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Meis family proteins are required for hindbrain development in the zebrafish

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SUMMARY

Meis homeodomain proteins function as Hox-cofactors by binding Pbx and Hox proteins to form multimeric complexes that control transcription of genes involved in development and differentiation. It is not known what role Meis proteins play in these complexes, nor is it clear which Hox functions require Meis proteins in vivo. We now show that a divergent Meis family member, Prep1, acts as a Hox co-factor in zebrafish. This suggests that all Meis family members have at least one shared function and that this function must be carried out by a conserved domain. We proceed to show that the Meinox domain, an N-terminal conserved domain shown to mediate Pbx binding, is sufficient to provide Meis activity to a Pbx/Hox complex. We find that this activity is separable from Pbx binding and resides within the M1 subdomain. This finding also presents a rational strategy for interfering with Meis activity in vivo. We accomplish this by expressing the Pbx4/Lzr N-terminus, which sequesters Meis proteins in the cytoplasm away from the nuclear transcription complexes. Sequestering Meis proteins in the cytoplasm leads to extensive loss of rhombomere (r) 3- and r4-specific gene expression, as well as defective rhombomere boundary formation in this region. These changes in gene expression correlate with impaired neuronal differentiation in r3 and r4, e.g. the loss of r3-specific nV branchiomotor neurons and r4-specific Mauthner neurons. We conclude that Meis family proteins are essential for the specification of r3 and r4 of the hindbrain.

Key words: Hox, Pbx, Meis, Prep, Hindbrain, Homeodomain, Rhombomere, Segmentation, Zebrafish

INTRODUCTION

Hox proteins are transcriptional regulators that specify cell fate during early embryonic development and organogenesis (reviewed by Krumlauf, 1994). However, Hox protein monomers display poor specificity and affinity for enhancer sequences, suggesting that they do not act in isolation. Recently, two families of Hox cofactors, Pbx and Meis, belonging to the TALE (Three Amino acid Loop Extension) homeodomain superfamily, were identified (reviewed by Mann and Affolter, 1998). In vitro analyses indicate that Meis and Pbx function by forming multimeric complexes with Hox proteins. In particular, Pbx binds to Hox proteins from paralog group 1-10 (Shen et al., 1997b) and Meis binds to Hox proteins from paralog group 9-13 (Shen et al., 1997a). Meis and Pbx also interact, via the Meinox domain (particularly the M1 and M2 subdomains) in Meis and the PBC-A and PBC-B domains in Pbx (reviewed by Mann and Affolter, 1998), to permit the formation of Meis/Pbx/Hox trimers (Berthelsen et al., 1998a; Jacobs et al., 1999; Ryoo et al., 1999; Shen et al., 1999; Vlachakis et al., 2000). The formation of multimeric complexes improves the affinity and specificity of Hox proteins for particular DNA sequences, potentially explaining the need for Pbx and Meis cofactors (reviewed by Mann and Affolter, 1998). However, given that Hox proteins are transcription factors it seems likely that Meis and Pbx might also contribute functions that regulate the transcriptional activity of the complexes. Indeed, Hox proteins contain activation domains (Di Rocco et al., 1997; Rambaldi et al., 1994; Vigano et al., 1998) that may interact with the coactivator CREB-binding protein (CBP)/p300 (a histone acetyl transferase) (Chariot et al., 1999; Saleh et al., 2000), and Pbx proteins reportedly interact with corepressors such as the histone deacetylases (HDACs) as well as N-CoR/SMRT (Asahara et al., 1999; Saleh et al., 2000). Although no transcription regulatory functions have been found for Meis proteins, the Meis homeodomain is not required for all Meis functions (e.g. Berthelsen et al., 1998a; Vlachakis et al., 2001), suggesting that Meis may also have roles beyond merely enhancing the affinity and specificity of Hox binding to DNA.

An in vivo role for Hox cofactors was first shown by analyzing mutations in the *Drosophila homothorax* (*hth*, the Meis ortholog) (Kurant et al., 1998; Pai et al., 1998; Rieckhof et al., 1997) and *extradenticle* (*exd*, the Pbx ortholog) (Rauskolb et al., 1993) genes. Mutations in either gene lead to posterior transformations of embryonic segments, without affecting the expression of Hox genes, showing that both Exd and Hth are required for Hox protein function during fly

development. Loss-of-function analyses in vertebrates have also revealed a requirement for *pbx* genes in segmentation processes during development. This is seen particularly clearly in the segmented hindbrain where disruption of the *pbx4* gene in the zebrafish *lazarus* mutant (Pöpperl et al., 2000) leads to abnormal segmentation. The *lazarus* phenotype is similar to that observed upon targeted deletion of Hox genes from paralog groups 1 and 2 in the mouse (e.g. Davenne et al., 1999; Gendron-Maguire et al., 1993; Goddard et al., 1996; Lufkin et al., 1991; Rijli et al., 1993; Studer et al., 1996), consistent with a role for Pbx proteins in regulating Hox function in the vertebrate hindbrain. By contrast, although several *meis* genes are expressed in the developing hindbrain (Sagerström et al., 2001; Salzberg et al., 1999; Zerucha and Prince, 2001), no loss-of-function analyses have been reported for *meis* genes to date. Instead, support for *meis* genes acting in hindbrain development come from ectopic expression analyses showing that Meis proteins posteriorize the rostral CNS in *Xenopus* (Salzberg et al., 1999) and cooperate with Pbx and Hox proteins to promote hindbrain fates in zebrafish (Vlachakis et al., 2001). Because vertebrates have several closely related, and perhaps functionally redundant, *meis* genes, loss-of-function analyses for *meis* may best be performed by using dominant negative constructs that interfere with all Meis family members. A basis for dominant negative strategies presents itself by the fact that Meis proteins act as part of larger complexes. These complexes are probably the functional units in vivo, as evidenced by dimers and trimers being detected by co-immunopreciptation from cell extracts (Chang et al., 1997; Ferretti et al., 2000; Knoepfler et al., 1997; Shen et al., 1999). Thus, Meis sites are found adjacent to Pbx and Hox sites in several Hox-dependent promoters (Ferretti et al., 2000; Jacobs et al., 1999; Ryoo et al., 1999) and the Pbx interaction domain of Meis is required for Meis function in vivo (Vlachakis et al., 2001). Therefore, expressing a Meis protein that retains its ability to bind Pbx, but lacks other essential functions, might interfere with endogenous Meis activity. However, attempts at accomplishing this by introducing point mutations into the homeodomain (thereby preventing DNA binding) of zebrafish Meis3 and *Drosophila* Hth (Ryoo et al., 1999; Vlachakis et al., 2001) did not generate a dominant negative protein. Similarly, expressing the Meinox domain of *Xenopus* Meis3 in vivo did not have a dominant negative effect (Salzberg et al., 1999), whereas expressing the Meinox domain of Hth only partially interfered with Hox function in *Drosophila* embryos (Ryoo et al., 1999).

Here we first demonstrate that highly divergent members of the Meis family display the same activity in promoting hindbrain fates, suggesting that conserved regions within Meis family members carry out this function. We proceed to define this essential region and find that it resides within the Meinox domain, a region previously implicated in Pbx binding. The activity of this region, M1, is independent of Pbx binding, suggesting that Meis proteins contribute a distinct activity to the complex. The M1 region does not encode a known motif and we hypothesize that it may interact with an auxiliary protein. This data predicts that, to inhibit Meis function the M1 domain must be removed from the Hox-cofactor complex, and we took advantage of the fact that nuclear localization of zebrafish Meis proteins is mediated by Pbx proteins (Vlachakis et al., 2001). We find that expressing the Pbx4/Lzr N-terminus in zebrafish embryos sequesters Meis proteins in the cytoplasm, thereby keeping them out of transcription complexes in the nucleus. Embryos without nuclear Meis displayed severe defects in hindbrain development. In particular, gene expression specific to rhombomere (r) 3 and r4 was largely lost and rhombomere boundaries do not form properly in this region. Neuronal differentiation in this region was also affected, e.g. nV branchiomotor neurons in r3 and Mauthner neurons in r4 were lost. Our results suggest that the Meis Meinox domain contributes an activity in addition to Pbx binding and show that Meis proteins are required for proper specification of r3 and r4 during hindbrain development.

