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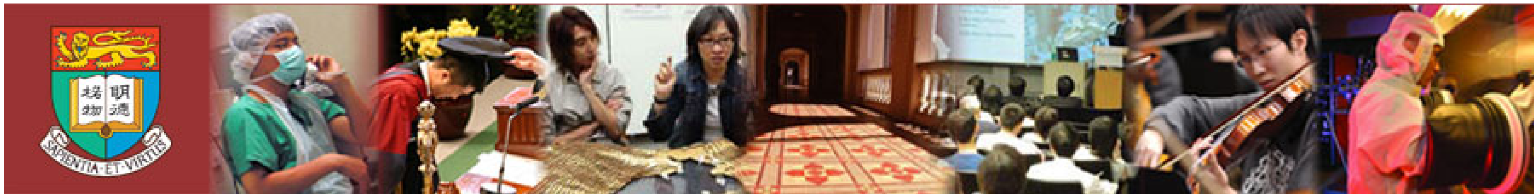

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Auto/Cross-Regulation of *Hoxb3* Expression in Posterior Hindbrain and Spinal Cord

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The complex and dynamic pattern of *Hoxb3* expression in the developing hindbrain and the associated neural crest of mouse embryos is controlled by three separate cis-regulatory elements: element I (region A), element IIIa, and the r5 enhancer (element IVa). We have examined the cis-regulatory element IIIa by transgenic and mutational analysis to determine the upstream trans-acting factors and mechanisms that are involved in controlling the expression of the mouse *Hoxb3* gene in the anterior spinal cord and hindbrain up to the r5/r6 boundary, as well as the associated neural crest which migrate to the third and posterior branchial arches and to the gut. By deletion analysis, we have identified the sequence requirements within a 482-bp element III482. Two Hox binding sites are identified in element III482 and we have shown that *in vitro* both Hoxb3 and Hoxb4 proteins can interact with these Hox binding sites, suggesting that auto/cross-regulation is required for establishing the expression of *Hoxb3* in the neural tube domain. Interestingly, we have identified a novel GCCAGGC sequence motif within element III482, which is also required to direct gene expression to a subset of the expression domains except for rhombomere 6 and the associated neural crest migrating to the third and posterior branchial arches. Element III482 can direct a higher level of reporter gene expression in r6, which led us to investigate whether *kreisler* is involved in regulating *Hoxb3* expression in r6 through this element. However, our transgenic and mutational analysis has demonstrated that, although kreisler binding sites are present, they are not required for the establishment or maintenance of reporter gene expression in r6. Our results have provided evidence that the expression of *Hoxb3* in the neural tube up to the r5/r6 boundary is auto/cross-regulated by *Hox* genes and expression of *Hoxb3* in r6 does not require *kreisler*. © 2002 Elsevier Science (USA)

Key Words: Hoxb3; hindbrain; rhombomere; *kreisler*; neural crest; cis-regulation.

INTRODUCTION

During embryogenesis, the vertebrate hindbrain develops a transient series of repeated morphological units called rhombomeres. The segmentation of the hindbrain into cell lineage-restricted rhombomeres is a crucial process in the specification of structures developing in the hindbrain (Fraser *et al.,* 1990; Lumsden and Krumlauf, 1996). In the neural tube, there is segment-specific differentiation of neuronal cell types (Lumsden and Keynes, 1989; Clarke and Lumsden, 1993; Theil *et al.,* 2002); through the segmental specification and migration of neural crest cells to the branchial arches, craniofacial structures are developed (Lumsden *et al.,* 1991; Trainor and Krumlauf, 2000, 2001; Sechrist *et al.,* 1993). The clustered *Hox* genes that are expressed in overlapping segmental domains in the hindbrain are key regulators for the anteroposterior specification and hindbrain segmentation (Sham *et al.,* 1993; McGinnis and Krumlauf, 1992; Krumlauf, 1994; Wilkinson, 1993). To understand how the rhombomere-restricted expression domains of *Hox* genes are established and maintained, transgenic analyses of cis-acting regulatory elements and studies of knockout mutant phenotype have revealed the cascade of interactions among Hox proteins and other transcription factors (Trainor and Krumlauf, 2000; Tümpel *et al.,* 2002). Based on the volume of information obtained from cis-regulatory mechanisms in *Hox* genes, it is possible to integrate and model the interaction and regulation of *Hox* genes *in silico* (Kastner *et al.,* 2002).

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Transgenic regulatory analyses of cis-elements show that the transcription factors Krox20, kreisler, and Hox proteins play crucial roles in directly regulating the rhombomeric expression of multiple *Hox* genes. The zinc finger transcription factor Krox20 is required for the development and maintenance of r3 and r5 (Schneider-Maunoury *et al.,* 1993, 1997; Swiatek and Gridley, 1993; Giudicelli *et al.,* 2001), and it directly regulates the transcription of *Hoxa2* and *Hoxb2* in these two rhombomeres through specific Krox20-binding sites (Sham *et al.,* 1993; Nonchev *et al.,* 1996a,b; Vesque *et al.,* 1996; Maconochie *et al.,* 2001). The *kreisler* gene which encodes a Maf/basic leucine zipper protein Krml1 is necessary for the formation of r5 in the mouse and it directly regulates the expression of *Hoxa3* and *Hoxb3* in this rhombomere using kreisler-binding sites in their r5 enhancer elements (Cordes and Barsh, 1994; Manzanares *et al.,* 1997, 1999a,b). In r5, where the expression of Krox20 and kreisler overlap, these two factors cooperate synergistically to activate the *Hoxb3* r5 enhancer (Manzanares *et al.,* 2002). In addition, auto- and cross-regulatory interactions among *Hox* genes have been demonstrated to be important mechanisms in maintaining the spatial patterns of *Hox* genes expression in the hindbrain and spinal cord. Auto- and/or cross-regulation have been described for Hoxb4, Hoxa1, Hoxb1, Hoxb2, and Hoxa3 together with other cofactors such as Pbx and Meis/Prep (Ferretti *et al.,* 2000; Gould *et al.,* 1997, 1998; Maconochie *et al.,* 1997; Manzanares *et al.,* 2001; Pöpperl *et al.,* 1995). Interestingly, expression of *Krox20* is also regulated by *Hox* genes. In r5, the expression of *Krox20* is initially repressed by Hoxa1 and Hoxb1, expression of *Krox20* occurs in r5 only after they have retreated from the hindbrain at around 8 dpc (Barrow *et al.,* 2000; Wilkinson *et al.,* 1989). It has been shown that, in r3, there is synergy between *Hoxa1* and *Krox20* in controlling rhombomere patterning (Helmbacher *et al.,* 1998). Therefore, there are intricate interactive loops among the transcription factors Krox20, kreisler and Hox proteins in the developing hindbrain to control the segmentation and specification processes.

In our transgenic regulatory analysis to investigate the cis-acting components that direct the dynamic pattern of *Hoxb3* expression in mouse embryos, we identified three separate elements which direct gene expression in different domains in the neural tube (Kwan *et al.,* 2001): element I (region A) shared between *Hoxb3* and *Hoxb4* controls posterior neural tube expression up to the r6/r7 boundary in the hindbrain (Aparicio *et al.,* 1995; Gould *et al.,* 1997, 1998; Morrison *et al.,* 1995; Whiting *et al.,* 1991); element IIIa directs expression in anterior spinal cord and hindbrain up to r5/r6 boundary (Kwan *et al.,* 2001); and element IVa regulates the most anterior expression specifically in a single rhombomere r5 (Manzanares *et al.,* 1997, 1999a,b). Comparing the group 3 paralog members *Hoxb3* and *Hoxa3*, it is interesting to note that, although their expression patterns are similar at 9.5 dpc, there are subtle differences in the regulation of their segmental expression in the hindbrain. *Hoxa3* is upregulated by *kreisler* in both r5 and r6 by the same cis-regulatory element, but *Hoxb3* is upregulated by *kreisler* only in r5 (Manzanares *et al.,* 1997, 1999a,b; Kwan *et al.,* 2001). The expression of *Hoxb3* in r6 is controlled by a separate cis-regulatory element IIIa, which is located more than 4 kb upstream of the r5 control region (Fig. 1A). This raises the interesting question of whether *kreisler* will be able to act on element IIIa and regulate *Hoxb3* expression in r6.

