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Trimeric Association of Hox and TALE Homeodomain Proteins Mediates *Hoxb2* Hindbrain Enhancer Activity

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Pbx/exd proteins modulate the DNA binding affinities and specificities of Hox proteins and contribute to the execution of Hox-dependent developmental programs in arthropods and vertebrates. Pbx proteins also stably heterodimerize and bind DNA with Meis and Pknox1-Prep1, additional members of the TALE (three-amino-acid loop extension) superclass of homeodomain proteins that function on common genetic pathways with a subset of Hox proteins. In this study, we demonstrated that Pbx and Meis bind DNA as heterotrimeric complexes with Hoxb1 on a genetically defined *Hoxb2* enhancer, r4, that mediates the cross-regulatory transcriptional effects of Hoxb1 in vivo. The DNA binding specificity of the heterotrimeric complex for r4 is mediated by a Pbx-Hox site in conjunction with a distal Meis site, which we showed to be required for ternary complex formation and Meis-enhanced transcription. Formation of heterotrimeric complexes in which all three homeodomains bind their cognate DNA sites is topologically facilitated by the ability of Pbx and Meis to interact through their amino termini and bind DNA without stringent half-site orientation and spacing requirements. Furthermore, Meis site mutation in the *Hoxb2* enhancer phenocopies Pbx-Hox site mutation to abrogate enhancer-directed expression of a reporter transgene in the murine embryonic hindbrain, demonstrating that DNA binding by all three proteins is required for trimer function in vivo. Our data provide in vitro and in vivo evidence for the combinatorial regulation of Hox and TALE protein functions that are mediated, in part, by their interdependent DNA binding activities as ternary complexes. As a consequence, Hoxb1 employs Pbx and Meis-related proteins, as a pair of essential cofactors in a higher-order molecular complex, to mediate its transcriptional effects on an endogenous Hox response element.

Hox genes encode homeodomain-containing transcription factors that play critical roles in specifying positional information along several embryonic axes. A growing body of evidence supports a role for several members of the TALE (three-amino-acid loop extension) class of homeoproteins as essential contributors to Hox developmental programs in arthropods and vertebrates. Hox interactions with TALE proteins of the Pbx/exd subtype have been most extensively studied (22); they result in enhanced DNA binding affinities or specificities in vitro (7–9, 16, 19, 28–30, 37, 40). Genetic analyses of both mice and *Drosophila melanogaster* provide strong in vivo support that Pbx and Hox function in concert during development (33) through response elements containing their cognate DNA binding sites (7, 20, 22, 32). In addition to cooperative DNA binding activity, the genetic interactions of Hox and Pbx/exd proteins appear to involve, in part, regulation of their subcellular distributions (1, 21).

Aside from Pbx-Hox interactions, a cooperative function among TALE family members has also been shown to be critical for transcriptional processes. Several recent reports have shown that Pbx proteins dimerize and bind DNA with Meis-hth (4, 10, 17) and Pknox1-Prep1 (2, 3, 17), members of an ancient subclass of TALE proteins evolutionarily related to but distinct from Pbx/exd (5, 11, 24). Indeed, heterodimeric TALE homeoprotein complexes are directly implicated by biochemical analyses in the regulated expression of several genes (2–4, 39). Further support for their convergent function comes from studies of *Drosophila*, where the homolog of Meis (homothorax) displays genetic epistatis with exd and regulates its nuclear entry (6, 27, 34).

An additional layer of complexity arises from genetic and biochemical evidence demonstrating Hox interactions with Meis-like proteins. In *Drosophila*, homothorax affects nuclear localization and functions in common genetic pathways with a subset of Hox proteins (6, 27, 34). In murine myeloid leukemias, both *MEIS* and *HOX* genes are activated by retroviral insertions, providing genetic support for the cooperative interactions of their respective gene products in mammalian neoplasias (25). Recent retroviral gene transfer experiments provide more direct genetic evidence that Meis1 and Hoxa9 collaborate in myeloid oncogenesis (18), and Hoxa9 and Meis1 have been shown in vitro to physically interact and bind DNA as heterodimers (38).

Thus far, investigations have primarily focused on the functional significance of dimeric Pbx-Hox, Pbx-Meis, and Meis-Hox interactions. These studies suggest two possible, but not necessarily mutually exclusive, models for integrating Hox and TALE protein functions. Representatives of the Pbx, Meis, and Hox homeoprotein families may compete with each other to establish a hierarchy of heterodimers or, alternatively, they may cooperatively enter into higher-order DNA binding complexes. In this report, we demonstrate that Hoxb1 binds native enhancers in vitro in a higher-order trimeric complex with Pbx and Meis. Unlike previous reports of Hox and TALE trimeric interactions, our results showed that the Meis component contributes to the DNA binding specificity of the heterotrimeric complex by binding a Meis cognate DNA site and is not simply tethered by Pbx to the complex. Furthermore, DNA binding by the Meis component appears to be essential for transcriptional activity of the trimeric complex in vivo, since the Meis site mutation phenocopies the Pbx-Hox site mutation to abrogate enhancer-directed expression of a reporter transgene in the

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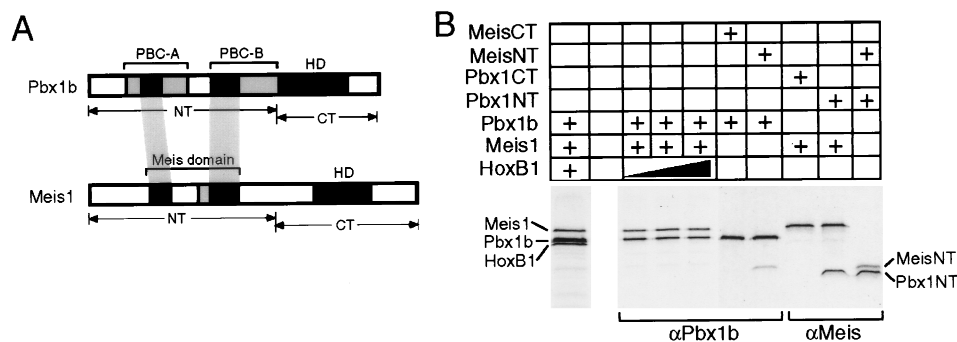

FIG. 1. The amino termini of Pbx and Meis proteins are necessary and sufficient for their heterodimerization in solution. (A) Schematic illustrations of Pbx and Meis proteins. The PBC-A and PBC-B domains consist of portions conserved in both the Pbx and Meis protein families (solid boxes) and portions conserved only within the Pbx or Meis subfamilies (lightly shaded areas). N-terminal and C-terminal deletion constructs are indicated by brackets. HD, homeodomain. (B) In vitro-produced proteins (indicated in schematic illustration above the gel lanes) were incubated together, immunoprecipitated with anti-Pbx1b antibodies (αPbx1b) or anti-Meis antibodies (αMeis) (indicated beneath the gel lanes), and then fractionated by SDS-PAGE. Coprecipitation of Meis-Pbx complexes was dependent on the amino termini of each protein and was not disrupted by the addition of increasing amounts of Hoxb1. CT, C terminus; NT, N terminus.

developing hindbrain. Therefore, Hoxb1 simultaneously binds DNA with Pbx and Meis-related proteins and employs these paired TALE homeoproteins as essential cofactors to mediate its transcriptional effects on hindbrain enhancers in vivo.