MATERIALS AND METHODS

Constructs

All genes used were derived from zebrafish and all constructs were verified by sequencing. *meis3, hoxb1b* and *pbx4* expression vectors were described previously (Vlachakis et al., 2001; Vlachakis et al., 2000). All Meis and Prep1 constructs were engineered to contain a MYC-epitope tag. A *prep1* cDNA was obtained as an expressed sequence tag (EST) database clone from Research Genetics (Huntsville, AL). The *prep1* open reading frame (ORF) was amplified by PCR using primers 5′-CCGACCGCTCGAGTTAGTCGCTG-ACGTCTAAACCCAGACCGGG-3′ and 5′-CCCGCCGGAATTCA-TGATGGCTGCCCAGTCTGTGTCC-3′ and subcloned via *Eco*RI/*Xho*I sites in the primers into *pCS2+MT*. In ΔNMeis3, the N-terminal 37 amino acids (aa) of the *meis3* ORF were deleted. Primers 5′-GCGAATTCAGTGCCTGACTCTCTGAAACAC-3′ and 5′-GCTCT-AGATTATCAGTGGGCATGTATGTC-3′ amplified the domain of the *meis3* ORF C-terminal to aa 37, which was subcloned via *Eco*RI and *Xba*I sites in the primers into the *pCS2+MT* vector. In ΔCMeis3, the C-terminal 93 aa of the *meis3* ORF were deleted. Primers 5′-CGGAATTCCATGGATAAGAGGTATGA-3′ and 5′-GCTCTAGA-TTCATGAGCGATTTGTTTGGTCAAT-3′ amplified the N-terminal 322 aa domain of the *meis3* ORF, which was subcloned via *Eco*RI and *Xba*I sites in the primers into the *pCS2+MT* vector. In ΔNCMeis3, both the N-terminal 37 aa and the C-terminal 93 aa of *meis3* ORF were deleted. Primers 5′-GCGAATTCAGTGCCTGACTCTCTGAA-ACAC-3′ and 5′-GCTCTAGATTCATGAGCGATTTGTTTGGTCA-AT-3′ amplified an aa 38-322 domain of *meis3* ORF, which was subcloned via *Eco*RI and *Xba*I sites in the primers into the *pCS2+MT* vector. In ΔHDCMeis3 the C-terminal 191 aa of the *meis3* ORF were deleted by digesting *pCS2+Meis3* with *Pst*I/*Xma*I, inserting oligonucleotide 5′-GATGATAATAGGCGGCCGC-3′ and then moving an *Eco*RI/*Nsi*I fragment into the *pCS2+MT* vector. In ΔNXCMeis3 the N-terminal 37 aa, the C-terminal 93 aa as well as an internal domain, aa 145-253, were deleted. Primers 5′-CCACT-AGTAACCTTTTCTAGTTCTAATAG-3′ and 5′-GGACTAGTAACA-ACAAGAAAAGAGGAATC-3′ amplified *pCS2+MTΔNCMeis3*, which was then digested with *Spe*I (site in the primers) and re-ligated. For ΔIMeis3 the M1 domain was amplified by primers 5′-CGGAATTCCATGGATAAGAGGTATGA-3′ and 5′-CGGCTCGAG-GGAGTCTCGTGGTGAGCAAGT-3′ and digested with *Eco*RI/*Xho*I. The region C-terminal to the I domain was amplified by primers 5′-CGGCTCGAGCTGGATAATCTGATCCAG-3′ and 5′-GCTCTAGA-TTATCAGTGGGCATGTATGTC-3′ and digested with *Xho*I/*Xba*I. The two fragments were then cloned into *pCS2+MT* digested with *Eco*RI/*Xba*I. For C→IMeis3 the C-terminal 56 aa of Prep1 (lacking any known activity) was amplified with primers 5′-CGGCTCGA-GGACGGCTTCCAGGCGCTTTCTTCA-3′ and 5′-CCGCTCGAG-GTCGCTGACGTCTAAACCCAGACC-3′ and cloned into the *Xho*I site of ΔIMeis3. In M1IM2Meis3 the N-terminal 37 aa, and aa 143-415 were deleted by digesting *pCS2+MTΔNXCMeis3* with *Spe*I/*Xba*I

and religating. In BMNPbx4 the N-terminal 171 aa of BM$^{M1/2}$Meis3 were fused in frame with the C-terminal aa 230-344 of the Pbx4 ORF. PCR primers 5′-GGTCTAGACCAGACGTAAGAGACGCAAC-3′ and 5′-GGTCT-AGATCATAGCCTGCCGTCAGGTGT-3′ amplified aa 230-344 of the Pbx4 ORF, which was subcloned into *pCS2+MT* (*pCS2+MTΔpbx4*) via *Xba*I sites in the primers. PCR primers 5′-CGGGATCCCCCGGGATGGCTCCAAAGAAGAAGCGTAAGGT-AAATC-3′ and 5′-GCTCTAGAGTCTTCCAGCACCAAATCAGTG-GG-3′ amplified aa 1-171 of BM$^{M1/2}$Meis3, which was subcloned into *pCS2+MTΔpbx4* via *Bam*HI/*Xba*I sites in the primers. For IPbx4 the I domain was amplified by primers 5′-GCTCTAGATTCTGGATT-TGATGAAAATATGG-3′ and 5′-CGGCTCGAGGAACTTGCCA-CTTGC-3′ and cloned via *Xho*I/*Xba*I sites together with a *Xba*I/*Not*I fragment from BMNPbx4 into the *pCS2+MT* vector cut with *Xho*I/*Not*I. For BM1IPbx4 a BM1+I fragment was amplified with primers 5′-CGGCTCGAGGTGCCTGACTCTCTGAAACAC-3′ and 5′-GCTCTAGATTCTGGATTTGATGAAAATATGG-3′ and cloned via *Xho*I/*Xba*I sites in the primers into IPbx4 cut with *Xho*I/*Xba*I. For ΔCPbx4 the N-terminus of Pbx4 was amplified with primers 5′-GGAATTCTATGGATGATCAGACCCGAATGCTG-3′ and 5′-GGGCTCGAGTCATTCGTGCCATTCGATTTTCTGAGCTTCGA-AGATGCTGTTCAGGCCGGACATGTCGAGGAAGCGGGAGCG-3′ digested with *Eco*RI/*Xho*I and cloned into *pCS2+* (for ΔCPbx4) or *pCS2+MT* (for MycΔCPbx4) digested with *Eco*RI/*Xho*I. This also introduces a biotin tag at the ΔCPbx4 C-terminus.

RNA injections, western blots, immunoprecipitations, in situ hybridization and immunostaining was performed as described previously (Vlachakis et al., 2001).

RESULTS

Divergent Meis family members share the ability to promote hindbrain fates

We have previously demonstrated that Meis3 cooperates with Hoxb1b and Pbx4 to induce hindbrain fates ectopically in the zebrafish (Vlachakis et al., 2001). To better understand the role of Meis proteins in this process we isolated the Meis family member Prep1 from zebrafish and compared it to Meis3. Analyses in mouse and human have shown that *prep1*, although clearly part of the Meis family, represents the most divergent family member identified to date, both in terms of its sequence and its expression pattern (Berthelsen et al., 1998a; Berthelsen et al., 1998b; Ferretti et al., 1999).

A search of the zebrafish EST database revealed several ESTs with sequence homology to murine Prep1. One of these, fc13f10, was obtained and sequenced. Sequence analysis revealed that zebrafish Prep1 has a similar domain structure to other Meis proteins (Fig. 1A; Prep1 Accession Number, AY052752). Prep1 is most similar to Meis3 in the homeodomain (71% identical at the amino acid level) and in the M1 and M2 domains (55% and 86% identical, respectively) that have been implicated in Pbx binding (Knoepfler et al., 1997). Other regions of Prep1, i.e. the N-terminus, the region between the M1 and M2 domains, the C-terminus and the region between the M2 domain and the homeodomain, were less than 26% identical. The fc13f10 Prep1 EST has been mapped to between 52.2 and 52.3 cM from the top of LG9 by the zebrafish mapping consortium.

prep1 transcripts are present in zebrafish embryos from the earliest stage analyzed (1 hour postfertilisation (hpf); Fig. 1B), suggesting that they are maternally deposited. *prep1* mRNA is detectable throughout the embryo, with highest levels at the germ ring during early gastrula stages (6 hpf; Fig. 1D) and dorsally and posteriorly at late gastrula stages (9 hpf; Fig. 1E). During segmentation stages (13 hpf, Fig. 1F; and 25hpf, Fig. 1H) *prep1* expression is detected throughout the embryo at low levels. This expression pattern is distinct from other *meis* genes that show very restricted expression (e.g. to the eyes, finbuds, hindbrain/spinal cord and somites) (Sagerström et al., 2001; Zerucha and Prince, 2001). Indeed, the expression pattern of *prep1* at gastrula and segmentation stages is more similar to that of *pbx4/lzr* (Pöpperl et al., 2000; Vlachakis et al., 2000). A *prep1* sense probe used as a control did not hybridize to embryos at any stage tested (Fig. 1C,G,I).