In this study, we examined the *Hoxb3* cis-acting regulatory element IIIa by transgenic and mutational analysis to determine the upstream trans-acting factors and mechanisms that are involved in controlling the expression of mouse *Hoxb3* in the anterior spinal cord and hindbrain up to r6 as well as the associated neural crest. By *in vitro* and transgenic *in vivo* analysis, we have shown that the expression of *Hoxb3* in the element IIIa domains is dependent on Hox auto- and/or cross-regulation as well as other trans-acting factors, but it is independent of kreisler.

MATERIALS AND METHODS

Generation of DNA Constructs

Transgenic constructs III and IIIa were described in Kwan *et al.* (2001). Construct IIIa contains a *Sac*I–*Stu*I fragment of construct III. Construct III482 was generated by cloning the 482-bp *Sau*3A fragment of construct IIIa (Fig. 1A; GenBank Accession No. AF529307) into the *Bam*HI site of the lacZ reporter cassette pB4ZA (Whiting *et al.,* 1991). This 482-bp *Sau*3A fragment was also cloned into pBluescript KS (Stratagene) to generate pTY1, which was used as template for all subsequent PCR amplifications. Construct III264 was generated by inserting a 264-bp PCR fragment as indicated in Fig. 1A into the *Hin*dIII–*Xba*I sites of pB4ZAL, which is similar to pB4ZA but with a linker of restriction enzyme sites. The primers used for amplification of the 264-bp fragment were: 5′-CGC TCT AGA ACT AGT GGA TC-3′ and 5′-TAT AGC TTG GGC CTC ACT GCT C-3′. To generate constructs 1, 2, and 3, which contained mutant binding sites, 393-bp DNA fragments (see Figs. 2 and 4A) were amplified by PCR using primers 5′-CGC TCT AGA ACT AGT GGA TC-3′ and 5′-TAT AAG CTT GGG CCT CAC TGC TC-3′. The mutant sites were introduced by PCR-based site-directed mutagenesis using the following mutant oligonucleotides (only forward primers are shown here, mutated nucleotides are underlined): for construct 1, 5′-ATT TAC TAC TCA GAC GGT ACC ATC CAT CCA AAA ATA GCA C-3′ (nucleotide position 159–197 of element III482) and 5′-TGC TCA GTG GTG AAT TCG GAA ACA TGT AAT TCC CAA GAG G-3′ (positions 305–344); construct 2, 5′-GGG CCC TAC AAG CGG CCG CTT TAC TAC TCA GAC TGC TGC-3′ (positions 141–179); construct 3, 5′-GTG AGA AGG AGA TCT TGG TAC AAT GGG CTT ATT GAT GCC T-3′ (positions 67–106). The DNA fragments containing the desired mutations as illustrated in Figs. 2 and 4A were cloned into pB4ZAL to generate constructs 1, 2, and 3. The DNA sequences of all the constructs were confirmed by sequencing reactions.

Transgenic Mice Analysis

Transgenic mice were generated as described in Kwan *et al.* (2001). Fertilized oocytes obtained from F_1 (CBA × C57BL/10) mice were used for DNA microinjection. Transgenic mice were geno-

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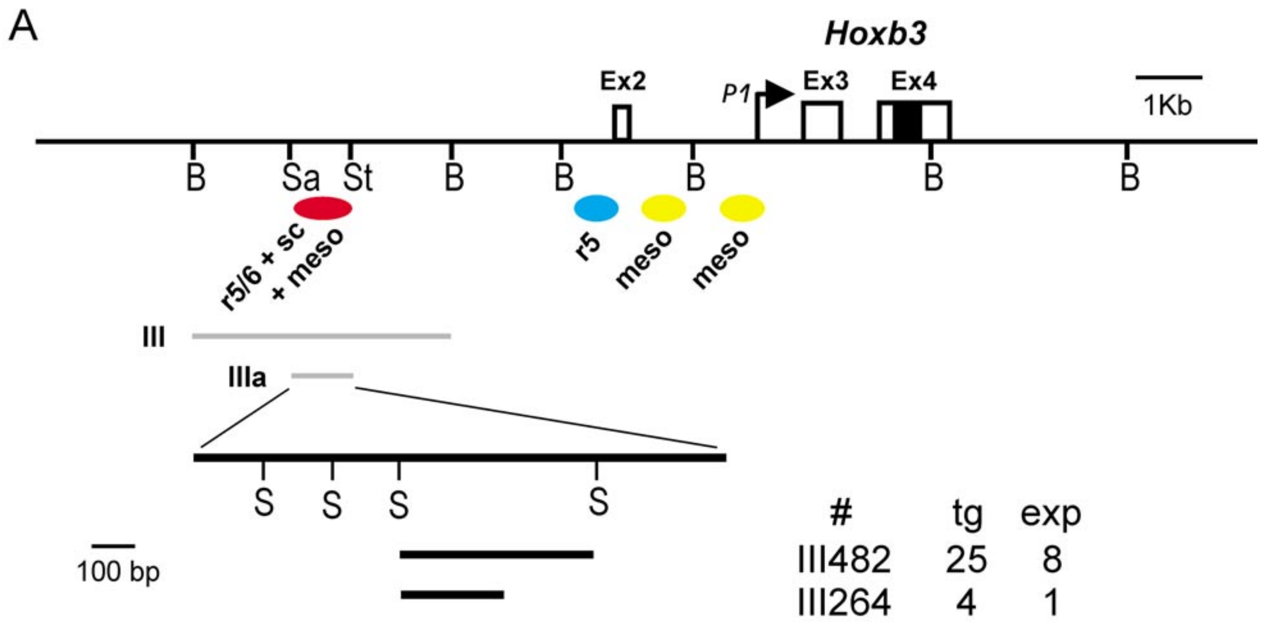