MATERIALS AND METHODS

Plasmid constructions and mutagenesis. Constructs for the expression of wild-type and mutant Pbx and Hox proteins under the control of SP6 or cytomegalovirus (CMV) promoters have been described in previous studies (8, 9). The full-length murine MEIS1 cDNA (25) was cloned into pCMV1 for use in transient-transcription assays. Deletion constructs of Pbx1 (Pbx1CT and Pbx1NT) and Meis1 (MeisCT and MeisNT) were constructed by standard cloning or PCR. A 900-bp (*Bam*HI to *Stu*I) upstream region of the *Hoxb2* r4 enhancer element was amplified by PCR and subcloned into the BGZ40 lacZ reporter vector (42) containing a minimal human β-globin promoter. The 900-bp element spanned the three Krox20 and consensus Pbx-Hox sites, as reported previously (20, 36). Mutations of the Meis and Pbx-Hox sites in the *Hoxb2* r4 element were performed by overlap extension PCR.

DNA binding and transcriptional assays. Proteins for DNA binding were produced in vitro from SP6 expression plasmids by using a coupled reticulocyte lysate system, as described previously (8). DNA binding reactions were performed at 4°C for 30 min in a 15-μl reaction mixture under conditions reported previously (8) and subjected to an electrophoretic mobility shift assay (EMSA) with 6% polyacrylamide gels in 0.25× Tris-borate-EDTA buffer. DNA probes consisted of gel-purified, end-labeled, double-stranded oligonucleotides encoding the *Hoxb2* r4 enhancer (20) or portions of the *Hoxb1* autoregulatory element (ARE) (32). Synthetic probes for the evaluation of Pbx-Meis DNA binding requirements contained consensus Pbx and Meis sites (underlined) in various configurations (e.g., 5′-CCCTGCCTTGATTGACAGTTGCGCCTG-3′ for nongapped sites and 5′-CTGCCTTGATGCCTGGTGACAGTTGCGC-3′ for N6-gapped sites). Actual sequences of DNA probes are available upon request.

Transient-transcription assays were performed essentially as described previously (12). COS-7 cells were seeded at 10^5 cells per 35-mm-diameter dish in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) 16 to 24 h prior to transfection. Cells were transfected by a calcium phosphate coprecipitation procedure with different combinations of expression plasmids encoding Pbx1a (2 μg), Hoxb1 (500 ng), or Meis1 (2 μg) along with reporter (1 μg) and internal control (pCMV-βgal) plasmids. The reporter plasmid consisted of the firefly luciferase gene, driven by a simian virus 40 early promoter, and one copy of the *Hoxb2* r4 enhancer or *Hoxb1* ARE. Total DNA concentration per dish was kept constant with nonspecific DNA. Luciferase activity was measured in light units with a Monolight 2010 luminometer; β-galactosidase activity was used to normalize luciferase activity to account for differences in transfection efficiency.

Immunoprecipitations and Western blots. In vitro-translated proteins (2 to 10 μl of reticulocyte lysates) were added to 200 μl of immunoprecipitation buffer (50 mM HEPES at pH 7.9, 250 mM NaCl, 5 mM dithiothreitol, 1% bovine serum albumin, 0.1% Triton X-100) and incubated at 4°C for 3 h with antibody (1 μg/100 μl) and an additional 2 h with 20 μl of protein G-Sepharose beads. Beads were precipitated and washed 10 times with immunoprecipitation buffer. The precipitated proteins were boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and analyzed by SDS-PAGE. Western blotting was performed as described previously (10) with an anti-Prep1 antiserum (Santa Cruz Biotechnology) on whole-cell lysates of hindbrain tissues microdissected from 9.5-embryonic-day embryos.

Transgenic mice. DNA constructs, as linearized inserts lacking vector sequences, were microinjected into fertilized mouse eggs generated from crosses of F_1 hybrids (C57BL/6J × CBA). Microinjected eggs were implanted into pseudopregnant females, and embryos were harvested at 9.5 days postconception (dpc). Embryos were fixed in 4% paraformaldehyde (in phosphate-buffered saline) for 30 minutes, washed in buffer W (phosphate-buffered saline, 0.01% deoxycholate, 0.02% Nonidet P-40), and stained for 1 to 4 h at 37°C in X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (1 mg/ml) with 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM $MgCl_2$ in buffer W.

RESULTS

The amino termini of Pbx and Meis are necessary and sufficient for their dimerization in solution. To distinguish among alternative models for the function of TALE homeodomain proteins Pbx and Meis, we evaluated several features of their dimeric interactions. Since Pbx and Meis-related proteins form stable complexes in solution in the absence of DNA (2, 10), dimerization was assessed by coprecipitation analyses. We first tested whether Hox proteins were capable of displacing Meis from Pbx in solution, a potential requirement for a competitive heterodimerization model. Increasing concentrations of Hoxb1 did not reduce the amount of Meis1 that coprecipitated with Pbx1 (Fig. 1B), indicating that Hoxb1 was unable to compete with Meis1a for Pbx1a interaction in solution. Furthermore, under these conditions, we were unable to demonstrate coprecipitation of Hoxb1 with the TALE heterodimer, consistent with previous observations that Pbx and Hox interactions are weak in solution but markedly stable in the presence of cognate DNA (8).

