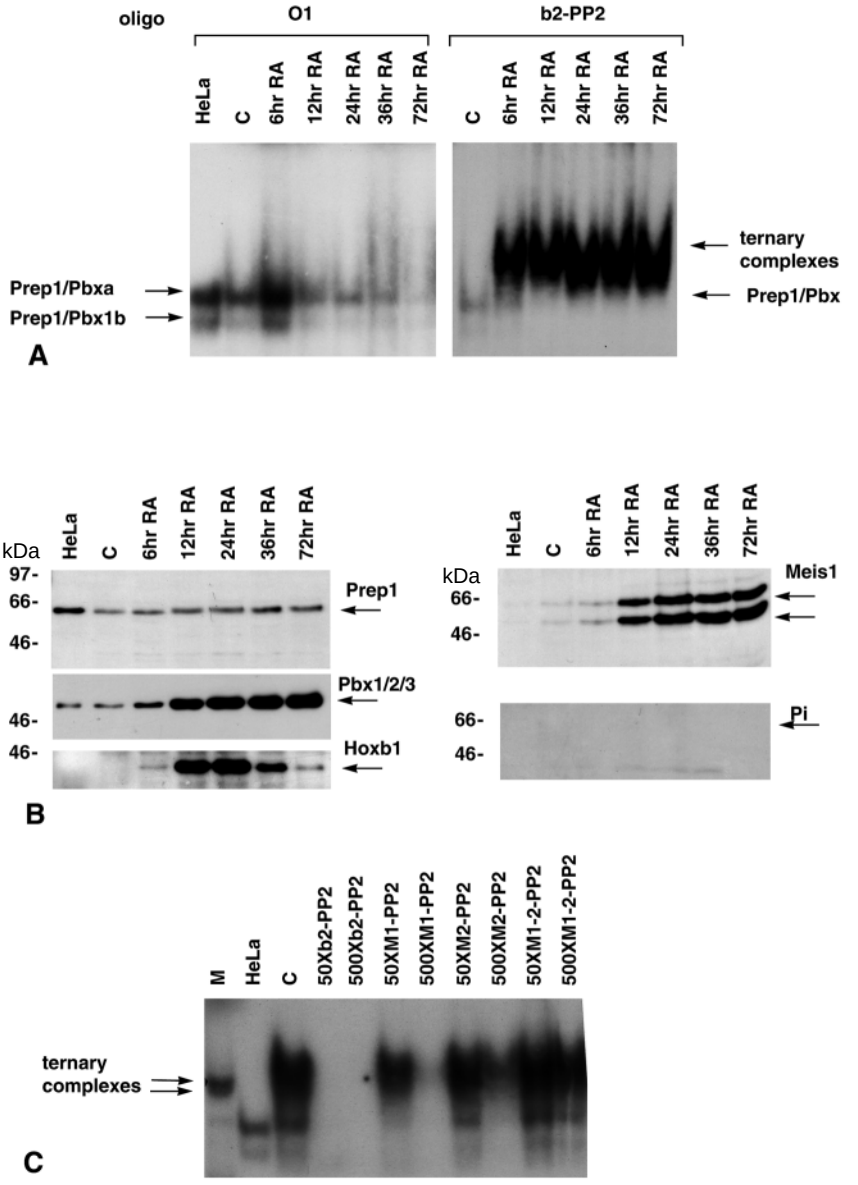

Fig. 9. A slower migrating complex binding the r4 *Hoxb2* enhancer is induced in P19 cells only by retinoic acid. Time course analysis of nuclear extracts prepared from control and induced P19 cells at different times after the addition of 10 μM all-trans retinoic acid (RA). Lanes C indicate extracts of uninduced cells. (A) EMSA with labeled O1 or b2-PP2 oligonucleotides. A slower migrating band binding to b2-PP2, but not to O1, is induced by RA. The O1-binding Prep1-Pbx complexes are increased at 6 hours and decrease thereafter. (B) Immunoblotting analysis (see Materials and Methods) of nuclear extracts from control (C) or RA-induced (0-72 hours) P19 cells. Prep1, Pbx1, Meis1 and Hoxb1 are induced by RA. (C) The RA-induced slower migrating complex has the same binding specificity as the in vitro-formed ternary Prep1-Pbx1-Hoxb1 complex. Binding-competition experiments with labeled b2-PP2 and unlabeled wild-type and mutated (50- and 500-fold excess) b2-PP2 oligonucleotides (sequence in Fig. 3). Extracts from 6 hour, 10 μM RA-induced cells. The