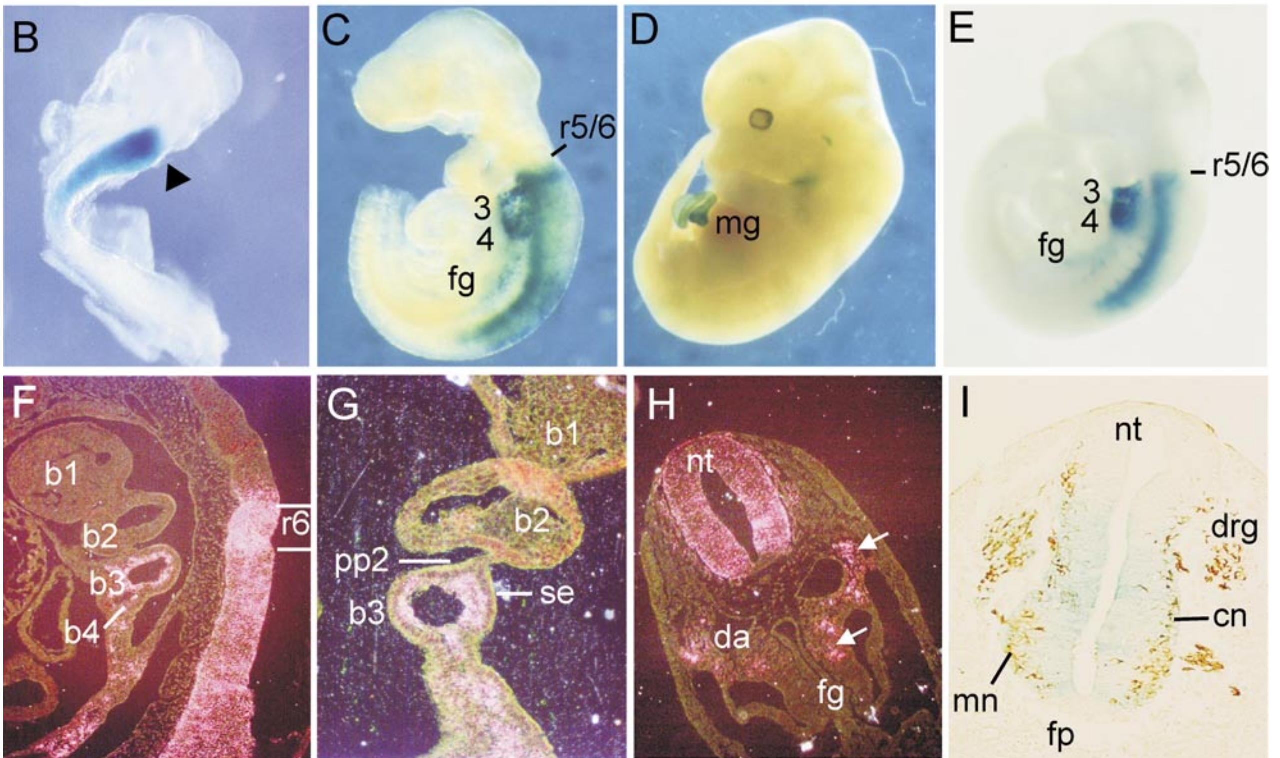

FIG. 1. Transgenic deletion analysis of Element III. (A) Schematic diagram to illustrate the location of the multiple regulatory elements and Elements III, IIIa, III482, and III264 in *Hoxb3*. Exons 2, 3, and 4 (Ex2, 3, and 4) of *Hoxb3* are indicated as open boxes; homeobox in exon 4 is represented by a filled box; the promoter P1 is indicated by an arrow. Yellow ovals, mesodermal-specific enhancers; blue oval, r5 enhancer; red oval, Element IIIa, which directs gene expression in the neural tube up to r5/6 boundary and in mesodermal derivatives. Exp, number of transgenic embryos showing consistent expression pattern of *lacZ* reporter; tg, number of transgenic embryos. Restriction sites: B, *Bam*HI; S, *Sau*3A; Sa, *Sac*I; St, *Stu*I. (B–E) Whole-mount *lacZ* staining of 8.5 (B), 9.5 (C), 12.5-dpc (D) transgenic embryos carrying Element III482; and 10.5 dpc (E) transgenic embryos carrying Element III264. Arrowhead, postotic sulcus; fg, foregut; mg, midgut; r5/6, rhombomeres 5 and 6; 3 and 4, branchial arches 3 and 4. (F–H) Dark-field illumination showing *lacZ* staining (pink) of sagittal (F), coronal (G), and transverse (H) sections of 9.5-dpc transgenic embryos carrying Element III482. Arrows indicate migrating neural crest cells; b1, b2, b3, and b4, branchial arches 1, 2, 3, and 4, respectively; r6, rhombomere 6; pp2, pharyngeal pouch 2; se, surface ectoderm; nt, neural tube; da, dorsal aorta; fg, foregut. (I) Transverse section showing neurofilament immunohistochemical staining of *lacZ*-stained 10.5-dpc transgenic embryo carrying Element III482. drg, dorsal root ganglion; mn, motor neuron; cn, commissural neuron; fp, floor plate.

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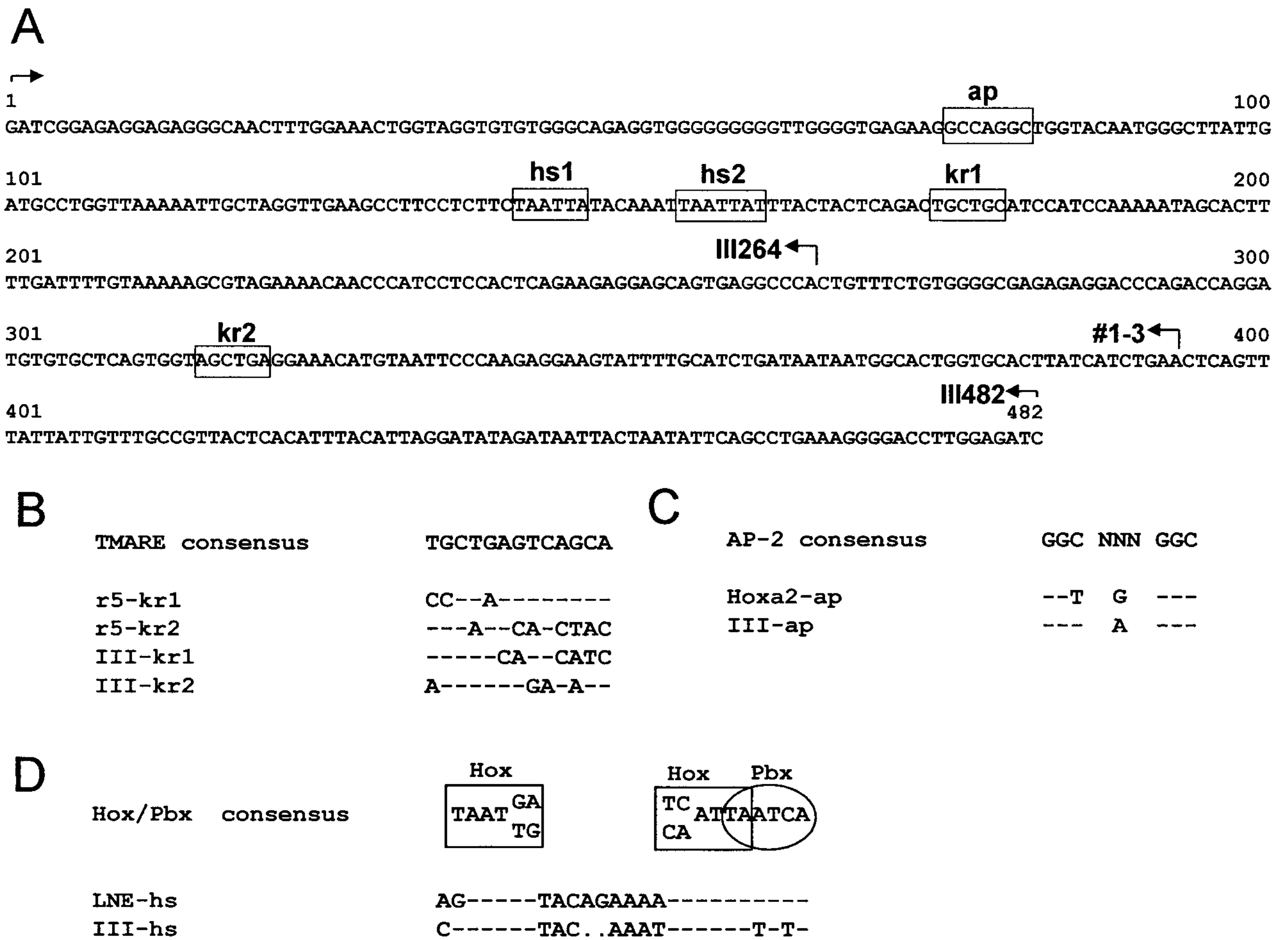

FIG. 2. (A) Sequence of mouse Element III482 and potential transcription factor binding sites. ap, GCCAGGC sequence motif; hs1 and hs2, Hox binding sites 1 and 2; kr1 and kr2, Krml1 binding sites 1 and 2; arrows indicate the boundaries of the enhancer elements (III264, III482 and constructs 1, 2, and 3) inserted into the *lacZ* reporters used in this study. (B) Sequence alignment of potential Krml1 binding sites. r5-kr1 and r5-kr2, Krml1 binding sites found in the r5-specific Element IVa of *Hoxb3* (Manzanares *et al.*, 1997, Kwan *et al.*, 2001); III-kr1 and III-kr2, Krml1 binding sites in Element III482 of *Hoxb3*. Dashes indicate nucleotides that are identical to the consensus. (C) Sequence alignment of AP-2-like binding site. Hoxa2-ap, AP-2 binding site found in *Hoxa2* (Maconochie *et al.*, 1999); III-ap, GCCAGGC sequence motif in Element III482. (D) Sequence alignment of Hox binding sites. Hox binding consensus is boxed in rectangle and Pbx binding consensus is circled in oval. LNE-hs, Hox/Pbx binding sites found in the late neural enhancer (LNE; Gould *et al.*, 1997) of *Hoxb4*; III-hs, Hox binding sites found in Element III482.

typed by PCR using DNA extracted from yolk sac. The β-galactosidase activity of transgenic embryos was analyzed by whole-mount staining as described in Kwan *et al.* (2001). Briefly, embryos were fixed in 1% formaldehyde, 0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM EGTA, 0.02% NP40 in PBS at 4°C for 30–90 min, depending on their size. The embryos were then washed in three changes of PBS with 0.02% NP40 at room temperature for 30 min each and stained in the dark in 1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP40 in PBS at room temperature. For preparation of paraffin sections, X-gal-stained embryos were postfixed in 4% paraformaldehyde overnight at 4°C, dehydrated, and embedded in paraffin wax before sections (6 μm) were prepared and counterstained with eosin.