Since Pbx-Hox interactions require sequences exclusively within the extended Pbx homeodomain (31), we next evaluated whether Pbx-Meis dimerization may also require one or both homeodomains. Deletion of either the Pbx1a or the Meis1a carboxy terminus containing the respective homeodomains did not abrogate their coprecipitation. In fact, coprecipitation of the amino termini was observed without either homeodomain. In contrast, removal of the amino-terminal regions of Pbx1a or Meis1a prevented coprecipitation of the Pbx-Meis complex (Fig. 1). Attempts to further refine Pbx1a dimerization requirements by the deletion of additional C-terminal sequences from Pbx1NT abrogated coprecipitation (data not shown). Therefore, the amino-terminal portions of both Pbx and Meis were necessary and sufficient for their dimerization in solution, as assessed by this coprecipitation assay (Fig. 1). The required amino-terminal regions encompassed amino acid segments with predicted helical features and constituted the only do-

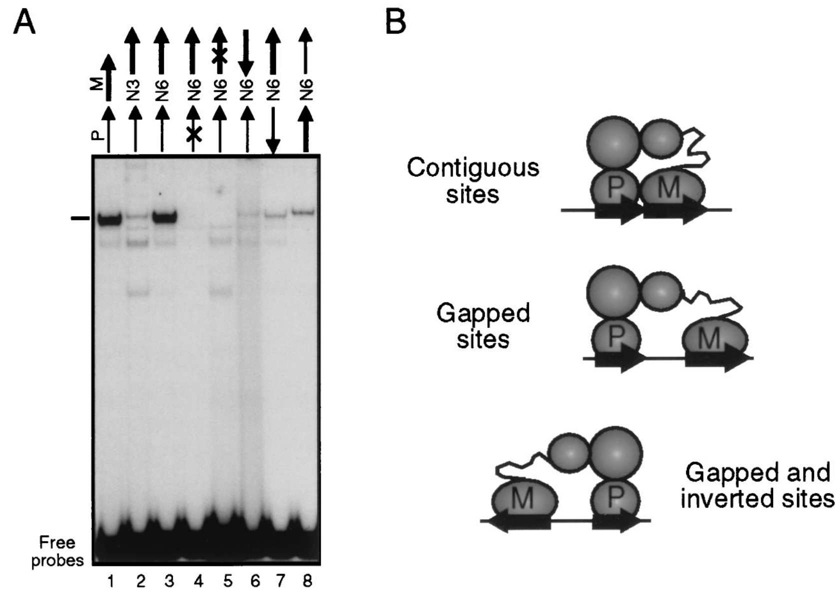

FIG. 2. Pbx and Meis heterodimers bind DNA without stringent half-site orientation and spacing requirements. (A) In vitro-synthesized Pbx1 and Meis1 proteins were subjected to EMSA with DNA probes (schematically illustrated above the gel lanes). Dimers that formed irrespective of half-site spacing and orientation are indicated to the left. P, Pbx half site; M, Meis half site; N, inserted nucleotides between half sites; ×, mutant half site. Arrows indicate half-site orientations. (B) Schematic depictions of Pbx-Meis heterodimers binding to various configurations of DNA half sites.

mains conserved between Pbx and Meis-Prep1 proteins outside of their homeodomains. This portion of Pbx1 is required for cooperative DNA binding with Meis and Prep1 (2, 10, 17) but is separate and distinct from that required for optimal interactions with Hox partners (8, 10).

Pbx and Meis bind DNA without stringent half-site orientation and spacing requirements. The observations that Pbx and Meis dimerize through their amino termini suggested the possibility that their DNA binding activity may not be constrained by stringent half-site spacing or orientation requirements. Thus, Pbx-Meis may be similar to MATα2, a yeast TALE homeodomain protein that dimerizes through its flexible amino terminus (15). Towards this end, Pbx-Meis heterodimeric binding activity was examined on synthetic oligonucleotides containing Pbx and Meis half sites with varied spacing and orientation. Pbx-Meis heterodimers tolerated separation of their half sites by 3 or 6 nucleotides (Fig. 2A, lanes 2 and 3). Steady-state binding was more robust on sites separated by 6 nucleotides and was dependent on intact half sites (Fig. 2A, lanes 3 to 5). Significant, but less robust, binding also occurred on DNA probes with half sites in various inverted orientations (Fig. 2A, lanes 6 to 8). Although an extensive analysis of site configurations was not conducted, at a minimum these studies demonstrated that Pbx-Meis heterodimers are more tolerant of alterations in half-site spacing and orientation (Fig. 2B) than are Pbx-Hox heterodimers (8). This flexibility is likely facilitated by the stable interactions of Pbx and Meis through their unique amino-terminal dimerization motifs.

Two hindbrain enhancers contain conserved Meis sites in proximity to Pbx-Hox sites. The flexible character of Pbx-Meis interactions raised the possibility that Pbx may simultaneously interact with Meis as well as Hox proteins in a higher-order molecular complex in which each component contacts DNA. Furthermore, the rigid half-site requirements of Pbx-Hox dimers, in contrast to the less stringent requirements of Pbx-Meis dimers, suggested that potential trimeric binding sites may consist of a core Pbx-Hox site separated from a Meis site by variable distances. This possibility was investigated by evaluating genetically characterized Hox enhancers for the presence of Meis sites flanking Pbx-Hox sites. Two enhancers were discovered to meet these criteria (Fig. 3). Both have been shown in genetic studies to mediate the in vivo transcriptional effects of Hoxb1 in conjunction with Pbx cofactors during hindbrain development. The *Hoxb1* ARE is necessary for the autoregulation of Hoxb1 expression in rhombomere 4 of the hindbrain (32). The *Hoxb2* r4 element functions to direct Hoxb1 cross-regulation of the *Hoxb2* gene in the same anatomic site (20). Both enhancers contain Pbx-Hox sites as previously reported (20, 32). However, they also both contain Meis sites which have strikingly similar configurations and which are upstream of, and in reverse orientation with respect to, their Pbx-Hox sites (Fig. 3).

Trimeric Meis-Pbx-Hox complexes assemble on the *Hoxb2* r4 enhancer and demonstrate a requirement for an intact Meis site. We performed EMSA with a DNA probe spanning the Pbx-Hox and Meis sites in the *Hoxb2* r4 enhancer to determine whether this element could support the formation of trimeric

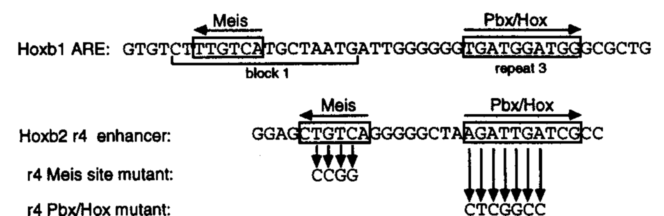

FIG. 3. Both the *Hoxb2* r4 and *Hoxb1* ARE enhancers contain Meis sites which are upstream and in reverse orientation with respect to Pbx-Hox sites. Nucleotide sequences are shown for *HOX* response elements employed as DNA probes for EMSA. Boxes and arrows indicate protein binding sites and their relative orientations. Mutations introduced into the Meis or Pbx-Hox sites of the *Hoxb2* r4 element are shown below.