first two lanes show the migration of control complexes: in vitro-formed ternary complex binding to b2-PP2 (M) and HeLa nuclear extract bound to the O1 oligonucleotide (HeLa). Lane C contains no competitor.

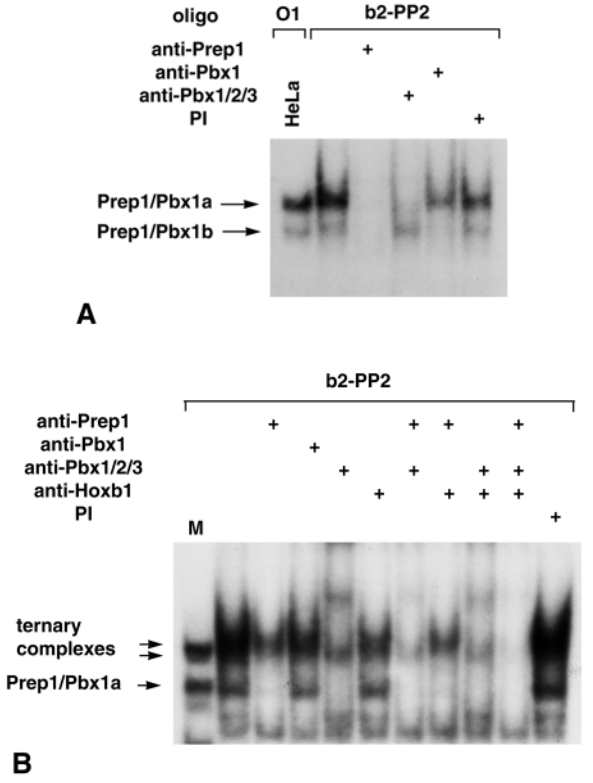

Fig. 10. The RA-induced slower migrating complex in P19 cells is a ternary complex containing Prep1, Pbx and Hoxb1. EMSA analysis with labeled b2-PP2 in the presence of specific antibodies as indicated. Lane 'HeLa' shows HeLa nuclear extracts incubated with O1 oligonucleotide; lane M, in vitro-translated Prep1-Pbx1a-Pbx1b-Hoxb1 mixture and labeled b2-PP2 oligonucleotides. The antibodies employed are indicated on top. PI, preimmune serum; anti-Pbx1/2/3 antibodies recognize C termini of all 50 kDa Pbx forms. Anti-Pbx1, anti-Hoxb1 and anti-Prep1 antibodies specifically recognize these proteins. (A) EMSA with nuclear extracts of uninduced P19 cells. (B) EMSA with nuclear extracts from P19 cells treated for 6 hours with 10 μM all-trans retinoic acid. The combination of anti-Prep1, anti-Pbx1/2/3 and anti-Hoxb1 antibodies inhibits all binding.