For immunohistochemistry with anti-neurofilament antibody 2H3 (Developmental Studies Hybridoma Bank), X-gal-stained embryos were refixed with 4% paraformaldehyde for 2 h and rinsed in PBS three times before the whole-mount immunostaining procedure as described in Mark *et al.* (1993). After color developing with DAB substrate, the double-stained embryo was further processed by paraffin sectioning as above.

Electrophoretic Mobility Shift Assays (EMSA)

Oligonucleotides were designed with 5′-overhangs after annealing with their complementary strands. Oligonucleotides were labeled with [α-^{32}P]dCTP (PB10205; Amersham) by end-filling 5′ overhangs using Klenow fragment. For analysis of the kreisler

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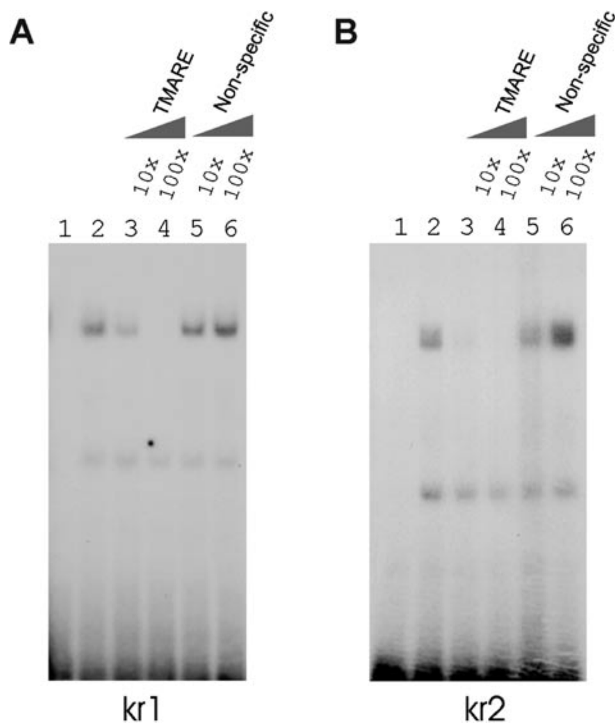

FIG. 3. Binding of recombinant Krml1 protein to the kr1 and kr2 sites in Element III482. Electrophoretic mobility shift assay (EMSA) showing MBP-Krml1 protein binds to (A) kr1 and (B) kr2 containing oligonucleotides. Lane 1, negative control (no protein added); lanes 2–6, with addition of MBP-Krml1 protein. Binding specificity is studied by competition with unlabeled T-MARE containing oligonucleotide at 10-fold (lane 3) and 100-fold molar excess (lane 4), or with nonspecific oligonucleotide at 10-fold (lane 5) and 100-fold molar excess (lane 6).

binding sites (underlined), the oligonucleotides used were: kr1, 5′-ACT ACT CAG ACT GCT GCA TCC ATC CAA-3′; kr2, 5′-GGA TGT GTG CTC AGT GGT AGC TGA GGA AAC ATG-3′. To determine binding specificity, competitor oligonucleotide T-MARE 5′-AGC TCG GAA TTG CTG ACG CAT TAC TC-3′ containing a consensus binding site with high affinity for Maf proteins (underlined) and nonspecific oligonucleotide 5′-GAG TAA TGA GGA CTC CTC AAT TCC GAG-3′ were added in 10-fold or 100-fold molar excess of the radiolabeled probe as described in Manzanares *et al.* (1997, 1999b).

For analysis of the Hox binding sites, the oligonucleotides used were as follows: wild type, 5′-AGC TTC CTC TTC TAA TTA TAC AAA TTA ATT ATT TAC TAC TC-3′; ms1, 5′-AGC TTC CTC TTC TAA TTA TAC AAA GGG CCC ATT TAC TAC TC-3′; ms2, 5′-AGC TTC CTC TT CTAG CGC TAC AAA TTA ATT ATT TAC TAC TC-3′; ms12, 5′-AGC TTC CTC TTC TAG CGC TAC AAA GGG CCC ATT TAC TAC TC-3′ (see Fig. 5E). GST-Hoxb3 protein contains GST fused to the first 260 amino acids of Hoxb3, including all of the N-terminal region, and the homeodomain. GST-Hoxb4 protein contains GST fused to the homeodomain of Hoxb4.

The DNA–protein binding reactions were set up as described by Pöpperl *et al.* (1995) with minor modifications. Briefly, a 20-μl binding reaction contained 20,000 cpm probe, 500 ng poly(dIdC-dIdC), protein (GST-Hoxb3, 0.2–1.6 μg; GST-Hoxb4, 2–50 ng), 20 mM Hepes–KOH (pH 7.9), 100 mM KCl, 0.25 μg/μl BSA, 2 mM DTT, 1 mM EDTA, 12% glycerol. The binding reaction was incubated at 24°C for 30 min. The samples were run in a 6% acrylamide glycerol gel (6% acrylamide, 2.5% glycerol, 0.075% APS, 0.5× TBE) in 0.5× TBE.

DNase I Footprinting Assays

Single-stranded ^{33}P-labeled DNA fragments were generated by PCR using pTY1 (see DNA constructs above) as template. The PCR primers used were: 5′-TAT AAG CTT CAG ATG ATA AGT GCA CCA G-3′ and 5′-CGC TCT AGA ACT AGT GGA TC-3′. The primers were end-labeled with [γ-^{33}P]dATP (BF1001; Amersham) using T4 polynucleotide kinase before using for PCRs. The PCR fragments generated were 393 bp in size and encompassed the same region as constructs 1, 2, and 3. The protein–DNA binding reactions were set up in the same manner as in the EMSA and were incubated at 24°C for 1 h. Then, 5 ng of DNase I (Sigma) was added to each reaction and incubated at 24°C for 30 min; after that, the reaction mixtures were separately on an 8% denaturing acrylamide gel. The DNase I footprints were analyzed on both DNA strands.

RESULTS

Identification of a Minimal Enhancer for Posterior Hindbrain and Spinal Cord

We have previously identified a 1.3-kb cis-acting enhancer element IIIa which can direct reporter gene expression in the hindbrain with an anterior limit at r5/r6, anterior spinal cord, and the associated neural crest (Kwan *et al.,* 2001). In order to define the specific sequences that can mediate the expression, we investigated a 482-bp Sau3A fragment within element IIIa by transgenic mice analysis using the *lacZ* reporter construct III482 (Fig. 1A). In 8.5-dpc transgenic embryos, expression of the *lacZ* gene was restricted to the anterior neural tube, with an anterior limit at the developing hindbrain at the posterior rhombomeric sulcus (Fig. 1B). In 9.5-dpc embryos, construct III482 was able to mediate *lacZ* gene expression in the hindbrain up to rhombomere 6, in anterior spinal cord, and the associated neural crest which migrated ventrally to the mesenchyme of the third, fourth, and posterior branchial arches as well as to the foregut (Figs. 1C, 1F–1H). At this stage, the level of *lacZ* expression was higher in rhombomere 6 than in the rest of the neural tube (Figs. 1C and 1F); a small number of cells at posterior r5 also expressed *lacZ* (Fig. 1F). Histological sections of the branchial region of 9.5-dpc transgenic embryos showed that the *lacZ* reporter is expressed in the neural crest cells populating the third, fourth, and posterior branchial arches, but not in the surface ectoderm (Figs. 1F and 1G). Transverse section of a transgenic embryo at the trunk level showed that the neural crest cells migrating ventrally to the dorsal aorta and the foregut also expressed the *lacZ* gene (Fig. 1H). Similar expression patterns were maintained in 10.5-dpc transgenic embryos; at this stage, the *lacZ*-marked vagal neural crest cells migrating to the