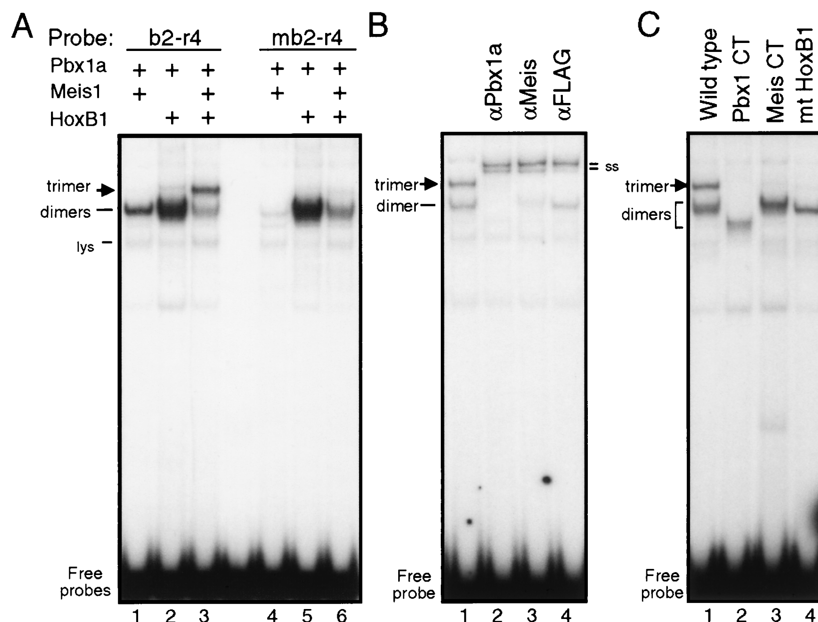

FIG. 4. A trimeric Pbx1-Meis1-Hoxb1 DNA binding complex requires an intact Meis site for its formation of the *Hoxb2* r4 element. (A) In vitro-synthesized proteins (indicated above the gel lanes) were subjected to EMSA with a radiolabeled *Hoxb2* r4 element (20) containing an intact (lanes 1 to 3) or a mutant (lanes 4 to 6) Meis site. A trimeric complex that formed in the presence of all three proteins is indicated by an arrow. (B) In vitro-translated proteins were incubated in DNA binding reaction mixtures in the presence of a radiolabeled probe and then subjected to EMSA. Antibodies were added to selected binding reaction mixtures as indicated above lanes 2 to 4. ss, antibody-protein complexes resulting from supershift analyses. (C) EMSA was performed with the *Hoxb2* r4 element and Hoxb1, Pbx1a, and Meis1a (lane 1). In lanes 2 to 4, a mutant protein (identity indicated above gel lanes) was substituted for the respective wild-type protein.

Meis-Pbx-Hoxb1 DNA binding complexes. On the *Hoxb2* r4 enhancer, Pbx1a-Meis1a heterodimers displayed measurable cooperative binding (Fig. 4A, lane 1). More robust dimeric binding was observed in reactions containing Pbx1a and Hoxb1 (Fig. 4A, lane 2). However, when all three proteins were present in the binding reaction, the predominant complex displayed a lower mobility than did heterodimers (Fig. 4A, lane 3). This was accompanied by a substantial reduction in the amount of dimeric complex detected, strongly suggesting that in the presence of all three proteins, simple heterodimeric binding of Hoxb1 with Pbx1a was not favored. Rather, the appearance of a slower-migrating complex suggested that Hoxb1 preferentially entered into a higher-order DNA binding complex containing both Pbx1a and Meis1a. Consistent with this possibility, the abundance of the slower-migrating complex (Fig. 4A, lane 3) was comparable to that of the Pbx-Meis complex (lane 1).

The requirement for DNA binding by each component of the heterotrimeric complex was evaluated by using probes containing mutations of the Meis or Pbx-Hox binding sites. Mutation of the Pbx-Hox site completely abrogated DNA binding by all heterodimeric and heterotrimeric complexes (data not shown). Mutation of the Meis site in r4 abrogated formation of the trimer but not the Pbx1a-Hoxb1 heterodimer (Fig. 4A, lane 5 versus 6), indicating that DNA binding by the Meis component was necessary to form a ternary complex on DNA. Interestingly, the amount of dimer observed on the mutant r4 element was markedly reduced in the presence of Meis1a (Fig. 4A, lane 6), but this was not associated with the concomitant appearance of the slower-migrating complex seen with the wild-type probe (lane 3). This observation suggested that, in solution, the three proteins formed a trimeric complex whose DNA binding requirements were more stringent than those of Pbx-Hox or Pbx-Meis heterodimers.

The presence of all three homeodomain proteins in the trimeric complex that formed on the wild-type r4 probe was verified by the inclusion of specific antibodies in the binding reactions. Supershifted complexes were observed with monoclonal antibodies directed against each of the input proteins, demonstrating that all three proteins were present in the slower-migrating complex (Fig. 4B, lanes 2 to 4). Small amounts of residual dimeric complexes in lane 4 represent Pbx-Meis heterodimers that do not contain Hoxb1 and thus were not supershifted by the anti-Flag antibody. Furthermore, the formation of a ternary complex was dependent on specific dimerization motifs in each protein (Fig. 4C). Heterodimeric Pbx-Hox complexes, but not trimeric complexes, were detected when EMSA was performed with mutants Pbx1CT (Fig. 4C, lane 2) or Meis1CT (lane 3), each of which is defective in Pbx-Meis dimerization (Fig. 1B). Similarly, a Hoxb1 hexapeptide mutant was incapable of entering into a trimeric complex with Pbx-Meis heterodimers (Fig. 4C, lane 4).

Heterotrimeric complexes also preferentially assemble on a subportion of the *Hoxb1* ARE that contains Meis and Pbx-Hox sites. As noted above, we discovered a strikingly similar configuration of Meis and Pbx-Hox consensus sites upon examination of a second genetically defined Hox enhancer, the *Hoxb1* ARE (Fig. 3). This enhancer contains three conserved Pbx-Hox consensus sites (r1 to r3), one of which, r3, has been shown to support the assembly of a trimeric Prep1-Pbx1-Hoxb1 complex in vitro (3). Consistent with these previous observations, we observed that the intact ARE supported the binding of a Meis1a-Pbx1a-Hoxb1 complex under our EMSA conditions (Fig. 5). A Pbx1a-Hoxb1 heterodimer formed on the ARE in the absence of Meis1a (Fig. 5, lane 2), similar to results obtained with the *Hoxb2* enhancer. With the addition of Meis1a, heterodimer binding was markedly reduced, and a trimeric complex was observed (Fig. 5, lane 3). To evaluate the specific sequences within the *Hoxb1* ARE that are required for the formation of a trimeric Hoxb1-Pbx1a-Meis1a complex, we