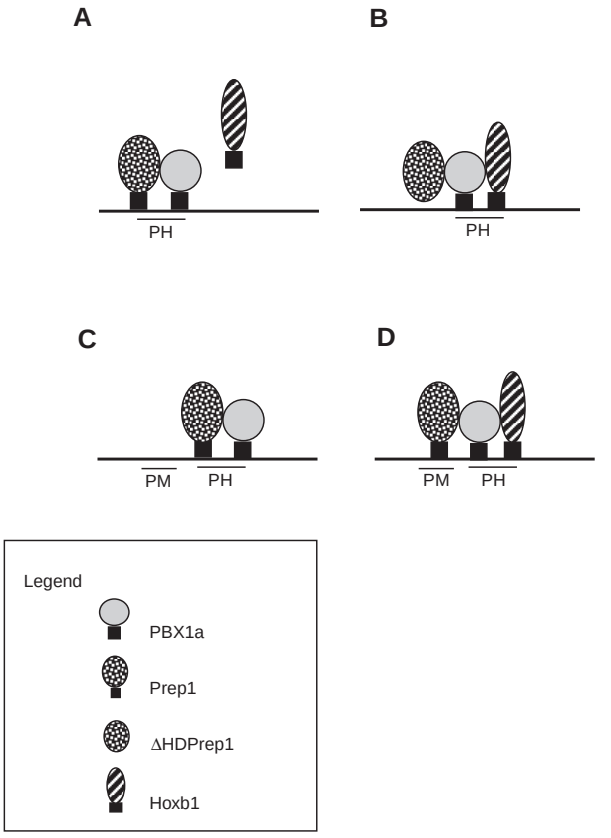

Fig. 11. Ternary complex formation on PH versus combined PM-PH sites in *Hoxb1* and *Hoxb2* enhancers. (A,B) Preformed Prep-Pbx complex binds the PH site. Since Hoxb1 does not bind Pbx in the absence of DNA, Prep-Pbx may at least in part hinder formation of Pbx-Hoxb1 complex on the PH site (A). In the experimental condition in which Prep1 is missing the homeodomain, a ternary complex can form (B). Note, however, that only two DNA binding domains are involved. (C,D) In the case of the combined PM-PH sites of the *Hoxb1* and *Hoxb2* enhancers, a preformed Prep-Pbx complex will bind to the PH site preferentially (A). Hoxb1 may displace the DNA binding domain of Prep1 to the PM site, and will form a dimeric complex with Pbx on the PH site (B). Notice that in this case three homeodomains are bound to DNA.

and Prep1 prevents its active export from the nucleus (Berthelsen et al., 1999; Abu-Shaar et al., 1999). Hence the nuclear presence of Pbx-Prep dimers will increase the chance of Hoxb1 'activation'.

Hox regulation and RA

RA treatment causes an anterior shift in both *Hoxa1* and *Hoxb1* expression (Conlon and Rossant, 1992; Marshall et al., 1994), resulting in reprogramming hindbrain development. Mutations in the PH site abolish the RA response of the *Hoxb2* gene, indirectly mediated by *Hoxb1* (Maconochie et al., 1997). Hence the bipartite PH motif of the *Hoxb2* r4 enhancer is required not only for r4-restricted expression but also for RA response. Using a combined PH-PM target (oligonucleotide b2-PP2) we find that ternary complex formation is induced by RA in embryonal carcinoma P19 cells. RA is known to induce Hoxb1, Meis and Pbx in P19 cells. We found that Prep1 and lower levels of Meis 1a/b are present in the nuclei of uninduced and RA induced P19 cells. The level of Prep1 was not drastically changed by the induction, while that of Meis1a/b was very low in uninduced and 6 hour-induced cells but strongly increased after 12 hours, i.e. after the induction of Hoxb1. The time course of induction indicates that Prep1 is a major constitutive, DNA-binding, TALE-class protein directly participating in ternary complexes, at least in uninduced and 6 hour-induced nuclear extracts (Fig. 10B). Indeed in ectopic expression studies in vivo, Prep1 and Meis1 are not the limiting reagents that restrict the expression and induction of the *Hoxb1* and and *Hoxb2* r4 enhancers (S. Nonchev, E. Ferretti and R. Krumlauf, data not shown). The participation of Prep1 in *Hoxb2* expression in vivo would be in agreement with the presence of *Prep1* throughout the hindbrain at the critical developmental stages (Ferretti et al., 1999).