Our sequence comparison (Fig. 1A) revealed that the M1 and M2 domains, which have been implicated in binding to Pbx, are well conserved between Meis3 and Prep1, suggesting that Prep1 may interact with Pbx proteins in a manner similar to Meis3. To determine whether Prep1 interacts with Pbx4/Lzr, the most prevalent Pbx protein during early zebrafish development (Pöpperl et al., 2000), we used an in vitro co-immunoprecipitation assay. Pbx4/Lzr was expressed alone or together with MYCMeis3 or MYCPrep1 and precipitated with anti-MYC antibody. We find that both MYCMeis3 (Fig. 1J, lane 2) and MYCPrep1 (lane 4) interact with Pbx4/Lzr. The anti-MYC antibody did not crossreact with Pbx4/Lzr (lane 6). We have previously shown that zebrafish Meis3 depends on Pbx proteins for its nuclear localization (Vlachakis et al., 2001), and that this requires an intact Meinox motif in Meis3, consistent with Meis3 interacting with Pbx proteins to access the nucleus in vivo. To determine if Prep1 behaves the same way, we tested its subcellular localization in the presence or absence of co-expressed Pbx4/Lzr. We find that at 5 hpf MYCPrep1 is primarily cytoplasmic in the absence of Pbx4/Lzr (Fig. 1K), but localizes to the nucleus when Pbx4/Lzr is co-expressed (Fig. 1L).

We have previously shown that, although Hoxb1b can interact with Pbx4/Lzr to induce ectopic expression of *hoxb1a* in r2 of the hindbrain, co-expression of Meis3 with Pbx4/Lzr and Hoxb1b leads to ectopic expression of both *hoxb1a* and *hoxb2* in a broad domain, resulting in transformation of the rostral CNS to a hindbrain fate (Vlachakis et al., 2001). To determine whether Prep1 can function to induce hindbrain fates in a manner similar to Meis3, we co-expressed Prep1 with Pbx4/Lzr and Hoxb1b in developing zebrafish embryos and scored for ectopic expression of the *hoxb1a* and *hoxb2* hindbrain genes. Western blot analysis showed that MYCMeis3 and MYCPrep1 were expressed at similar levels (Fig. 1P). Expression of MYCPrep1 or MYCMeis3 by themselves had no effect on *hoxb1a* or *hoxb2* expression (not shown). By contrast, expressing MYCMeis3 or MYCPrep1 together with Pbx4/Lzr and Hoxb1b resulted in massive ectopic expression of both *hoxb1a* (not shown) and *hoxb2* (Fig. 1M-O) anterior to their normal expression domains, leading to anterior truncations. Because Prep1 represents the most divergent Meis family member known, these results suggest that all known members of the zebrafish Meis family, despite differences in sequence and expression pattern, share the ability to promote hindbrain fates.

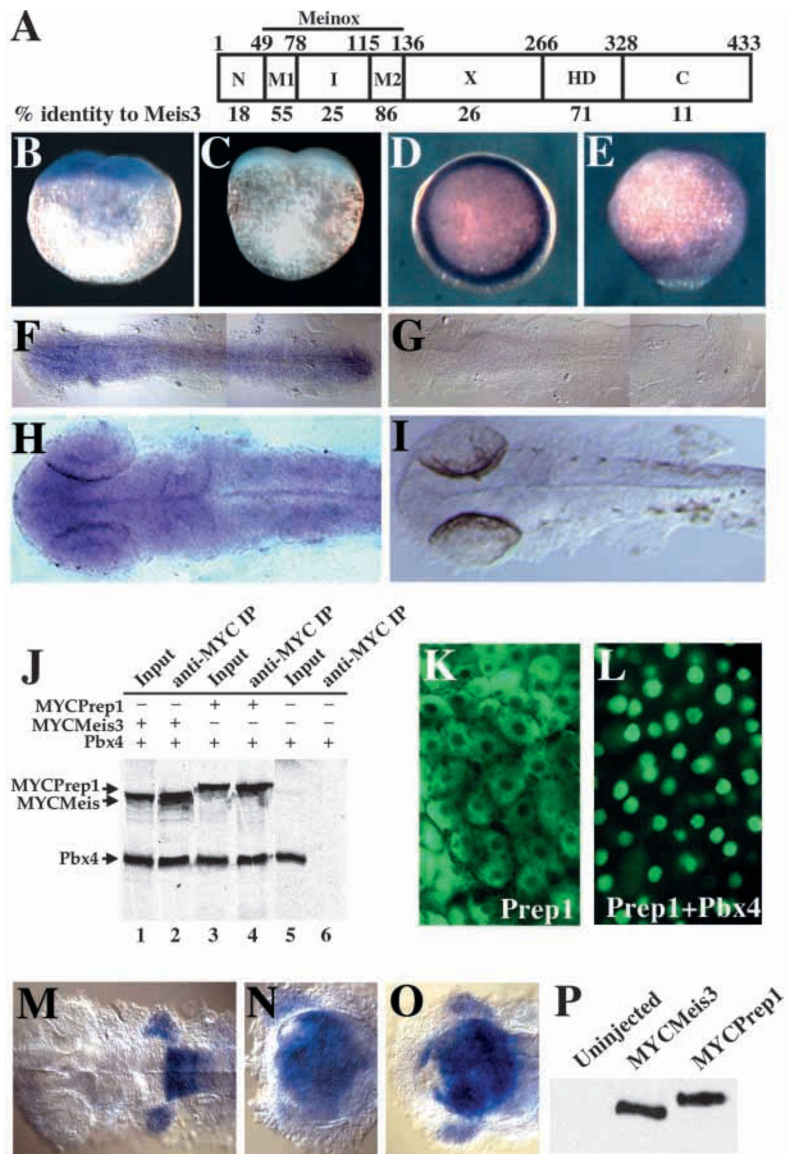

Fig. 1. Prep1 retains functions similar to Meis3. (A) Prep1 protein. Letters indicate the name of individual domains; the Meinox domain includes the M1, I and M2 domains. Numbers on top represent amino acid positions in Prep1 and numbers on the bottom indicate percent identity of each domain between Prep1 and Meis3. (B-I) Expression pattern of *prep1* during zebrafish embryogenesis. An antisense (B,D,E,F,H) or sense (C,G,I) probe for *prep1* was hybridized to zebrafish embryos at the two-cell stage (1 hpf; B,C), early gastrula (6 hpf; D), late gastrula (9 hpf; E), early segmentation (13 hpf; F,G) and late segmentation (25 hpf; H,I). (B,C) Lateral views with animal pole towards the top. (D) An animal pole view. (E) A lateral view with dorsal towards the right and anterior towards the top. (F-I) Dorsal views with anterior towards the left. (J) Prep1 binds to Pbx4/Lzr in vitro. Pbx4/Lzr was in vitro transcribed in the presence of ^{35}S-methionine together with MycMeis3 (lanes 1, 2), MycPrep1 (lanes 3, 4) or by itself (lanes 5, 6), immunoprecipitated with anti-Myc antibody, resolved on a 10% SDS-PAGE gel and exposed to film. (K,L) Prep1 is brought to the nucleus by Pbx4/Lzr. One- to two-cell stage embryos were injected with 300 pg *MycPrep1* mRNA by itself (K) or together with 300 pg *pbx4/lzr* mRNA (L), raised to 5 hpf and stained with anti-Myc antibody. (M-O) Prep1 induces hindbrain fates in the same way as Meis3. One- to two-cell stage embryos were injected with 500 pg *lacZ* RNA (M), *meis3+pbx4+hoxb1b* mRNA (N; 165 pg each), or *prep1+pbx4+hoxb1b* mRNA (O; 165 pg each), raised to 25 hpf and analyzed for *hoxb2* expression by in situ hybridization. All three embryos are dorsal views with anterior to the left. (P) MycMeis3 and MycPrep1 are expressed at similar levels. One- to two-cell stage embryos were injected with 300 pg *MycMeis3* mRNA or *MycPrep1* mRNA, raised to 5 hpf, lysed, resolved on a 10% SDS-PAGE gel, western blotted and probed with anti-Myc antibody.