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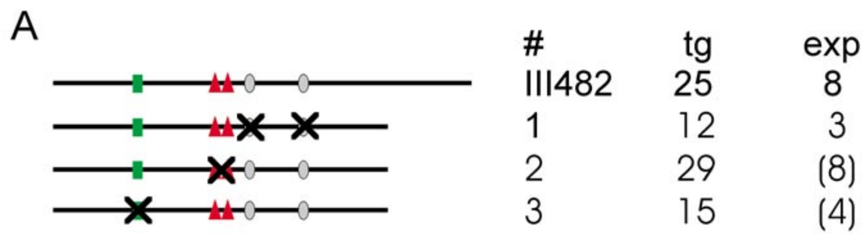

FIG. 4. Functional study of Krml1, Hox, and GCCAGGC binding sites by site-directed mutagenesis and transgenic mice analysis. (A) Summary of the mutant constructs generated by site-directed mutagenesis used in transgenic analysis. Sites mutated: green rectangle, GCCAGGC sequence binding site; red triangles, Hox binding sites; gray ovals, Krml1 binding sites. Tg, total number of transgenic embryos; Exp, number of transgenic embryos showing consistent expression pattern of *lacZ* reporter; brackets indicate transgenic embryos showing only subset of expression domains directed by Element III482. (B–I) *lacZ*-stained 10.5-dpc transgenic embryos generated with constructs containing wildtype and mutant binding sites. (B, D, F, H) Lateral view. (C, E, G, I) Dorsal view. (B, C) Transgenic embryos carrying Element III482. (D, E) Transgenic embryos carrying construct 1, with both kr1 and kr2 sites mutated. (F, G) Transgenic embryos carrying construct 2, with both hs1 and hs2 sites mutated; (H, I) Transgenic embryos carrying construct 3, with the GCCAGGC site mutated. r5/6, rhombomeres 5 and 6 boundary; 3, branchial arch 3; 4, branchial arches 4 and posterior branchial arches, fg, foregut.

foregut could be observed (see Fig. 4B). By 12.5 dpc, *lacZ* expression in the neural tube was completely down-regulated, but in the midgut, the reporter gene continued to express in the neural crest derivatives (Fig. 1D). Immunohistochemical analysis of *lacZ*-stained 10.5-dpc embryo using the neurofilament-specific antibody 2H3 in histological sections showed that the neurofilament and *lacZ* staining overlapped in the ventral half of the neural tube as well as in the dorsal root ganglia (Fig. 1I). Therefore, element III482 could direct reporter gene expression in the neural tube and the associated neural crest, contributing to the central and peripheral nervous systems.

The transgene expression patterns directed by construct III482 were similar to those of element III or IIIa (Kwan *et al.,* 2001), except for the absence of *lacZ* expression in the surface ectoderm of the branchial arches at 9.5 dpc and the absence of transient expression in posterior somites at 8.5 dpc. Therefore, this 482-bp fragment contained sequences necessary for directing gene expression to the neural tube and associated neural crest. We have examined the activity of a smaller fragment of 264 bp (construct III264, Fig. 1A) in transgenic embryos and found that among four transgenic embryos only one of them expressed the reporter, the patterns of expression at 10.5 dpc were identical to those derived from construct III482 (Fig. 1E). Hence, this 264-bp fragment represented the minimal enhancer region.

kreisler Is Not Required for the Activity of the 482-bp Enhancer

We determined the sequence of the 482-bp enhancer and identified a number of transcription factor binding sites, including those with sequence homology to consensus Krml1 and Hox, as well as a GCCAGGC motif which resembles binding site for AP-2 family of transcription factors (Fig. 2). Two potential Krml1 binding sites with homology to the consensus T-MARE site as well as kreisler-binding sites in the *Hoxb3* r5 enhancer (Figs. 2A and 2B) (Manzanares *et al.,* 1997) could be identified. The kr1 site is located within the 264-bp minimal region; and kr2 is out of the minimal enhancer (Fig. 2A). As Krml1 is essential for upregulating *Hoxb3* expression in r5 and for *Hoxa3* in both r5 and r6 (Manzanares *et al.,* 1999a,b, 2001), we tested by *in vitro* and *in vivo* experiments whether Krml1 could also regulate *Hoxb3* in r6. We first examined whether the Krml1 protein could bind to the kr1 and kr2 sites by EMSA. We found that the DNA-binding region of the Krml1 fusion protein (MBP-Kr) could form specific complex with double-stranded oligonucleotides containing sequences of either the kr1 site (Fig. 3A) or the kr2 site (Fig. 3B). The binding of the Krml1 fusion protein to these two sites were blocked by addition of an excess of oligonucleotides containing a consensus binding site for Maf proteins (Fig. 3, T-MARE), but was not affected by the addition of excess random oligonucleotides (Figs. 3A and 3B). The results of the EMSA experiment suggested that Krml1 protein could interact specifically with both the kr1 and kr2 sites *in vitro*.

We then carried out site-directed mutagenesis experiments and tested the mutated kr1 and kr2 sites in the context of a 393-bp subfragment within enhancer III482 by transgenic mice analysis. Using the reporter construct 1 (Fig. 4A), we generated 12 transgenic embryos and 3 of them expressed lacZ in the hindbrain, spinal cord, and branchial arches; the expression patterns at 10.5 dpc were unaffected by the mutant sites and were the same as the normal control (Figs. 4D and 4E). Therefore, the activity of enhancer III does not require any of the two Krml1 sites, and we conclude that these sites are not required for the activity of enhancer III482 to direct gene expression in r6.

Hox Binding Sites Suggest Auto- and Cross-Regulation

Based on sequence analysis, we identified two consensus Hox binding sites (TAATTA) which are 7 bp apart; we designated these two sites hs1 and hs2 (Figs. 2A and 2D). Compared with the binding sites in the LNE (late neural enhancer) of *Hoxb4* (Gould *et al.,* 1997), the hs1 site here is almost identical to the Hox binding site in LNE, but the hs2 site only weakly resembles the Hox/Pbx complex binding site (Fig. 2D). We have investigated the protein binding activity of the hs1 and hs2 sites further by *in vitro* EMSA and DNase footprinting analyses. Using oligonucleotides containing both hs1 and hs2 sites (Fig. 5E, wt), we tested the binding of two recombinant Hox proteins, GST-Hoxb3 and GST-Hoxb4. When GST-Hoxb3 was used in EMSA, we found that specific complex was formed in a concentration-dependent manner (Fig. 5A, lanes 2–5). Addition of excess unlabeled wild type oligonucleotides could compete out the binding (Fig. 5A, lanes 6–8); but oligonucleotides containing mutated binding sites (Fig. 5E, ms12) could not compete with the labeled wild type oligonucleotides for complex formation (Fig. 5A, lane 11). This indicated that the GST-Hoxb3 protein could bind to the predicted Hox protein binding sites hs1 and hs2. To test the relative binding specificity of the two sites hs1 and hs2, we used oligonucleotides with either hs1 site mutated (ms1) or hs2 site mutated (ms2) in the EMSA experiment. We found that either ms1 or ms2 could inhibit complex formation between wild type oligonucleotides and the GST-Hoxb3 protein at a similar concentration, with ms1 being slightly more effective in competing out the binding (Fig. 5A, lanes 9 and 10).