In conclusion, this work highlights the key role that interactions between TALE, Pbx and Hox proteins play in integrating the information required for spatial restriction of *Hox* expression and response on complex target sites. They add to

our understanding of the specificity of Hox interactions with DNA and enhance our ability to evaluate and search for potential *Hox* target sites and genes in the *Hox* cascade of AP patterning.

The authors are especially grateful to Jens Berthelsen for starting this whole project, and to Valérie Ferreira, Tim Jinks, Richard Mann, Miguel Manzanares, Fulvio Mavilio, Stefan Nonchev, Martina Palazzolo and Vincenzo Zappavigna for many stimulating discussions. We thank Amanda Hewett, Peter Mealyer and Rosemary Murphy for help in animal husbandry. Dr Arthur M. Buchberg kindly provided anti-Meis 1 antibodies. This work was performed with the aid of grants from Telethon and the Italian Association for Cancer Research (AIRC) to F.B. and also by an EEC Biotechnology Network grant (#BIO2 CT-930060) and Medical Research Council (MRC) support to R.K. H.P. was funded by an HFSP fellowship, M.M. by an MRC Training fellowship and E.F. by an EMBO short-term fellowship to visit R.K.'s group.

REFERENCES

Abu-Shaar, M., Ryoo, H. D. and Mann, R. S. (1999). Control of the nuclear localization of extradenticle by competing nuclear import and export signals. *Genes Dev*. **13**, 935-945.

Alexandre, D., Clarke, J. D., Oxtoby, E., Yan, Y.-L., Jowett, T. and Holder, N. (1996). Ectopic expression of Hoxa1 in the zebrafish alters the fate of the manibular arch neural crest and phenocopies a retinoic acid induced phenotype. *Development* **122**, 735-746.

Bell, E., Wingate, R. J. T. and Lumsden, L. (1999). Homeotic Transformation of Rhombomere Identity After Localized Hoxb1 Misexpression. *Science* **284**, 2168-2171.

Berthelsen, J., Kilstrup-Nielsen, C., Blasi, F., Mavilio, F. and Zappavigna, V. (1999). The subcellular localization of PBX1 and EXD proteins depends on nuclear impoprt and export signals and is modulated by association with PREP1 and HTH. *Genes Dev*. **13**, 946-953.

Berthelsen, J., Vandekerkhove, J. and Blasi, F. (1996). Purification and characterization of UEF3, a novel factor involved in the regulation of the urokinase and other AP-1 controlled promoters. *J. Biol. Chem.* **271**, 3822-3830.

Berthelsen, J., Zappavigna, V., Ferretti, E., Mavilio, F. and Blasi, F. (1998a). Prep1, a novel partner of Pbx proteins, modifies Pbx-Hox protein cooperativity. *EMBO J.* **17**, 1434-1445.

Berthelsen, J., Zappavigna, V., Mavilio, F. and Blasi, F. (1998b). Prep1, a novel functional partner of Pbx proteins. *EMBO J.* **17**, 1423-1433.

Burglin, T. (1997). Analysis of TALE superclass homeobox genes (MEIS, PBC, KNOX, Iroquois, TGIF) reveals a novel domain conserved between plants and animals. *Nucleic Acids Res.* **25**, 4173-4180.

Carpenter, E. M., Goddard, J. M., Chisaka, O., Manley, N. R. and Capecchi, M. R. (1993). Loss of Hoxa1 (Hox1.6) function results in the reorganization of the murine hindbrain. *Development* **118**, 1063-1075.

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. 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**, 5225-5228.

Chan, S.-K., Pöpperl, 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., Brocchieri, L., Shen, W. F., Largman, C. and Cleary, M. L. (1996). Pbx modulation of Hox homeodomain amino-terminal arms establishes different DNA-binding specificities across the Hox locus. *Mol. Cell. Biol.* **16**, 1734-1745.