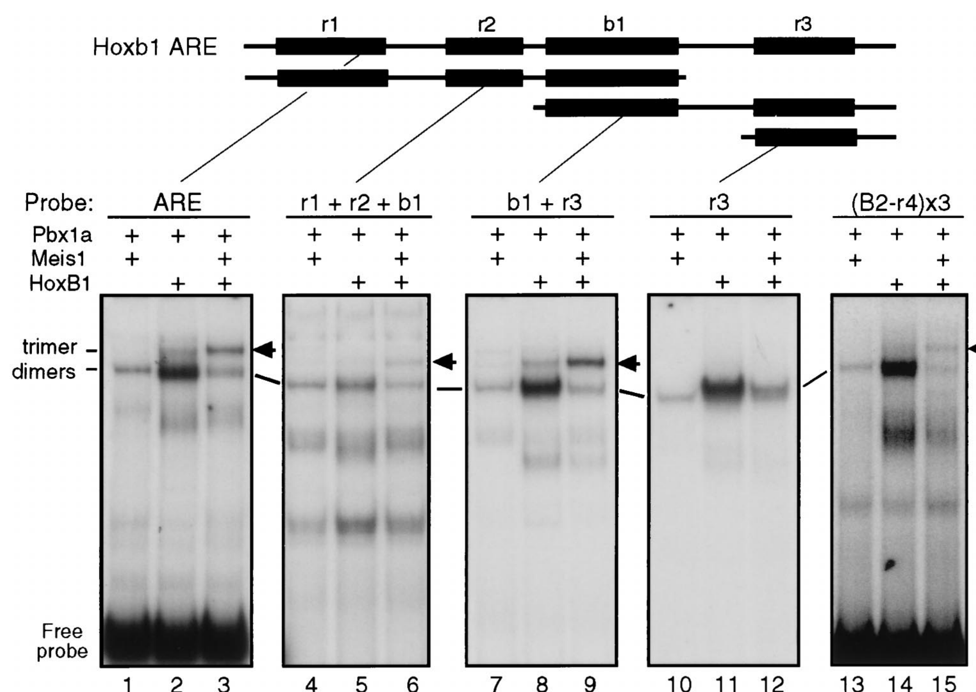

FIG. 5. Trimeric Hoxb1-Pbx1-Meis1 DNA binding complexes preferentially bind to a subportion of the *Hoxb1* ARE that contains consensus Meis and Pbx-Hox sites. EMSA was performed on DNA binding complexes containing various combinations of Pbx1, Meis1, or Hoxb1 proteins (indicated above the gel lanes). The DNA probes consisted of radiolabeled oligonucleotides containing subfragments of the *Hoxb1* ARE (32), indicated schematically at the top. r1 to r3 were previously identified by their similarity to the Pbx consensus site. Migrations of dimer and trimer complexes are indicated to the left. Arrow heads denote trimeric complexes. Free DNA probes are not shown in the middle panels because they are smaller and migrate faster than the full-length ARE. The probe used in the rightmost panel consisted of a multimerized Pbx-Hox site from the *Hoxb2* r4 enhancer (20).

performed EMSA with ARE subfragments as probes. Trimer formation was found to be most robust on a probe containing the conserved block 1 (b1) and the adjacent Pbx-Hox consensus repeat element r3 (Fig. 5). In contrast to previous studies (3), the r3 element alone did not support trimer binding, although Pbx1a-Hoxb1 heterodimers bound robustly to this element (Fig. 5, lanes 11 and 12). The amount of Pbx1a-Hoxb1 dimer detected on the r3 element was markedly reduced in the presence of Meis1a (Fig. 5, lane 12), but this was not associated with the concomitant appearance of a slower-migrating complex, as seen with the b1-r3 subfragment (lane 9). These findings again suggested that in the presence of all three proteins, Hox-Pbx heterodimers converted in solution to trimers with increased DNA binding requirements. Such trimers, however, did not appear to be sufficiently stable to withstand immunoprecipitation under our conditions (Fig. 1B). Since no trimeric complex formed on the r3 element alone, the sequences in b1 were deduced to be critical for trimer binding and, in fact, to encode the consensus Meis site described above (Fig. 3). Taken together, these data suggested that heterotrimer formation on DNA was dependent on the recognition of cognate DNA sites by each respective homeodomain in the complex. Therefore, two genetically defined response elements for Hoxb1 support the in vitro binding of higher-order molecular complexes containing Hoxb1 and the TALE class homeodomain proteins Pbx1a and Meis1a. Our analyses indicated that all three proteins are capable of binding DNA as a trimeric complex on these enhancer elements and that the Meis component contributes substantially to the DNA binding specificity of the ternary complex.

Trimeric complexes display Meis-dependent transcriptional activity. The potential functional consequences of trimeric Hox-Pbx-Meis interactions on the *Hoxb2* r4 enhancer were first tested in transient-transcription assays. For these studies, we employed a reporter gene containing a single copy of the *Hoxb2* r4 enhancer upstream from a simian virus 40 early promoter. When coexpressed, Pbx1a and Meis1a displayed no activation above background levels, comparable to the results obtained with Hoxb1 alone or with Hoxb1 coexpressed with Pbx1a (Fig. 6). In contrast, cotransfection of all three homeodomain proteins resulted in a severalfold increase in transcription above the baseline (Fig. 6). When similar analyses were performed with a reporter gene containing a mutant Meis site in the *Hoxb2* enhancer, no activation above the background was observed when all three homeodomain proteins were cotransfected. This provides in vivo evidence that trimer-enhanced transcriptional function requires the recognition of an appropriate binding site by the Meis component, consistent with our observations that the assembly of a trimeric complex on this enhancer in EMSA is dependent upon an intact Meis site. Previous studies employing reporter genes containing multiple copies of the r3 element of the ARE provided evidence that the Meis-related protein Prep1 enhanced transcriptional activation of a Pbx1-Hoxb1 complex (2). We obtained similar evidence for an accessory role of Meis1a with a reporter gene containing a single ARE. This reporter showed 10-fold-higher transcriptional activity upon cotransfection of all three proteins than Pbx1a and Meis1a alone (Fig. 6). On this element, coexpressed Pbx1a and Hoxb1 (in the absence of exogenous Meis1a) were also capable of activating transcription, consistent with previous observations (12). However, this activity was reproducibly two- to threefold lower and probably reflects the presence of endogenous Meis-Prep1 proteins in COS-7 cells (Fig. 7C) that are limiting for the nuclear entry of

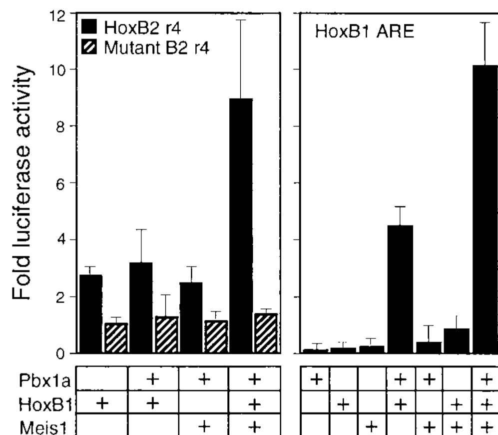

FIG. 6. Transcriptionally active trimeric DNA binding complexes display Meis site-dependent activity on the *Hoxb2* enhancer. Luciferase activity was assayed from transiently transfected COS-7 cells. Cotransfection assays were performed in the presence (+) or absence (−) of the indicated expression plasmids encoding Pbx1a, Hoxb1, or Meis1 with reporter plasmids indicated at the tops of the respective panels. Reporter constructs contained a single 30-bp *Hoxb2* enhancer element with Meis-Pbx-Hox sites (left panel) or the *Hoxb1* ARE (right panel). The mutant *Hoxb2* enhancer differed from the wild type by four nucleotide substitutions in the Meis site, as shown in Fig. 3. Data are expressed as the fold difference in luciferase activity obtained in comparison to activities obtained with a parental expression plasmid that did not contain coding sequences and a reporter plasmid that did not contain the enhancer element. Bars represent the means (plus standard deviations) of three to five independent experiments performed in duplicate.