When the same EMSA experiment was performed by using the GST-Hoxb4 protein in similar conditions, a specific complex clearly formed (Fig. 5B, lanes 4 and 5) which could be competed out by wild type oligonucleotides (Fig. 5B, lanes 6–8) but not by mutant ones (lane 11). When either mutant oligonucleotide ms1 or ms2 was added to the assay mixture, complex formation between wild type oligonucleotides and the GST-Hoxb4 protein was greatly reduced (Fig. 5B, lanes 9 and 10), suggesting that the GST-Hoxb4 protein could bind to hs1 or hs2 sites with similar affinity.

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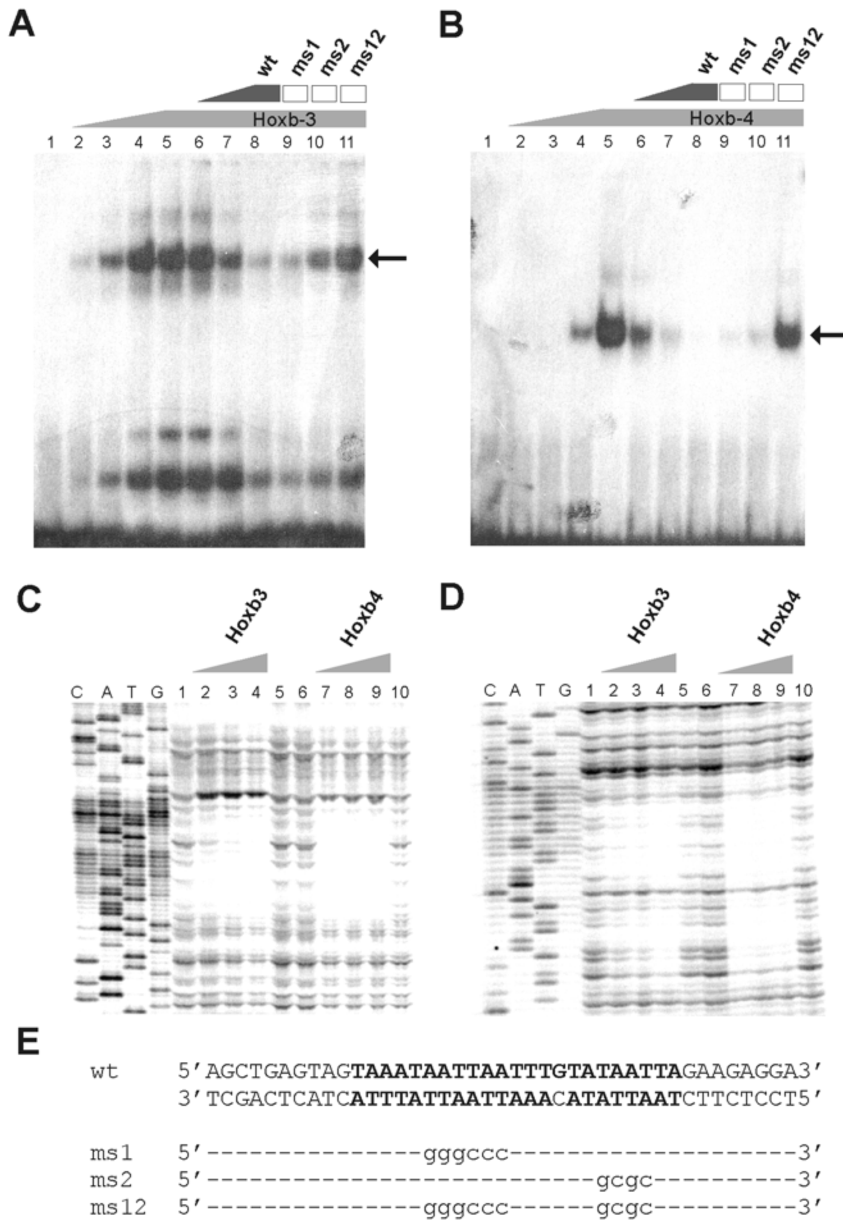

FIG. 5. Binding of recombinant Hoxb3 and Hoxb4 proteins to hs1 and hs2 sites in Element III482. EMSA showing binding of GST-Hoxb3 fusion protein (A) and GST-Hoxb4 fusion protein (B) to hs1 and hs2 sites. Lane 1, GST only; lanes 2–5, with increasing amount of GST-Hoxb3 (A) or GST-Hoxb4 (B) proteins; lanes 6- , with 20-, 100-, and 500-fold of unlabeled wild type oligonucleotides; lanes 9–11, with 500-fold of ms1, ms2, and ms12 oligonucleotides. Arrow indicates specific complex formed. (C, D) DNaseI footprinting analysis of Hoxb3 and Hoxb4 proteins on the sense strand (C) and anti-sense strand (D) of Element III482. Lanes C, A, T, G: DNA sequencing reactions of the sense strand. Lanes 1, 5, 10: no protein added; lane 6: GST only; lanes 2–4: increasing amount of GST-Hoxb3; lanes 7–9: increasing amount of GST-Hoxb4. (E) DNA sequences of the oligonucleotides used in the EMSA. The sequences protected by the recombinant Hoxb3 and Hoxb4 proteins in the DNaseI footprinting analysis are shown in bold type. wt, wild type oligonucleotide containing hs1 and hs2 sites; ms1, mutant oligonucleotide with mutated hs1 site shown in lowercase; ms2, mutant oligonucleotide with mutated hs2 site; ms12, mutant oligonucleotide with mutated hs1 and hs2 sites.

In addition to EMSA experiments using oligonucleotides, we performed DNAse I footprinting analysis using the 393-bp DNA fragment within element III482. When DNA fragment with the sense strand end-labeled was used, a single region covering 21 bp was protected by both the GST-Hoxb3 and the GST-Hoxb4 proteins (Fig. 5C). When the antisense strand was

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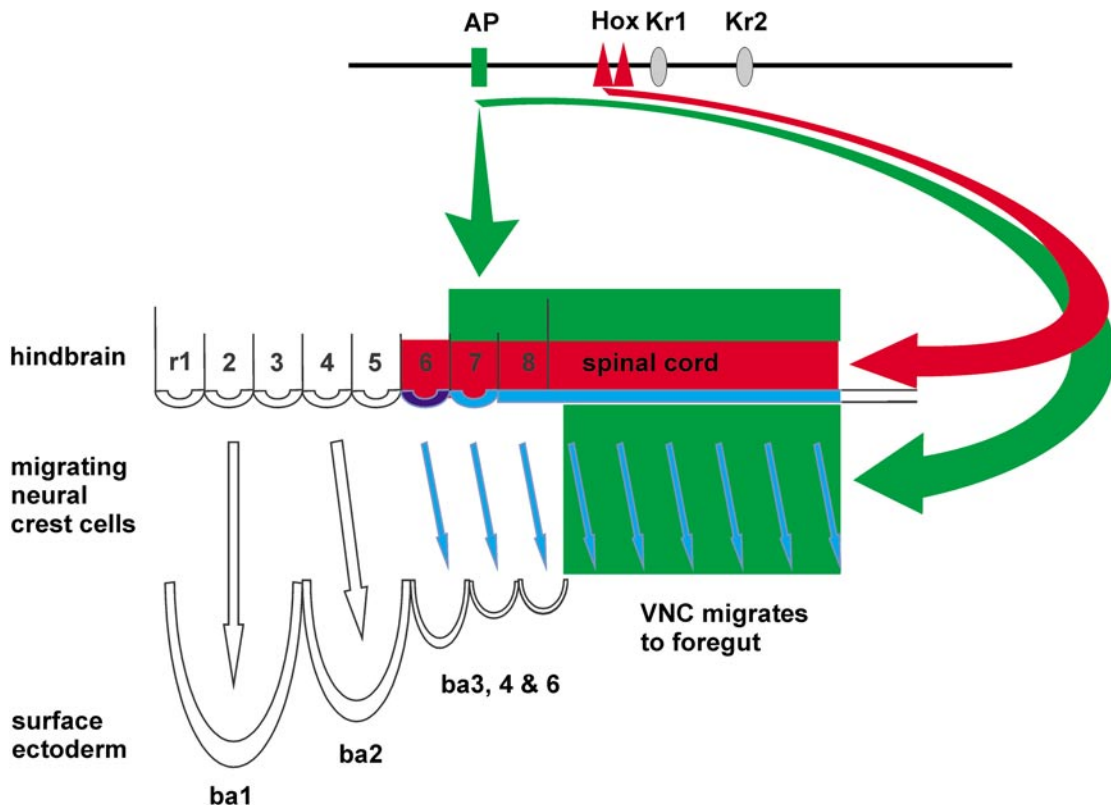