Chang, C. P., Shen, W. F., Rozenfeld, S., Lawrence, H. J., Largman, C. and Cleary, M. L. (1995). Pbx proteins display hexapeptide-dependent cooperative DNA binding with a subset of Hox proteins. *Genes Dev.* **9**, 663-**74.**

Chang, C. P., Jacobs, Y., Nakamura, T., Jenkins, N., Copeland, N. G. and Cleary, M. L. (1997). Meis proteins are major in vivo DNA binding partners for wild-type but not chimeric Pbx proteins. *Mol. Cell. Biol.* **17**, 5679-5687.

Conlon, R. A. and Rossant, J. (1992). Exogenous retinoic acid rapidly induces anterior ectopic expression of murine Hox-2 genes in vivo. *Development* **116**, 357-368.

Di Rocco, G., Mavilio, F. and Zappavigna, V. (1997). Functional dissection of a transcriptionally active, target-specific HOX/PBX complex. *EMBO J.* **16**, 3644-3654.

Dolle, P., Lufkin, T., Krumlauf, R., Mark, M., Duboule, D. and Chambon, P. (1993). Local alterations of Krox-20 and Hox gene expression in the hindbrain of Hoxa1 (Hox1.6) homzygote null mutant embryos. *Proc. Natl. Acad. Sci. USA* **90**, 7666-7670.

Dupé, V., Davenne, M., Brocard, J., Dollé, P., Mark, M., Dierich, A., Chambon, P., and Rijli, F. (1997). In vivo functional analysis of the *Hoxa1* 3′ retinoid response element (3′ RARE). *Development* **124,** 399-410.

Ferretti, E., Schulz, H., Talarico, D., Blasi, F. and Berthelsen, J. (1999). The Pbx-Regulating Protein Prep-1 is present in a Pbx-complexed form throughout mouse embryogenesis. *Mech. Dev.* **83**, 53-64.

Frasch, M., Chen, X. and Lufkin, T. (1995). Evolutionary-conserved enhancers direct region-specific expression of the murine *Hoxa-1* and *Hoxa-2* loci in both mice and *Drosophila*. *Development* **121**, 957-974.

Gavalas, A., Studer, M., Lumsden, A., Rijli, F., Krumlauf, R. and Chambon, P. (1998). *Hoxa1* and *Hoxb1* synergize in patterning the hindbrain, cranial nerves and second pharyngeal arch. *Development* **125**, 1123-1136.

Goddard, J., Rossel, M., Manley, N. and Capecchi, M. (1996). Mice with targeted disruption of Hoxb1 fail to form the motor nucleus of the CIIth nerve. *Development* **122**, 3217-3228.

Hill, J., Clarke, J. D. W., Vargesson, N., Jowett, T. and Holder, N. (1995). Exogenous retinoic acid causes specific alterations in the development of the midbrain and hindbrain of the zebrafish embryo including positional respecification of the Mauthner neuron. *Mech. Dev*. **50**, 3-16.

Jacobs, Y., Schnabel, C. A. and Cleary, M. L. (1999). Trimeric association of Hox and TALE homeodomain proteins mediates *Hoxb2* hindbrain enhancer activity. *Mol. Cell Biol*. **19**, 5134-5142.

Kamps, M. P., Look, A. T. and Baltimore, D. (1991). The human t(1;19) translocation in pre-B ALL produces multiple nuclear E2A-Pbx1 fusion proteins with differing transforming potentials. *Genes Dev.* **5**, 358-368

Kamps, M. P., Murra, C., Sun, X. and Baltimore, D. (1990). A new homeobox gene contributes the DNA binding domain of the t(1:19) translocation protein in pre-B ALL. *Cell* **60**, 547-555.

Knoepfler, P. S., Calvo, K. R., Chen, H., Antonarakis, S. E. and Kamps, M. P. (1997). Meis 1 and pKnox1 bind DNA cooperatively with Pbx1 utilizing an interaction surface discrupted in oncoprotein E2a-Pbx1. *Proc. Natl. Acad. Sci. USA* **94**, 14553-14558.