Pbx, which requires Meis-related proteins, as demonstrated with orthologous proteins in *Drosophila* (27, 34).

***Hoxb2* enhancer function in the hindbrain requires Meis in addition to Pbx-Hox consensus binding sites.** We next evaluated whether Meis-related cofactors contribute to function of the Hoxb2 r4 enhancer in directing rhombomere-restricted expression in the mouse hindbrain. *lacZ* reporter transgenes were constructed with 5′ flanking regions of the *Hoxb2* gene (Fig. 7A) containing *cis*-acting elements required for its upregulated expression in rhombomeres 3 to 5 (called r3 to r5), following 8.5 dpc of development (20, 36). In addition to the 180-bp r4 enhancer which directs r4-restricted expression, we included three Krox20 sites that direct *Hoxb2* expression in r3 and r5 to serve as internal controls for comparing transgene expression in r4. Transgenic embryos were evaluated at 9.5 to 10 dpc, when r3 expression is beginning to wane but r4 expression is maximal (36, 41). Embryos microinjected with constructs containing wild-type sequences displayed intense reporter staining in r4 and its associated neural crest, which migrates into the second branchial arch (Fig. 7B). Less staining was seen in r3 and r5, as was expected at this stage of development (36, 41). Embryos that were transgenic for constructs containing mutations in the Pbx-Hox consensus site of the r4 enhancer showed persistent *lacZ* expression in r3 and r5 but no expression in r4 (Fig. 7B). This is consistent with previous studies (20) reporting the contributions of a direct cross-regulatory interaction of Hoxb1 and Pbx cofactors to *Hoxb2* expression in r4. A similar loss of r4 and branchial-arch staining, but not r3 or r5 staining, was seen in transgenic embryos microinjected with constructs containing Meis site mutations (Fig. 7A and B) that abrogate in vitro DNA binding by Meis-Pbx-Hoxb1 trimers as well as by Meis-enhanced transcription. Therefore, mutations of the Meis site in the *Hoxb2* r4 enhancer phenocopy Pbx-Hox site mutations, indicating that appropriate transgene expression requires DNA binding by Meis-like proteins as well as Pbx and Hox proteins.

DISCUSSION

These studies provide in vitro and in vivo evidence that Hoxb1 binds DNA simultaneously with Pbx and Meis-related TALE homeodomain proteins as cofactors to mediate its transcriptional effects on the *Hoxb2* r4 hindbrain enhancer. Although trimeric associations of Hox and TALE proteins have recently been reported on two other enhancer elements (3, 39), our studies are the first to demonstrate that DNA binding by the Meis component contributes to the specificity of ternary Hox-TALE homeoprotein complexes and is obligatory for their in vivo functions (Fig. 8). We had previously hypothesized that Pbx may simultaneously interact with and bind DNA with Hox and Meis partners, based on observations that Pbx employs distinctly different domains for interactions with Hox versus Meis-related proteins (10). However, the preferences of both Hox and Meis partners to assume a position on DNA immediately 3′ to Pbx in heterodimeric complexes appeared to be inconsistent with the formation of higher-order complexes in which all three homeodomains contact their cognate DNA sites. In the current study, we provided a solution to this topological predicament by demonstrating that Pbx-Meis heterodimers are capable of binding DNA without stringent half-site orientation and spacing requirements. The amino-terminal portions of Pbx and Meis are necessary and sufficient for their stable heterodimerization, presumably leaving their respective homeodomains free to bind DNA half sites in various configurations. This property allows Pbx-Meis-Hox heterotrimeric complexes to assemble on DNA sites consisting of a Pbx-Hox consensus core sequence flanked by a distant Meis site. This configuration satisfies the stringent half-site DNA binding requirements of the Pbx-Hox component, while simultaneously permitting DNA binding by the more flexible Meis component.

One role we observed for Meis is to increase the DNA binding requirements of the heterotrimeric complex. This was most evident by the suppression of Pbx-Hox binding to dimeric sites upon the inclusion of Meis in DNA binding reaction mixtures, presumably due to an increased requirement for DNA recognition by all three proteins. Thus, ternary complexes were not observed on DNA probes consisting of Pbx-Hox sites alone. On two natural enhancers, site-directed mutation or deletion of the flanking Meis site abrogated the in vitro DNA binding of trimeric Meis-Pbx-Hox complexes. These data demonstrate that Meis makes essential contributions to the binding specificity of the ternary complex, thereby preventing its recognition of simple dimeric Pbx-Hox sites. The specific mechanisms by which Meis affects the DNA binding requirements of the ternary complex were not established by our studies. Preliminary studies suggest that Meis does not measurably affect the DNA binding selectivity of Hoxb1, which appears to be comparable whether Hoxb1 is acting as a trimer with Pbx-Meis or as a heterodimer with Pbx (14). Furthermore, deletion of Meis's homeodomain does not completely impair its ability to suppress Pbx-Hox binding to dimeric sites (14), indicating that the increased binding specificity of trimeric complexes is not due solely to their requirements for a cognate Meis site. One possibility is that Meis modulates the DNA binding properties of Pbx, particularly since portions of Pbx that are necessary for dimeric interactions with Meis in solution are directly upstream of the Pbx homeodomain. In the absence of a cognate Meis site, the conformation of the Pbx homeodomain N-terminal arm may be sufficiently altered by a tethered but non-DNA-bound Meis partner to abrogate ter-