The Meinox domain is sufficient to mediate the activity of Meis family proteins

Because Prep1 and Meis3 can both promote hindbrain fates, the sequences responsible for this activity must be shared between the two proteins. Meis3 and Prep1 have highest sequence identity in the Meinox domain (consisting of the M1, I and M2 regions) and in the homeodomain. Although this is consistent with Meis proteins mediating their in vivo effects solely by binding Pbx and DNA, thereby perhaps stabilizing Pbx/Hox complexes, it remains possible that other domains in Meis proteins are essential for function, or that the Meinox and homeodomain have activities in addition to Pbx and DNA binding. To determine which domains are necessary for Meis protein function, we generated a series of Meis3 deletion constructs (Fig. 2A) and tested whether they could promote hindbrain fates upon co-expression with Pbx4/Lzr and Hoxb1b in zebrafish embryos.

All constructs shown in Fig. 2A are expressed at comparable levels in vivo as determined by western blotting of lysates from injected embryos (Fig. 3A, lanes 2-10). To determine whether the deletion constructs can still interact with Pbx, we tested to see if they localized to the nucleus following co-expression with Pbx4/Lzr. All constructs shown in Fig. 2A translocated to the nucleus in the presence of Pbx4/Lzr, except for ΔNXCMeis3 (Fig. 3D,E) and ΔIMeis3 (Fig. 3F,G), both of which remained at least partly cytoplasmic. We conclude that, although most constructs interact well with Pbx4/Lzr, ΔNXCMeis3 and ΔIMeis3 do so inefficiently or not at all. We do not think that the Pbx interaction domain was removed in the ΔNXCMeis3 or ΔIMeis3 constructs; rather, that the Pbx binding motif (i.e. the Meinox domain) was interfered with indirectly. This is supported by the observation that removing the homeodomain (HD) from ΔNXCMeis3 (to generate M1IM2Meis3) and inserting an unrelated sequence in place of the I domain of ΔIMeis3 (to generate C→IMeis3) restored Pbx-dependent nuclear localization (Fig. 3H-K).

When expressed alone in zebrafish embryos, none of the constructs in Fig. 2A lead to ectopic expression of *hoxb1a* and *hoxb2*, nor do they affect endogenous gene expression in the hindbrain, showing that they do not have a dominant negative

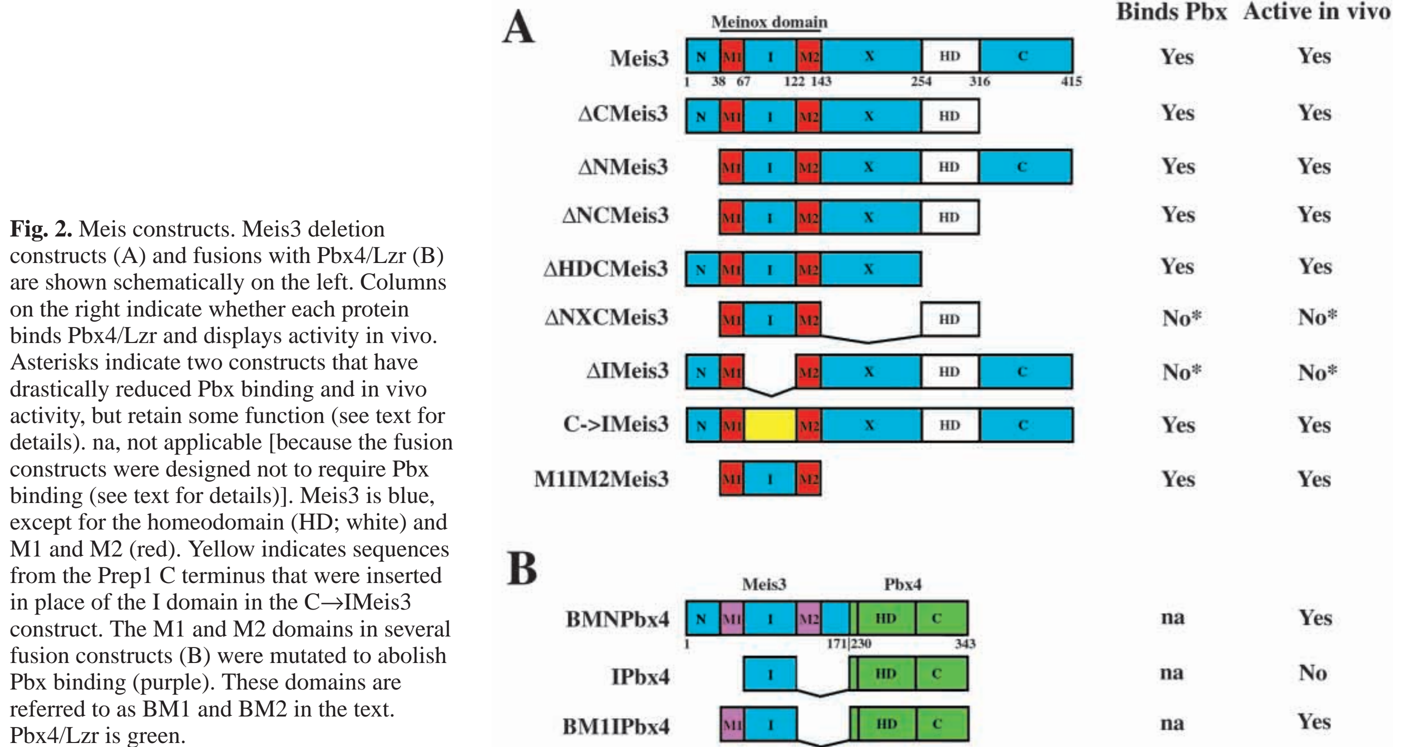

Fig. 2. Meis constructs. Meis3 deletion constructs (A) and fusions with Pbx4/Lzr (B) are shown schematically on the left. Columns on the right indicate whether each protein binds Pbx4/Lzr and displays activity in vivo. Asterisks indicate two constructs that have drastically reduced Pbx binding and in vivo activity, but retain some function (see text for details). na, not applicable [because the fusion constructs were designed not to require Pbx binding (see text for details)]. Meis3 is blue, except for the homeodomain (HD; white) and M1 and M2 (red). Yellow indicates sequences from the Prep1 C terminus that were inserted in place of the I domain in the C→IMeis3 construct. The M1 and M2 domains in several fusion constructs (B) were mutated to abolish Pbx binding (purple). These domains are referred to as BM1 and BM2 in the text. Pbx4/Lzr is green.

effect (not shown). When co-expressed with Pbx4/Lzr and Hoxb1b, each of the constructs generated phenotypes quantitatively and qualitatively similar to those seen when wild-type Meis3 is co-expressed with Pbx4/Lzr and Hoxb1b. In particular, they promote ectopic *hoxb1a* and *hoxb2* expression as well as anterior truncations (Fig. 3; compare P,T with O,S; Table 1). However, the ΔNXCMeis3 and ΔIMeis3 constructs were less effective and rarely displayed the type of anterior truncations indicative of the rostral CNS being transformed to a hindbrain fate (Table 1). This result is probably due to reduced Pbx binding by these constructs (see above), rather than to the homeodomain or I domain being required for function. Indeed, the M1IM2Meis3 (with the HD deleted) and C→IMeis3 (with the I domain replaced) constructs, which bind Pbx4/Lzr well, retain high activity (Fig. 3P,T; Table 1). We conclude that the Meinox domain is sufficient to provide Meis activity in this ectopic expression system. Because we find that the sequence of the I region is irrelevant for Meis activity, we also suggest that the I region serves primarily to space the M1 and M2 domains properly, and that the sequences essential for Meis activity reside within the M1 or M2 domains, or both.

The Meinox domain contributes a function in addition to Pbx binding

Our results show that the Meinox domain is sufficient to confer Meis activity to Pbx/Hox complexes, but it is unclear exactly what function is provided by this domain. Because Meis proteins use the Meinox domain to bind Pbx, it is possible that the function provided by the M1IM2Meis3 construct is simply Pbx binding, perhaps because it thereby stabilizes the Pbx/Hox complex.

To test this possibility, we set out to determine if a Meinox domain lacking the ability to bind Pbx still retains activity. To carry out this experiment it became necessary to devise a means for the Meinox domain to participate in Pbx/Hox complexes without being able to interact with Pbx (Fig. 2B). We replaced the N-terminus of Pbx4/Lzr (containing the PBC-A and PBC-B domains required for Meis binding) with the Meis N-terminus (containing the Meinox domain). This eliminates the normal interaction between the Meinox domain and Pbx4/Lzr, but as the chimeric protein retains the Hox interaction motif in Pbx4/Lzr, it still ensures that the Meinox domain is part of the Pbx/Hox transcription complex bound to DNA. Notably, as this construct lacks the PBC-A and PBC-B domains, it can not bind endogenous Meis proteins. To also eliminate the ability of this construct to bind endogenous Pbx proteins, we used a Meinox domain that contains multiple amino acid substitutions in the M1 (aa 64-67 KCEL→NNSQ) and M2 (L141→A; E142→A) motifs. We have previously shown that this mutated Meinox domain can not bind to Pbx4/Lzr in vivo (Vlachakis et al., 2001) and we confirmed that the resulting fusion protein, BMNPbx4, does not bind endogenous Pbx by performing co-immunoprecipitations on lysates from embryos expressing BMNPbx4 (Fig. 3V). To ensure that BMNPbx4 localizes to the nucleus, we also introduced a nuclear localization signal (NLS) at its N-terminus.