Knoepfler, P. S. and Kamps, M. P. (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.

Knoepfler, P. S. and Kamps, M. (1997a). The highest affinity DNA element bound by Pbx complexes in t(1:19) leukemic cells fails to mediate cooperative DNA-binding or cooperative transactivation by E2A-Pbx1 and Class I Hox proteins – evidence for selective targetting of E2A-Pbx1 to a subset of Pbx-recognition elements. *Oncogene* **14**, 2521-2531.

Knoepfler, P. S. and Kamps, M. (1997b). The Pbx family of proteins is strongly upregulated by a post-transcriptional mechanism during retinoic acid-induced differentiation of P19 embryonal carcinoma cells. *Mech. Dev*. **63**, 5-14.

Krumlauf, R. (1993). *Hox* genes and pattern formation in the branchial region of the vertebrate head. *Trends Genet* **4**, 106-112.

Krumlauf, R. (1994). *Hox* genes in vertebrate development. *Cell* **78,** 191-201.

Lu, Q. and Kamps, M. P. (1996). Structural determinants within Pbx1 that mediate cooperative DNA binding with pentapeptide-containing Hox proteins: Proposal for a model of a Pbx-Hox-DNA complex. *Mol. Cell. Biol.* **16**, 1632-1640.

Lu, Q., Knoepfler, P. S., Scheele, J., Wright, D. D. and Kamps, M. P. (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.

Lumsden, A. and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. *Science* **274,** 1109-1115.

Maconochie, M. M., Nonchev, S., Studer, M., Chan, S.-K., Pöpperl, H., Sham, M. H., Mann, R. S. and Krumlauf, R. (1997). Cross-regulation in the mouse HOXB complex: the expression of Hoxb2 in rhombomere 4 is regulated by Hoxb1. *Genes Dev.* **11**, 1885-1895.

Mann, R. S. (1995). 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.

Mann, R. S. and Abu-Shaar, M. (1996). Nuclear import of the homeodomain protein Extradenticle in response to Wg and Dpp signaling. *Nature* **383**, 630-633.

Mark, M., Lufkin, T., Vonesch, J.-L., Ruberte, E., Olivo, J.-C., Dolle, P., Gorry, P., Lumsden, A. and Chambon, P. (1993). Two rhombomeres are altered in Hoxa1 mutant mice. *Development* **119**, 319-338.

Marshall, H., Nonchev, S., Sham, M. H.,Muchamore, I., Lumsden, A. and Krumlauf, R. (1992). Retinoic acid alters hindbrain Hox code and induces transformation of rhombomeres 2/3 into a 4/5 identity. *Nature* **360**, 737-741.

Marshall, H., Studer, M., Pöpperl, H., Aparicio, S., Kuroiwa, A., Brenner, S. and Krumlauf, R. (1994). A conserved retinoic acid response element required for early expression of the homeobox gene Hoxb-1. *Nature* **370**, 737-741.

Monica, K., Galili, N., Nourse, J., Saltman, D. and Cleary, M. L. (1993). PBX2 and PBX3, new homeobox genes with extensive homology to the human proto-oncogene PBX1. *Mol. Cell. Biol.* **11**, 6149-57.

Morrison, A. (1998). 1+1=4 and much much more. *BioEssays* **20,** 794-797.

Moskow, J. J., Bullrich, F., Huebner, K., Daar, I. O. and Buchberg, A. M. (1995). Meis1, a PBX1-related homeobox gene involved in myeloid leukemia in BXH-2 mice. *Mol. Cell. Biol.* **15**, 5434-43.

Nourse, J., J. D., Mellentin, Galili, N., J., Wilkinson, Stanbridge, E., S. D., Smith and Cleary, M. L. (1990). Chromosomal translocation t(1:19) results in synthesis of a homeobox fusion mRNA that encodes for a potential chimeric transcription factor. *Cell* **60**, 535-545.