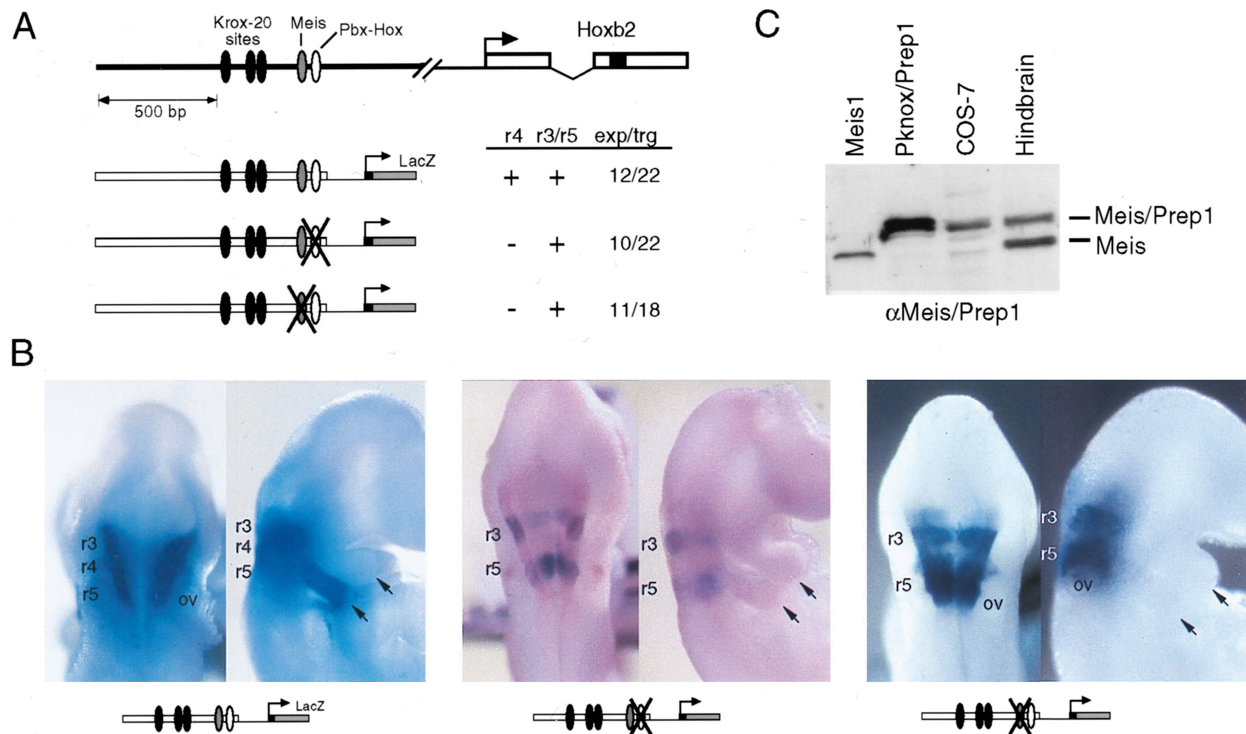

FIG. 7. Both the Meis and Pbx-Hox consensus sites are required for *Hoxb2* r4 enhancer function in the hindbrain. (A) The *Hoxb2* 5′ flanking region is shown above, and transgene expression constructs are shown below. Krox20 sites are shown as dark filled ovals, Meis sites are shown as shaded ovals, and Pbx-Hox consensus sites are shown as open ovals. The domain(s) and frequency of expression for each construct are provided at the right (exp, expressing; trg, transgenic). The number of embryos with detectable expressions of the transgene are indicated along with the total number of embryos examined by *lacZ* staining. (B) Dorsal and lateral views of *lacZ* staining patterns in transgenic embryos. Constructs are indicated beneath the panels. Arrows indicate branchial arches. ov, otic vesicle. (C) Western blot analysis demonstrates the expression of Meis and Prep1 proteins in the hindbrain at embryonic day 9.5. In vitro translates of Meis and Prep1-Pknox1 are shown in the first two lanes, respectively. Extracts of COS-7 cells and microdissected hindbrain are shown in the right lanes. Immunoreactive proteins were detected with a commercial rabbit antiserum against Prep1 that also cross-reacts with Meis proteins.

nary complex formation on Pbx-Hox sites. The DNA binding site requirements of Meis in a ternary complex with Pbx1a and Hoxb1 were not addressed in detail. Both enhancers contained similarly configured Meis sites, which showed inverted orientations with respect to nearby consensus Pbx-Hox sites. Interestingly, the distance separating Meis and Pbx-Hox sites was precisely one (r4) or two (ARE) DNA helical turns. It is not yet clear how rigid this spacing requirement may be in accommodating the possible topological features of the ternary homeodomain protein complex.

Our observation that Meis contributes to the DNA binding requirements of ternary complexes differs from previous observations and raises the possibility that its contributions may vary with different enhancers or under different cellular conditions. Berthelsen et al. (3) showed by EMSA that a trimeric complex consisting of Pbx1, Hoxb1, and the Meis-related protein Prep1 formed on the r3 element of the *Hoxb1* ARE. Since the DNA binding selectivity of the ternary complex was identical to that of Pbx-Hoxb1, the findings of Berthelsen et al. were most consistent with tethering of Prep1 to a DNA-bound Pbx-Hoxb1 heterodimer without a requirement for Prep1 homeodomain binding to DNA (Fig. 8). However, it is notable that DNA binding by the Prep1-containing ternary complex was enhanced upon deletion of the Prep1 homeodomain, suggesting that the homeodomain partially suppressed Pbx-Hoxb1 binding and thus contributed at some level to the selectivity of the ternary complex. Using Meis1 instead of Prep1, we were unable to demonstrate the formation of a comparable complex on r3 alone. We observed formation of a trimeric complex on the *Hoxb1* ARE, but the sequences within block 1 as well as the consensus Pbx-Hox site in r3 were required. The discrepant results likely reflect variations in EMSA conditions and/or protein preparations but not inherent differences between Prep1 and Meis1, since we obtained comparable results with either protein under our DNA binding conditions (14). Swift et al. (39) have also observed tethering of Meis to a DNA-bound heterodimer with the B element of the elastase enhancer, which mediates the effects of homeodomain protein PDX1 in pancreatic acinar cells (Fig. 8). Notably, ternary Pbx-Meis-PDX1 complexes were detected in the nuclear extracts of acinar cell lines by EMSA with the B element as a probe. Unfortunately, comparable studies have not detected endogenous Hoxb1-containing complexes in the nuclear extracts of embryos with probes representative of either Pbx-Hoxb1 sites (10) or the *Hoxb2* r4 enhancer (14). These studies consistently detect heterodimeric Pbx-Meis or Pbx-Prep1 complexes; the lack of ternary complexes presumably reflects the low abundance of Hoxb1 in embryonic extracts. Nevertheless, in vitro studies leave open the possibility that tethering of the Meis-Prep1 component of a heterotrimer can occur under some conditions, but the potential functional implications of this effect in vivo remain to be determined.