BMNPbx4 is expressed at similar levels to Meis3 following microinjection (Fig. 3A, compare lanes 2 and 11) and localizes to the nucleus (Fig. 3L), as expected. Expression of BMNPbx4 alone resulted in embryos with normal expression of *hoxb1a* and *hoxb2* (not shown), whereas co-injection with Hoxb1b resulted in embryos exhibiting ectopic *hoxb1a* (Fig. 3Q) and *hoxb2* (Fig. 3U). This phenotype was qualitatively and quantitatively similar to the phenotype produced by expressing

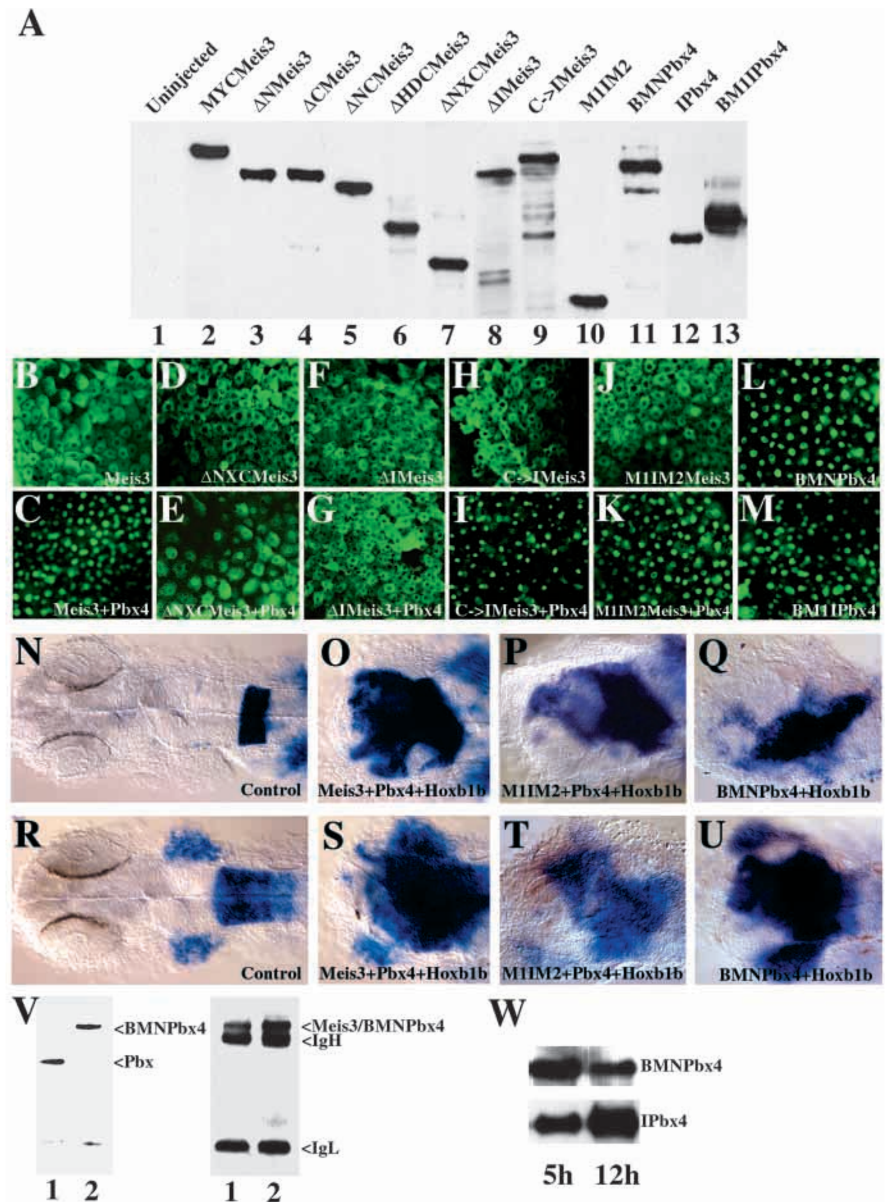

Fig. 3. The M1 domain is sufficient to confer Meis activity. (A) All constructs used are expressed at comparable levels in embryos. One- to two-cell stage embryos were injected with 300 pg of each mRNA encoding Myc-tagged constructs as indicated at the top of each lane. Embryos were raised to 5 hpf, lysed, resolved on a 10% SDS-PAGE gel, western blotted and probed with anti-Myc antibody. (B-M) Analysis of Pbx4/Lzr-mediated nuclear localization of Meis constructs. One- to two-cell stage embryos were injected with 300 pg of each mRNA as indicated at the bottom right of each panel, raised to 5 hpf and stained with anti-Myc antibody. All Meis constructs were Myc-tagged, whereas Pbx4/Lzr was untagged. (N-U) Analysis of in vivo activity of Meis constructs. One- to two-cell stage embryos were injected with 500 pg *lacZ* RNA (control) or 165 pg of each mRNA as indicated in the lower right corner of each panel, raised to 25 hpf and analyzed for expression of *hoxb1a* (N-Q) or *hoxb2* (R-U) by in situ hybridization. All embryos are dorsal views with anterior to the left. (V) Meis3-Pbx4 fusion constructs do not bind endogenous Pbx. One- to two-cell stage embryos were injected with 300 pg *MycMeis3* (lane 1) or *MycBMNPbx4* (lane 2) and raised to 10 hpf. Embryos were lysed, immunoprecipitated with anti-Myc, resolved on a 10% SDS-PAGE gel, western blotted and probed with anti-Pbx4 antiserum (left panel) or anti-Myc antiserum (right panel). Note that the BMNPbx4 fusion protein in lane 2 of the left-hand panel is detected by the anti-Pbx4 antiserum. MycMeis3 and BMNPbx4 are the same size. IgH, antibody heavy chain; IgL, antibody light chain. (W) Meis3-Pbx4 fusion proteins remain stable at 12 hpf. One- to two-cell stage embryos were injected with 300 pg *MycBMNPbx4* or *MycIPbx4* mRNA and harvested at 5 hpf or 12 hpf. Embryos were lysed, and three embryo equivalents were resolved on a 10% SDS-PAGE gel, western blotted and probed with anti-Myc antiserum.

the Meinox domain together with Pbx4/Lzr and Hoxb1b (Fig. 3P,T; Table 1). This result indicates that the BMNPbx4 chimera now contains the combined activities of Pbx4/Lzr and Meis3.

Additional constructs were generated to better delineate the region of the Meis3 N-terminus required for this activity. We first generated a construct containing only the I domain fused to Pbx4/Lzr. This construct (IPbx4; Fig. 2B) is expressed at the same level as Meis3 following injection (Fig. 3A, lane 12) and localizes to the nucleus (not shown). IPbx4 lacks in vivo activity (Table 1), confirming that the I domain is not required for function and also showing that simply fusing sequences to the Pbx4/Lzr C-terminus is not sufficient for activity. We then added the M1 domain (containing the same amino acid substitutions as in BMNPbx4) onto the IPbx4 construct to generate BM1IPbx4 (Fig. 2B). This construct is expressed at the same level as other constructs (Fig. 3, lane 13) and localizes to the nucleus (Fig. 3M). BM1IPbx4 has no effect when expressed by itself (not shown), but leads to ectopic *hoxb1a* and *hoxb2*, as well as anterior truncation similar to those seen with the BMNPbx4 construct, when co-expressed with Hoxb1b (Table 1). On the basis of the data from the deletion analysis and the chimeric constructs, we conclude that the Meinox domain has a function in addition to Pbx binding and that the M1 domain is sufficient for this function, at least in our ectopic expression system. We do not think that the M1 domain acts by stabilizing the fusion protein, because a fusion protein lacking the M1 domain (IPbx4) does not appear to be less stable over time in vivo than one that retains the M1 domain (BMNPbx4; Fig. 3W). Instead, we speculate that the M1 domain may serve as a binding site for an auxiliary protein.