FIG. 6. Multiple transcription factor binding sites are required for regulating the expression patterns mediated by Element III482. The schematic line on the top represents Element III482; green rectangle, AP-2-like binding site GCCAGGC; red triangles, hs1 and hs2 sites; gray ovals, kr1 and kr2 sites. Blue color represents β-galactosidase activities detected in the neural tube and migratory neural crest (blue arrows) in transgenic embryos carrying Element III482; dark blue represents stronger β-galactosidase activity in r6. The green blocks, pointed out by green arrows, represent the neural tube and neural crest expression domains which are dependent on the GCCAGGC site; the red block, pointed by the red arrow, represents expression domains which are dependent on functional Hox binding sites hs1 and hs2, hence auto-/cross-regulated; the kreisler-binding sites kr1 and kr2 are not required for regulating gene expression mediated by Element III482. ba1, branchial arch 1; VNC, vagal neural crest.

end-labeled, two regions separated by a single G nucleotide and spreading over the same 21 bp were protected by both GST-Hoxb3 and GST-Hoxb4 proteins (Fig. 5D). The 21-bp protected region, as shown in Fig. 5E, overlapped exactly with the predicted Hox binding sites hs1 and hs2.

Hox Binding Sites Are Required for Controlling Neural Expression

Both the EMSA and DNase footprinting experiments have shown that Hoxb3 and Hoxb4 proteins could bind to the hs1 and hs2 sites *in vitro*. To test whether these Hox binding sites are required for controlling gene expression *in vivo*, we tested the activities of these binding sites using *lacZ* reporter constructs (Fig. 4). In the *lacZ* reporter construct 2, both hs1 and hs2 sites are mutated by site-directed mutagenesis. Among 29 transgenic embryos we generated and analyzed, 8 of them expressed the reporter gene in the 3rd, 4th, and posterior branchial arches; some cells in the developing foregut and the dorsal root ganglia were also positively stained (Fig. 4F), suggesting that the reporter gene was expressed in the neural crest cells originated from the posterior hindbrain and adjacent spinal cord domain. However, no *lacZ* expression could be detected in the neural tube of the transgenic embryos; the normal activity of this DNA fragment in rhombomeres 6, 7, and 8 and anterior spinal cord was abolished when the Hox binding sites were mutated. Our *in vivo* transgenic experiment clearly showed that the Hox binding sites are required for gene expression in the neural tube, but are not necessary for mediating gene expression in neural crest derived tissues. Other separate regulatory sequences will be required to control gene expression in the neural crest.

GCCAGGC Binding Site Is Required for Neural Tube but Not Neural Crest Expression

It has been shown that the *Hoxa2* enhancer has an AP-2 binding site which can mediate rhombomere independent

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gene expression in the neural crest (Maconochie *et al.,* 1999). Within the III482 enhancer, we have identified a GCCAGGC sequence which resembles the AP-2 binding site in *Hoxa2* (Figs. 2A and 2C). To test the importance of this potential AP-2 binding site in directing gene expression in the neural crest, we performed *in vivo* transgenic assay using a mutant construct in which the potential binding site was changed to GAGATCT (Fig. 4A, construct 3). Among 15 transgenic embryos generated using this construct, there were 4 which expressed the *lacZ* reporter gene. Interestingly, in all 4 transgenic embryos, the expression of *lacZ* was maintained in the neural crest of the third and posterior branchial arches, and only in rhombomere 6 in the neural tube. When the GCCAGGC binding site was mutated, no *lacZ* expression could be detected in rhombomeres 7 and 8 and adjacent anterior spinal cord, nor in neural crest in the more posterior region. Our results suggest that the GCCAGGC sequence is important for controlling the segmental expression of reporter gene in r7, r8, and the more posterior neural tube and associated neural crest domain, but it is not a neural crest-specific regulatory site. Also, previous *in vitro* studies had indicated that AP-2 family members of proteins would only bind to consensus sequence of GCCN(3/4)GGC (Mohibullah *et al.,* 1999). Therefore, based on cellular specificity and sequence characteristics, it is possible that the activity of this GCCAGGC site may not be mediated by the AP-2 family of proteins. Other yet-to-be-identified factors would be involved in mediating the activity of this site.

DISCUSSION

In this study, our analysis on the cis-acting enhancer element III has identified the sequence requirements for *Hoxb3* gene expression in the posterior hindbrain and adjacent spinal cord domain, as well as the associated neural crest encompassing the cardiac and vagal neural crest cells (summarized in Fig. 6). We have provided evidence that the reporter gene expression in the neural tube is dependent on Hox binding sites, and *in vitro* both Hoxb3 and Hoxb4 proteins can interact with the Hox binding sites, suggesting that auto/cross-regulation is required for establishing the expression of *Hoxb3* in the hindbrain up to the r5/r6 boundary and in the anterior spinal cord. We have identified a GCCAGGC sequence motif which is required for directing gene expression in r7, r8, and spinal cord as well as the migratory vagal neural crest. As illustrated in Fig. 6, for controlling the reporter gene expression in the posterior hindbrain at r7, r8, and the adjacent spinal cord (the domain where the red and green overlap), either the Hox binding sites or the GCCAGGC sequence motif will need to be intact and functional. Mutation of either of the two sequences will abolish reporter gene expression in this neural domain, suggesting that these two sequence regions cannot functionally compensate for each other and they are both required. We have also identified two kreisler binding sites, kr1 and kr2. However, our transgenic and mutational analysis has indicated that these kreisler binding sites are not required for the establishment or maintenance of reporter gene expression in r6. Our results further confirm previous findings on the differential regulatory mechanisms for *Hoxa3* and *Hoxb3* expression in r5 and r6 by *kreisler* (Manzanares *et al.,* 1997, 1999a,b, 2001).

Auto/Cross-Regulation of Hoxb3 Gene Expression

From the element III482 sequence, we have identified two closely linked Hox binding sites with the TAAT core sequence (Figs. 2A and 2D); these binding sites are highly conserved among the mouse, human, and zebrafish *Hoxb3* loci (data not shown). In our transgenic mutational study, we have demonstrated that the hs1 and hs2 sites are essential for controlling gene expression in the neural tube (Figs. 4F and 4G). In the absence of functional hs1 and hs2 sites, no reporter gene expression could be detected in the hindbrain and spinal cord, but gene expression in the neural crest cells migrated from the same neural domain are not affected (Figs. 4F, 4G, and 5). Therefore, separate elements are required to regulate gene expression in the neural crest. Although it was previously believed that the *Hox* gene identity of the neural crest is preprogrammed, carrying positional information acquired in the hindbrain to the branchial arches, recent neural crest cell transposition experiments have clearly shown that cranial neural crest is not prepatterned but complex cellular and tissue interactions are involved in craniofacial development (Trainor and Krumlauf, 2000, 2001; Trainor *et al.,* 2002). Here, we showed that, in terms of cis-regulatory mechanism, the expression of *Hoxb3* in the neural tube is Hox-dependent, but the establishment and maintenance of gene expression in the migratory cardiac and vagal neural crest are not dependent on the Hox auto/cross-regulatory circuit. Our results further support the idea that the functional maintenance of *Hox* gene identity in the neural crest is independent from the neural tube.