Pai, C.-Y., Kuo, T.-S., Jaw, T. J., Kurant, E., Chen, C.-T., Bessarab, D. A., Salzberg, A. and Sun, Y. H. (1998). The homothorax protein activates the nuclear localization of another homeoprotein, extradenticle, and suppresses eye development in Drosophila. *Genes Dev*. **12**, 435-446.

Peltenburg, L. T. C. and Murre, C. (1996). Engrailed and Hox homeodomain proteins contain a related Pbx interaction motif that recognizes a common structure present in Pbx. *EMBO J.* **15**, 3385-3393.

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., Bienz, M., Studer, M., Chan, S. K., Aparicio, S., Brenner, S., Mann, R. S. 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.

Rieckhof, G. E., Casares, F., Ryoo, H. D., Abu-Shaar, M. and Mann, R. S. (1997). Nuclear translocation of Extradenticle requires homothorax, which encodes an extra-denticle related homeodomain protein. *Cell* **91**, 171-183.

Sham, M. H., Vesque, C., Nonchev, S., Marshall, H., Frain, M., Das Gupta, R., Whiting, J., Wilkjinson, D., Charnay, P. and Krumlauf, R. (1993). The zing finger gene Krox20 regulates Hoxb-2 (Hox2.8) during hindbrain segmentation. *Cell* **72**, 183-196.

Simeone, A., Acampora, D., Arcioni, L. and Boncinelli, E. (1990). Sequential activation of Hox2 homeobox genes by retinoic acid in human embryonal carcinoma cells. *Nature* **346**, 763-766.

Studer, M., Gavalas, A., Marshall, H., Ariza-McNaughton, L., Rijli, F., Chambon, P. and Krumlauf, R. (1998). Genetic interaction between *Hoxa1* and *Hoxb1* reveal new roles in regulation of early hindbrain patterning. *Development* **125,** 1025-1036.

Studer, M., Lumsden, A., Ariza-McNaughton, L., Bradley, A. and Krumlauf, R. (1996). Altered segmental identity and abnormal migration of motor neurons in mice lacking Hoxb1. *Nature* **384**, 630-634.

Studer, M., Pöpperl, H., Marshall, H., Kuroiwa, A. and Krumlauf, R. (1994). Role of a conserved retinoic acid response element in rhombomere restriction of *Hoxb-1*. *Science* **265,** 1728-1732.

Van Dijk, M. A., Peltenburg, L. T. and Murre, C. (1995). *Hox* gene products modulate the DNA binding activity of Pbx1 and Pbx2. *Mech. Dev.* **52**, 99-108.

Vesque, C., Maconochie, M., Nonchev, S., Ariza-McNaughton, L., Kuroiwa, A., Charnay, P. and Krumlauf, R. (1996). Hoxb-2 transcriptional activation by Krox20 in vertebrate hindbrain requires an evolutionary concerved cis-acting element in addition to the Krox-20 site. *EMBO J.* **15**, 5383-5896.

Whiting, J., Marshall, H., Cook, M., Krumlauf, R., Rigby, P. W. J., Stott, D. and Allemann, R. K. (1991). Multiple spatially specific enhancer are required to reconstruct the patten of Hox-2.6 gene expression. *Genes Dev*. **5**, 2048-2059.

Yee, S.-P. and Rigby, P. W. J. (1993). The regulation of *myogenin* gene expression during the embryonic development of the mouse. *Genes Dev.* **7**, 1277-1289.

Zhang, M., Kim, H.-J., Marshall, H., Gendron-Maguire, M., Lucas, A. D., Baron, A., Gudas, L. J., Gridley, T., Krumlauf, R. and Grippo, J. F. (1994). Ectopic Hoxa1 induces rhombomere transformation in mouse hindbrain. *Development* **120**, 2431-2442.