Expression of the Pbx4/Lzr N-terminus sequesters Meis proteins in the cytoplasm

Our finding that the M1 domain is sufficient for Meis activity provides a rationale for a dominant negative strategy. In

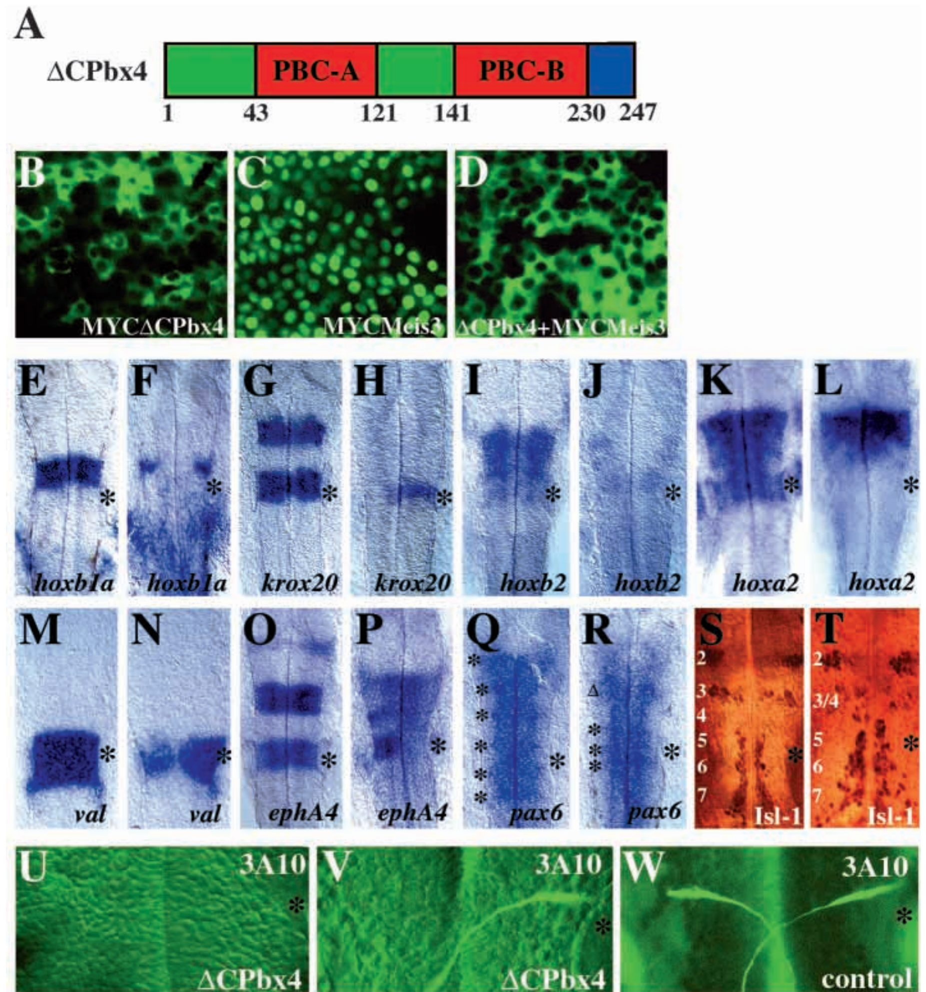

Fig. 4. Loss of Meis function disrupts hindbrain development. (A) ΔCPbx4 construct with amino acid positions indicated at the bottom. The red boxes indicate the PBC-A and PBC-B domains. The blue domain represents a biotin tag introduced at the C terminus. (B-D) ΔCPbx4 sequesters Meis3 in the cytoplasm. One- to two-cell stage embryos were injected with 300 pg of *MycΔCPbx4* (B), *MycMeis3* (C) or *ΔCPbx4 +MycMeis3* (D), raised to 12 hpf and stained with anti-Myc antibody. (E-R) ΔCPbx4 affects gene expression in the hindbrain. One- to two-cell stage embryos were injected with 300 pg of *ΔCPbx4* mRNA (F,H,J,L,N,P,R) or *lacZ* mRNA (E,G,I,K,M,O,Q), raised to 14 hpf (M,N) or 24 hpf (E-L,O-R) and analyzed by in situ hybridization for the genes indicated at the bottom of each panel. Black asterisks indicate the level of the otic vesicle on the right side of each embryo. Black asterisks on left side in Q, and R indicate rhombomere boundaries. Black triangle in R indicates region of strong *pax6* expression. (S-W) ΔCPbx4 affects neuronal differentiation. One- to two-cell stage embryos were injected with 300 pg of *ΔCPbx4* mRNA (S,U,V) or *lacZ* mRNA (T,W), raised to 48 hpf (S,T) or 28 hpf (U-W) and stained with anti-islet (S,T) or 3A10 (U-W) antibody. Black asterisks indicate the otic vesicle and rhombomeres are numbered on the left.

particular, it might not be sufficient to eliminate the DNA binding capacity of Meis to generate a dominant negative construct because such a construct will retain the M1 domain. Instead, we set out to devise a strategy where the M1 domain is kept out of Pbx/Hox complexes. Specifically, as the M1 domain is also involved in Pbx binding, we hypothesized that expressing a construct that sequesters Meis proteins away from Pbx/Hox complexes might act in a dominant negative fashion. To test this possibility we generated a construct expressing only the N-terminus of Pbx4/Lzr, containing the PBC-A and PBC-B domains required for binding to Meis, but lacking the motifs required for binding Hox proteins and for nuclear localization (Fig. 4A). We observed that this construct (MycΔCPbx4) was cytoplasmically located at 12 hpf following expression in zebrafish embryos (Fig. 4B). By contrast, injected MycMeis3 is found exclusively in the nucleus at this stage of development (Fig. 4C), probably as a result of nuclear transport by endogenous Pbx, which has become highly expressed by this stage (Vlachakis et al., 2001). Strikingly, when ΔCPbx4 is co-expressed with MycMeis3, MycMeis3 is found primarily in the cytoplasm (Fig. 4D). These data are consistent with ΔCPbx4 competing with endogenous Pbx proteins for binding to Meis3 in the cytoplasm and subsequently retaining Meis3 in the cytoplasm. This result raises the possibility that ΔCPbx4 might act in a dominant negative fashion by keeping Meis proteins out of nuclear Pbx/Hox complexes.

Meis function is required for proper formation of r3 and r4 during hindbrain development

To test if cytoplasmic retention of endogenous Meis proteins results in developmental defects, we expressed ΔCPbx4 in developing zebrafish embryos. Because Meis3 acts together with Pbx4/Lzr and Hoxb1b to promote r4 fates when expressed ectopically (Vlachakis et al., 2001), we first tested whether ΔCPbx4 interfered with endogenous gene expression in r4. We find that *hoxb1a* (Fig. 4F) expression was reduced or absent in 93% of *ΔCPbx4*-injected embryos (Table 2), consistent with a role for Meis proteins in regulating gene expression in r4, whereas embryos injected with an equivalent amount of *lacZ* RNA (Fig. 4E) were unaffected. Expression of *hoxb1a* was affected in 83% (72/87; not shown) of *ΔCPbx4* injected embryos already at the end of gastrulation, suggesting that Meis proteins are required for *hoxb1a* expression soon after its onset. This is consistent with reports that expression of murine *hoxb1* (the ortholog of zebrafish *hoxb1a*) is dependent on Hox activity (Pöpperl et al., 1995). By contrast, expression of *hoxb1b*, which precedes *hoxb1a* expression and is the earliest *hox* gene expressed in zebrafish, was unaffected by ΔCPbx4 (not shown), indicating that expression of *hoxb1b* is independent of Meis