The most compelling evidence that DNA binding by a Meis-related protein is required for the in vivo function of ternary complexes is provided by our analysis of the requirements for function of the *Hoxb2* r4 enhancer in rhombomere 4 of the

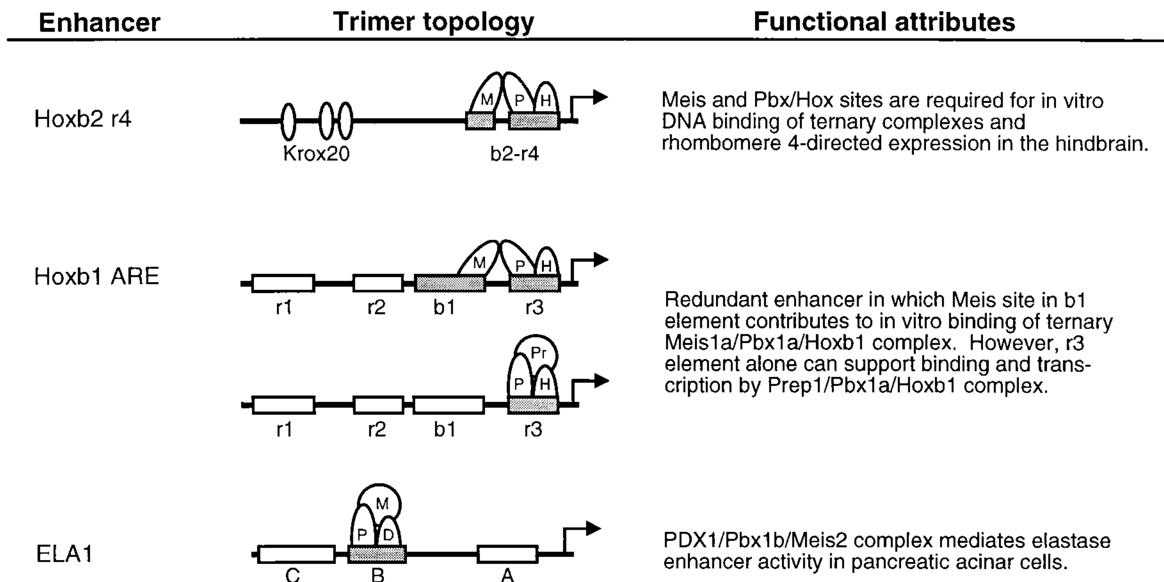

FIG. 8. Schematic representations of TALE homeoprotein trimeric complexes on various enhancer elements. Different trimeric complexes containing Hox, Pbx, and Meis-Prep1 components are depicted on enhancer elements whose in vivo functions have been reported in previous studies. There are two contrasting types of complexes: those in which the Meis-Prep1 component is simply tethered without binding to DNA and those in which DNA binding by the Meis component is essential.

developing hindbrain. Elegant genetic studies have demonstrated that this enhancer directs the appropriate expression of the *Hoxb2* gene in response to Hoxb1 cross-regulation in rhombomere 4 at approximately 8.5 to 10 days of hindbrain development (20). Extensive mapping showed that a consensus Pbx-Hox site was essential for r4 enhancer-mediated expression in rhombomere 4, but not in rhombomeres 3 and 5, which was consistent with our own observations. However, these earlier studies also indicated that the Pbx-Hox site was sufficient for r4-directed expression, a conclusion that conflicts with our current findings that mutation of the flanking Meis site phenocopies Pbx-Hox site mutation. This disparity may be accounted for by the fact that the previous studies employed synthetic elements that were not in a natural configuration and that contained iterated copies of Pbx-Hox sites (20). Since Meis crossbinds to Pbx-Hox consensus sites (10), synthetic elements containing them in tandem resemble the natural tripartite Meis-Pbx-Hox elements identified here and, in fact, weakly support DNA binding by ternary Hoxb1 complexes in vitro (Fig. 5, lane 15). This may also account for the Meis-mediated enhancement of the expression of reporter genes containing similar multimerized configurations of the ARE r3 site (2). Our studies demonstrate a consistent requirement for the Meis site in vitro and in vivo, but it is not yet clear which of the various Meis-Prep1 family members may be directly responsible for r4 enhancer function in the developing hindbrain. Both Meis and Prep1 proteins are expressed in the hindbrain at embryonic day 9.5 by Western blot analyses (Fig. 7C). Since *Meis* genes display dynamic expression profiles during embryonic development (26), a more precise determination of the in vivo roles of individual Meis-related proteins in r4 functions will require studies with mice that are nullizygous for one or more of the *Meis* genes.

The r4 and ARE enhancers contain strikingly similar configurations of Pbx-Hox and flanking Meis sites that were found to be essential for in vitro DNA binding by ternary Meis-Pbx-Hoxb1 complexes. However, our in vivo studies focused on the r4 enhancer because the ARE is a complex regulatory element with multiple potential homeoprotein binding sites (32). Although we found that heterotrimer binding was most robust on the b1-r3 subportion of the ARE, weak binding was also detected on other portions as well. The in vivo requirements for each of the several conserved sequence motifs in the ARE for its function in the developing mouse hindbrain have been extensively evaluated (32). When individually mutated, none of the conserved elements of this enhancer was found to be essential for ARE activity in rhombomere 4. Mutation of repeat 3 had the most significant effect but only partially reduced enhancer function, to approximately 60% of wild-type activity, whereas b1 (containing a conserved Meis site) was dispensable for ARE function. These in vivo data appear to be consistent with our observations that Meis-Pbx-Hoxb1 ternary complexes bind in vitro, with differing affinities, to at least two portions of the ARE. This contrasts with the *Hoxb2* r4 enhancer, in which mutation of either the Pbx-Hox or the Meis sites results in the complete loss of expression in rhombomere 4. Therefore, the ARE appears to be redundant in its composition, which complicates attempts to correlate the in vivo and in vitro contributions of individual elements.

The functional interrelationships of TALE proteins were initially suggested by studies of *Drosophila* showing that the Meis ortholog homothorax regulates the activation of the Pbx homolog exd through a posttranslational mechanism involving nuclear translocation (6, 27, 34). There is evidence for a similar mechanism in mammals (13, 35). These observations suggest that obligate heterodimerization with Meis-related proteins may be a generalized feature of Pbx/exd protein transcriptional function. In further support of this hypothesis, heterodimers consisting of Pbx paired with Meis or Pknox1-Prep1 are present in many adult and embryonic tissues (2, 10) and are implicated in the regulated expression of several genes (2–4, 10, 17, 39). Our studies demonstrate, however, that the heterodimerization of TALE proteins does not preclude their interaction with Hoxb1, thereby allowing DNA binding as a trimeric complex in which each component binds its cognate DNA site. These observations support a model in which Hoxb1

functionally interacts with preformed TALE protein heterodimers, perhaps serving as a specificity factor to direct ternary complexes to a subset of enhancers with appropriate binding sites (23).

In summary, our studies provide support at the molecular level for previous observations that each component of the TALE heterodimer interacts and functions on common genetic pathways with a subset of Hox proteins. Although its generality for Hox function remains to be determined, a trimeric model invoking a higher-order assembly of Hox and TALE proteins provides a molecular framework for integrating the functions of these developmentally important proteins.

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