To explain how Hox proteins recognize and regulate their target genes, including *Hox* genes in auto- and cross-regulation through cis-acting elements, two models have been proposed. The "widespread binding" model suggests that Hox proteins bind to multiple monomer sites and cooperatively increase their binding and occupancy of cis-regulatory elements. On the other hand, the "co-selective binding" model suggests that Hox proteins could interact with protein cofactors that increase their DNA binding affinities for larger compound binding sites (Biggin and McGinnis, 1997; Phelan *et al.,* 1995; Galant *et al.,* 2002). Most of the vertebrate auto- and cross-regulated *Hox* gene cis-regulatory elements have been shown to involve Hox/PBC complexes (Chan *et al.,* 1997; Ferretti *et al.,* 2000; Frasch *et al.,* 1995; Gould *et al.,* 1997; Li and McGinnis, 1999; Maconochie *et al.,* 1997; Manzanares *et al.,* 2001; Pöpperl *et al.,* 1995). Although we have identified within element III482 two functional Hox binding sites and the hs2

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site has some homology to a Hox/PBC consensus sequence, the sequences of the hs1 and hs2 sites are more similar to a consecutive series of Hox binding sites with TAAT motif (Figs. 2A and 2D). In the DNase I footprinting experiments, we have shown that the entire hs1 and hs2 region was fully protected by Hoxb3 and Hoxb4 proteins (Figs. 3C–3E). Therefore, in element III482, Hox proteins may interact with the multiple Hox-binding sites in a "widespread binding" model, similar to the cis-regulation of some of the *Hox* genes in *Drosophila* (Beachy *et al.,* 1993; Galant *et al.,* 2002).

In examining the functions of members of paralogous group 3 by targeted gene replacement and mutations, it has been shown that the three group 3 genes, *Hoxa3*, *Hoxb3*, and *Hoxd3*, can functionally compensate for each other (Condie and Capecchi, 1993; Greer *et al.,* 2000; Manley and Capecchi, 1995, 1997, 1998). Each of the group 3 gene members may not have a unique role, but individual functional specificity is dictated by the subtle differences in their expression domains (Greer *et al.,* 2000). In the developing hindbrain, the expression patterns of members of the paralogous groups 2, 3, and 4 are overlapping with the expression domains driven by element III482, suggesting that these Hox members could be involved in the auto/cross-regulation of *Hoxb3* gene expression through interactions with the Hox binding sites in element III482.

kreisler Is Not Required for *Hoxb3* Expression in r6

The dynamic changes of *Hoxb3* expression patterns in the hindbrain rhombomeres from 9.25 to 11 dpc (Sham *et al.,* 1992; Kwan *et al.,* 2001) suggest that there are critical temporal controls on the activities of cis-regulatory elements resulted in changes in levels of gene expression in the rhombomeres at different stages. For the upregulation of *Hoxb3* in r5 at 9.25 dpc, the temporal regulation is contributed by the transient overlapping expression of two transacting factors, Krox20 and kreisler, in r5 at this stage of embryogenesis. Detailed analysis of the complex binding sites in the *Hoxb3* r5-element has demonstrated the synergistic interaction of Krox20 and kreisler proteins in the upregulation of *Hoxb3* expression in r5 (Manzanares *et al.,* 2002). This upregulated r5 domain of Hoxb3 expression last for a duration of about 1 day, during which time the expression of all three genes overlap. By 10.5 dpc, when the expression of Krox20 and kreisler are both downregulated, the level of *Hoxb3* expression in r5 is much reduced (Gould *et al.,* 1997).

Our transgenic analysis of the *Hoxb3* element III482 showed that the expression of reporter gene is at a higher level in r6, and we showed that there are two kreisler binding sites, kr1 and kr2, within the element which could bind to recombinant krml1 protein *in vitro*. However, our transgenic mutational study clearly demonstrated that these two kreisler binding sites are not required *in vivo* for the activity of element III482 and will not be involved in regulating *Hoxb3* expression in r6. Our results agree with previous analysis of gene expression in a double transgenic embryo carrying Hoxb3 element III in a homozygous *kreisler* mutant background, which demonstrated that the expression of the reporter gene in r6 was not affected by the absence of functional kreisler (Manzanares *et al.,* 1999b; Kwan *et al.,* 2001).

Although *kreisler* is expressed in both r5 and r6 (Cordes and Barsh, 1994), the characteristic segmentation process for these two rhombomeres appears to be different. Analysis of the phenotype of *kreisler* mutant has demonstrated that the mouse kreisler gene is involved in the formation of r5, but the generation of a definitive r6 territory is independent of both *kreisler* and r5 (Manzanares *et al.,* 1999a,b). This segmentation mechanism is different from that of the zebrafish *valentino* mutant in which *val* is required for subdividing a prorhombomere into two distinct rhombomeres r5 and r6 (Moens *et al.,* 1998, Prince *et al.,* 1998). Therefore, in the mouse embryo, *kreisler* has distinct roles in r5, which is independent of its function in r6. *kreisler* is active only in r5 for upregulating its target *Hoxb3* expression, and it will not interact with the binding sites in element III to maintain *Hoxb3* expression in r6. For the paralogous member *Hoxa3*, a different mechanism is operated; upregulation of *Hoxa3* expression in both r5 and r6 at 9.5 dpc is dependent on *kreisler* as the same r5/r6 enhancer is able to read out the presence of kreisler in these two rhombomeres. Also, for *Hoxa3*, only kreisler is necessary and Krox 20 is not required (Manzanares *et al.,* 1999a, 2002).

Additional Factors Controlling Hoxb3 in Posterior Hindbrain and Spinal Cord

Our transgenic analysis of reporter gene expression directed by element III482 has shown that this element controls gene expression in both the neural tube and the associated neural crest cells which migrate and populate the third and posterior branchial arches as well as the cardiac and vagal neural crest cells. Previous study on cis-regulation of the *Hoxa2* gene, which demonstrated that the AP-2 family of transcription factors are involved in regulating *Hoxa2* expression in the cranial neural crest cells independent from the control of rhombomere expression (Maconochie *et al.,* 1999), has led us to examine whether the AP-2 family members could also be regulating *Hoxb3* expression in the neural crest. Our examination of the element III482 sequence has identified a GCCAGGC motif which is similar to the *Hoxa2* AP-2 binding site GGTGGGC (Figs. 2A and 2C; Maconochie *et al.,* 1999) but is different from the consensus binding site GGCN(3/4)GGC in having only a single A instead of three or four nucleotides between the GC blocks (Mohibullah *et al.,* 1999). Our analysis from the TRANSFAC transcription factor database has shown that, overlapping with this GGCAGGC motif, the sequence of GTGAGAAGGCCAGG also resembles the binding site sequence of GaaNGAAGGa(a/g)AGG (lowercase indicates mismatches) for the Krüppel-box transcrip-

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tion factor KLF4, which has been identified to be a downstream target gene of AP-2α (Pfisterer *et al.,* 2002). In addition, overlapping with this same GGCAGGC motif, there is also sequence homology with binding sites for retinoid receptors, including those for RAR, RXR-α, RAR-γ, and RAR-α (TRANSFAC Database Accession Nos. I00040, I00038, I00405, and I00401). It is not clear whether these or some other factors yet to be identified are involved in the transactivation of gene expression through the GGCAGGC motif in element III482. However, our results have shown that this GGCAGGC motif is essential for regulating the segmental gene expression in r7, r8 of the hindbrain, and spinal cord together with the associated neural crest.

A study of the conserved cis-regulatory elements in the amphioxus *Hox* complex has identified a control region Element 3B from *AmphiHox-3*. In transgenic mice, this element is shown to direct reporter gene expression in the neural tube and hindbrain up to r6, with lower level of expression in r6 than in r7, r8, and the spinal cord (Manzanares *et al.,* 2000). The expression patterns driven by amphioxus Element 3B in the neural tube of transgenic mice are remarkably similar to the expression domains controlled by the GGCAGGC motif in mouse *Hoxb3* element III482, except for the expression in the neural crest (see green box in Fig. 6). Considering that migratory neural crest cells have not been reported from amphioxus, the amphioxus Element 3B may lack sequence information for mediating gene expression in the neural crest in the mouse. Therefore, through an evolutionary conservation approach, it may be possible to identify the trans-acting factor(s) which mediate the activity of element III482 in the more posterior hindbrain domains by comparing conserved cis-regulatory elements from more diverse species.

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