Table 1. Activity of Meis deletion and fusion constructs

	Outcome (%)			
Injected RNA*	Unaffected	Ectopic staining†	Ectopic staining/ truncated axis‡	Probe
pbx4+hoxb1b	37 (18/49)	63 (31/49)	0 (0/49)	*hoxb1a*
	93 (40/43)	7 (3/43)	0 (0/43)	*hoxb2*
meis3+pbx4+hoxb1b	7 (4/61)	49 (30/61)	44 (27/61)	*hoxb1a*
	3 (2/57)	46 (26/57)	51 (29/57)	*hoxb2*
ΔNMeis3+pbx4+hoxb1b	10 (8/80)	40 (32/80)	50 (40/80)	*hoxb1a*
	12 (11/93)	34 (32/93)	54 (50/93)	*hoxb2*
ΔCMeis3+pbx4+hoxb1b	7 (6/85)	62 (53/85)	31 (26/85)	*hoxb1a*
	20 (22/110)	49 (54/110)	31 (34/110)	*hoxb2*
ΔNCMeis3+pbx4+hoxb1b	15 (34/228)	63 (143/228)	22 (51/228)	*hoxb1a*
	16 (31/192)	48 (92/192)	36 (69/192)	*hoxb2*
ΔHDCMeis3+pbx4+hoxb1b	25 (36/141)	42 (59/141)	33 (46/141)	*hoxb1a*
	34 (40/117)	37 (43/117)	29 (34/117)	*hoxb2*
ΔNXCMeis3+pbx4+hoxb1b	40 (46/116)	58 (68/116)	2 (2/116)	*hoxb1a*
	77 (63/82)	23 (19/82)	0 (0/82)	*hoxb2*
ΔIMeis3+pbx4+hoxb1b	24 (13/54)	74 (40/54)	2 (1/54)	*hoxb1a*
	69 (37/54)	31 (17/54)	0 (0/54)	*hoxb2*
C→IMeis3+pbx4+hoxb1b	40 (33/83)	29 (24/83)	31 (26/83)	*hoxb1a*
	52 (33/64)	27 (17/64)	22 (14/64)	*hoxb2*
M1IM2Meis3+pbx4+hoxb1b	21 (37/175)	58 (101/175)	21 (37/175)	*hoxb1a*
	51 (61/119)	34 (40/119)	15 (18/119)	*hoxb2*
BMNPbx4+hoxb1b	27 (48/179)	60 (108/179)	13 (23/179)	*hoxb1a*
	55 (96/176)	38 (67/176)	7 (13/176)	*hoxb2*
BM1IPbx4+hoxb1b	38 (31/82)	39 (32/82)	23 (19/82)	*hoxb1a*
	40 (27/68)	25 (17/68)	35 (24/68)	*hoxb2*
IPbx4+hoxb1b	100 (194/194)	0 (0/194)	0 (0/194)	*hoxb1a*
	99 (202/203)	0 (0/203)	1 (1/203)	*hoxb2*

*One- to two-cell stage embryos were injected with the indicated mRNAs, fixed at 25 hpf and analyzed by in situ hybridization for *hoxb1a* and *hoxb2* expression.

†Embryos showing normal morphology but ectopic gene expression. Note that *pbx4+hoxb1b* induces ectopic expression of *hoxb1a* in r2, but not elsewhere, and has a minimal effect on *hoxb2* expression or embryo morphology.

‡Embryos with anterior truncations and ectopic gene expression.

function. ΔCPbx4 also interfered with gene expression in r3 at a frequency similar to r4, as illustrated by *krox20*, which was affected in r3 in 81% of *ΔCPbx4*-injected embryos (Fig. 4G,H; Table 2). Other genes whose expression domains include r3 and r4 were also affected. For instance, *hoxb2* expression was affected in r3 and r4 in 95% (Fig. 4I,J; Table 2) and *hoxa2* expression was affected in r3-r5 in 72% (Fig. 4K,L; Table 2) of *ΔCPbx4*-injected embryos. Other rhombomeres appear to be less affected. In particular, although *hoxa2* expression (Fig. 4K,L; Table 2) is affected in r3-r5, it is largely normal in r2 of *ΔCPbx4*-injected embryos. In addition, although *krox20* and *ephA4* expression (Fig. 4G,H,O,P; Table 2) is strongly affected in r3 (42% and 16% lack expression, respectively), these genes are less affected in r5 (only 1-2% lack expression). Furthermore, *hoxb3* and *valentino* expression is only mildly affected in r5 and r6 and no *ΔCPbx4*-injected embryos lacked expression of these genes (Fig. 4M,N; Table 2). Analysis of gene expression outside the hindbrain showed that the forebrain and midbrain (*otx2*), midbrain-hindbrain boundary (*pax2.1*) and somites (*MyoD, hoxb3*) were essentially normal (not shown). We conclude that r3 and r4 do not develop properly in the presence of ΔCPbx4, which is consistent with the formation of these rhombomeres requiring Meis proteins.

We next analyzed expression of *pax6*, which is present throughout the hindbrain, but also outlines rhombomere boundaries (black asterisks on left in Fig. 4Q). Expression of *pax6* reveals six boundaries in control embryos (Fig. 4Q), but in most ΔCPbx4-injected embryos only three boundaries are observed (black asterisks on left in Fig. 4R). Using the otic vesicle as a landmark (black asterisk on right), we conclude that these boundaries correspond to r4/r5, r5/r6 and r6/r7. Sometimes we also observed a strongly staining region in the rostral hindbrain (black triangle in Fig. 4R) of ΔCPbx4-injected embryos. This domain may correspond to the r2/r3 boundary, in agreement with r2 retaining normal *hoxa2* expression. Thus, boundary formation in the rostral hindbrain is affected. We also observed that *ephA4* expression was occasionally (~10% of affected embryos) found at low levels throughout the hindbrain of ΔCPbx4 injected embryos (compare Fig. 4P with 4O). This expression level is similar to that normally seen in r1 and may indicate that r1-specific gene expression expands caudally when rhombomere formation is interrupted, although this remains speculative in the absence of r1-restricted markers.

To explore further the effect of ΔCPbx4 on r3 and r4 development, we analyzed neuronal differentiation in this

Table 2. Effect of ΔCPbx4 on hindbrain gene expression*

		Effect (%)		
Gene	Rhombomere	Normal	Partial†	Absent‡
ephA4	r3	28 (30/106)	56 (59/106)	16 (17/106)
	r5	78 (83/106)	21 (22/106)	1 (1/106)
hoxa2	r2	99 (106/107)	1 (1/107)	0 (0/107)
	r3-r5	28 (30/107)	66 (71/107)	6 (6/107)
krox20	r3	19 (17/88)	39 (34/88)	42 (37/88)
	r5	49 (43/88)	49 (43/88)	2 (2/88)
hoxb2	r3-r5	5 (4/78)	78 (61/78)	17 (13/78)
hoxb1a	r4	7 (4/60)	80 (48/60)	13 (8/60)
hoxb3	r5-r6	33 (26/78)	67 (52/78)	0 (0/78)
valentino	r5-r6	26 (49/192)	74 (143/192)	0 (0/192)

*300 pg of *ΔCPbx4* mRNA was injected at the one- to two-cell stage, embryos were harvested at 24 hpf (except for *valentino* and *hoxb3*, which were harvested at 14 hpf) and assayed by in situ hybridization for the expression of the indicated gene. For genes expressed in more than one nonadjacent rhombomere, the rhombomeres are scored separately. For genes expressed in more than one adjacent rhombomere, the rhombomeres are scored together because of the difficulty in unequivocally assigning rhombomere boundaries, except for *hoxa2* where the anteriormost domain (r2) was clearly regulated differently. A comparable number of embryos injected with 300 pg control mRNA (*lacZ*) and assayed for expression of each gene showed >98% normal staining.

†Partial gene expression is defined as loss of gene expression within a portion of a rhombomere.

‡Absence of gene expression indicates that no expression was detectable within a rhombomere.

region. Both the primary reticulospinal neurons and the branchiomotor neurons display a segment-specific distribution in the hindbrain, permitting us to characterize the effect of ΔCPbx4 on neuronal differentiation in individual rhombomeres. We find that 73% (30/41) of ΔCPbx4-injected embryos lack one or both r4-specific Mauthner neurons (Fig. 4U-W). Using an anti-Islet1 antibody we also observed an effect on branchiomotor neurons in 70% (21/30) ΔCPbx4-injected embryos. This effect is strongest in r3, as most embryos lack nV branchiomotor neurons on at least one side of the midline in r3 (Fig. 4S,T). Because there are only a few islet-1 positive cells in r4 it is difficult to determine whether it is affected, although this region occasionally seems to be reduced in size, in agreement with the observed loss of r4 Mauthner neurons. nVII neurons in r6 and r7 are also affected, although less severely, perhaps as a result of these neurons originating in r4 before migrating to r6 and r7 (Chandrasekhar et al., 1997). By contrast, nV neurons in r2 are largely unaffected. These results are consistent with the observed effect of ΔCPbx4 on gene expression and suggest that specification of r3 and r4 is particularly dependent on Meis function.

To confirm that this phenotype is specific, we attempted to rescue *ΔCPbx4*-injected embryos by co-expressing *pbx4/lzr* mRNA. We expected Pbx4/Lzr to compete with ΔCPbx4 for Meis binding in the cytoplasm and bring Meis proteins to the nucleus where they could interact with Hox proteins and activate transcription. We find that expressing *pbx4/lzr* mRNA, along with *ΔCPbx4* mRNA, rescued *hoxb1a* expression to virtually normal levels in all embryos (43/43). We attribute this high frequency of rescue to ΔCPbx4 not entering the nucleus. Thus, once Meis proteins have entered the nucleus together with Pbx4/Lzr, they are inaccessible to the ΔCPbx4 dominant negative protein. We also used the *BMNPbx4* construct to rescue *ΔCPbx4*-injected embryos. Because BMNPbx4 does not interact with Pbx, it should not be affected by the ΔCPbx4 dominant negative construct. Furthermore, as it contains the M1 domain it should be able to rescue Meis activity in ΔCPbx4-expressing embryos. We find that expression of *BMNPbx4* together with *ΔCPbx4* restores *hoxb1a* expression in all embryos (30/30), but that the rescued expression is less complete than following rescue with *pbx4/lzr*. We attribute this difference to BMNPbx4 being less active than wild-type Meis3 in vivo (Table 1). This result further shows that the effect of ΔCPbx4 is because of its interference with endogenous Meis activity.

DISCUSSION

Meis family proteins have been implicated as Hox cofactors (reviewed by Mann and Affolter, 1998), but a requirement for Meis proteins during vertebrate embryonic development has not been established, primarily because of the lack of an appropriate loss-of-function approach. We first showed that two divergent members of the Meis family display similar activities in vivo. We then showed that the M1 domain is sufficient for this function. The M1 domain resides within the Meinox domain, in close proximity to the Pbx interaction domain, but this activity is independent of Pbx binding. We used the Pbx4/Lzr N-terminus, containing the Meis interaction domain, to sequester Meis family proteins in the cytoplasm, thereby preventing them from acting in transcriptional complexes in the nucleus. We found that sequestering Meis proteins in the cytoplasm leads to developmental defects in the hindbrain. In particular, gene expression, boundary formation and neuronal differentiation was disrupted in r3 and r4. Our results are consistent with Meis family proteins being required for development of the hindbrain, particularly r3 and r4.

What role do Meis proteins play in the multimeric transcription complexes?

Several reports have shown that Meis, Pbx and Hox proteins can interact to form trimeric complexes (Berthelsen et al., 1998a; Ferretti et al., 2000; Jacobs et al., 1999; Ryoo et al., 1999; Shen et al., 1999; Vlachakis et al., 2000) and that Hox and Meis need to interact with Pbx to function in vivo (Vlachakis et al., 2001). Although these data suggest that Meis/Pbx/Hox complexes exist in vivo, the role that each protein plays within the complex remains unclear. Possible roles for Hox and Pbx proteins derive from their interaction with transcriptional coactivators (Chariot et al., 1999; Saleh et al., 2000) and corepressors (Asahara et al., 1999; Saleh et al., 2000). The absence of such interactions for Meis proteins has led to the suggestion that they stabilize Pbx/Hox complexes by binding both to DNA and to Pbx. In possible disagreement with this hypothesis, it has been found that, although Meis proteins require an intact Pbx interaction domain, they do not require an intact homeodomain to synergize with Pbx and Hox proteins (e.g. Berthelsen et al., 1998a; Vlachakis et al., 2001), although this has only been analyzed during conditions of Meis overexpression. In this report we identify a domain essential

for function near the Pbx interaction motif of Meis3. By mutating residues required for Pbx binding and transferring the domain from Meis3 onto Pbx4/Lzr, we show that this activity is retained even when Pbx binding is abolished. We interpret our results to mean that Meis proteins contribute an activity to the multimeric complexes in addition to stabilization. Because this domain does not contain any known motifs we hypothesize that it serves as a binding site for an auxiliary protein required for transcription activity.

Furthermore, if Meis proteins serve only to stabilize Pbx/Hox complexes it should be possible to generate a dominant negative form of Meis by disrupting DNA binding while retaining Pbx binding. We did not observe reproducible dominant negative phenotypes using such constructs (Vlachakis et al., 2001) (N. V. and C. G. S., unpublished), and although a similar construct does not have an effect in *Xenopus* embryos (Salzberg et al., 1999), expressing a homeodomain-less Hth construct in *Drosophila* has a mild dominant negative effect on Hox-dependent functions (Ryoo et al., 1999). Our identification of a required domain adjacent to the Pbx interaction domain explains these results given that constructs lacking the homeodomain will retain the M1 domain and will not be strongly dominant negative. Our results instead support the idea that to interfere with Meis function, this essential domain must be kept out of the multimeric complexes.

For what Hox-dependent processes are Meis proteins required?

Our experiments reveal a role for Meis proteins in the development of the hindbrain, particularly r3 and r4. Notably, this region of the hindbrain expresses Hox genes only from paralog group 1 and 2, and the phenotype we observe is similar to that of mice lacking paralog group 1 and 2 Hox genes (Barrow and Capecchi, 1996; Davenne et al., 1999; Studer et al., 1996). Because expression of paralog group 1 and 2 Hox genes is controlled by Hox proteins acting in an auto- and cross-regulatory fashion, we suggest that Meis proteins are essential cofactors for Hox proteins in this capacity. Although both murine *hoxb1* and *hoxb2* have Meis binding sites adjacent to Hox and Pbx binding sites in their enhancers (Ferretti et al., 2000; Jacobs et al., 1999), the Meis site in the *hoxb1* enhancer is not essential for expression (Ferretti et al., 2000). These data may indicate that, although Meis proteins are required for both *hoxb1* and *hoxb2* expression, binding to the Meis site is dispensable for *hoxb1* expression.

Our results also indicate that *hoxb1a* and *hoxb2* expression is dependent on Meis, whereas *hoxb1b* expression is not. This finding correlates with the fact that *hoxb1b* (the zebrafish counterpart to murine *hoxA1*) is the earliest Hox gene expressed in zebrafish. Because there are no other Hox proteins present to regulate initial *hoxb1b* expression, it is possible that its expression is regulated by a Hox-independent mechanism, and that Meis proteins are therefore not required. Once *hoxb1b* is expressed it may then act with *meis* and *pbx* to crossregulate the transcription of later expressed Hox genes. Indeed, we have shown that co-expression of Hoxb1b with Meis3 and Pbx4/Lzr is sufficient to induce ectopic *hoxb1a* and *hoxb2* expression in zebrafish (Vlachakis et al., 2001) and murine *hoxA1* probably regulates directly the expression of *hoxB1* (the murine counterpart to zebrafish *hoxb1a*) (Pöpperl et al., 1995).

Meis proteins may also be required for the proper formation of other structures. For instance, although r2 retains *hoxa2* expression in *ΔCPbx4*-injected embryos, it occasionally also expresses ectopic *ephA4* and there may be similar subtle effects on more caudal rhombomeres, as well as on regions outside the hindbrain. Furthermore, because our dominant negative approach relies on the ΔCPbx4 construct binding to Meis, any Meis functions that are independent of Pbx binding would not be detected in our experiments.

The phenotype we observe as a result of interfering with Meis activity is also qualitatively similar to that of the *lazarus* mutant (which carries a mutation in the *pbx4* gene) (Pöpperl et al., 2000). Particularly, in both cases gene expression is affected primarily in r3 and r4 and less in r1, r2 or r5-r7. This suggests that Pbx and Meis function in the same pathway during hindbrain development. This is consistent with work in *Drosophila*, where the phenotypes of *hth* and *exd* mutants are largely indistinguishable (Kurant et al., 1998; Pai et al., 1998; Rieckhof et al., 1997) and the genes are thought to act in the same pathway. An explanation for Meis and Pbx acting in the same pathway in the hindbrain probably comes from Meis proteins not interacting directly with Hox proteins expressed in the hindbrain (primarily paralog group 1-4), whereas Pbx proteins do. Therefore, Meis proteins can only act as Hox cofactors in the hindbrain by binding to Pbx. Our finding that Meis and Pbx loss-of-function give similar hindbrain phenotypes is therefore consistent with all hindbrain Hox functions that require Pbx also requiring Meis. However, although the *meis* loss-of-function and *lazarus* phenotypes are qualitatively similar, they differ quantitatively. Surprisingly, we observe both a higher frequency and a more severe effect on hindbrain gene expression in the absence of Meis function than reported for the *lazarus* mutant. We speculate that this is unlikely to be a result of Pbx-independent effects of Meis proteins on Hox function, but may instead stem from the presence of maternal *pbx4/lzr* transcript, as well as additional *pbx* genes expressed in the *lazarus* mutant (Pöpperl et al., 2000). If this is correct, complete removal of Pbx activity might be required to conclusively define the relative roles of Pbx and Meis in regulating Hox function.

Note added in press

While this work was under review two other manuscripts reporting Meis loss of function phenotypes were published (Dibner et al., 2001; Waskiewicz et al., 2001).

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