Regulatory Analysis of the Mouse Hoxb3 Gene: Multiple Elements Work in Concert to Direct Temporal and Spatial Patterns of Expression

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The expression pattern of the mouse Hoxb3 gene is exceptionally complex and dynamic compared with that of other members of the Hoxb cluster. There are multiple types of transcripts for Hoxb3 gene, and the anterior boundaries of its expression vary at different stages of development. Two enhancers flanking Hoxb3 on the 3' and 5' sides regulate Hoxb2 and Hoxb4, respectively, and these control regions define the two ends of a 28-kb interval in and around the Hoxb3 locus. To assay the regulatory potential of DNA fragments in this interval we have used transgenic analysis with a LacZ reporter gene to locate cis-elements for directing the dynamic patterns of Hoxb3 expression. Our detailed analysis has identified four new and widely spaced cis-acting regulatory regions that can together account for major aspects of the Hoxb3 expression pattern. Elements Ib, IIIa, and IVb control gene expression in neural and mesodermal tissues; element Va controls mesoderm-specific gene expression. The most anterior neural expression domain of Hoxb3 is controlled by an r5 enhancer (element IVa); element IIIa directs reporter expression in the anterior spinal cord and hindbrain up to r6, and the region A enhancer (in element I) mediates posterior neural expression. Hence, the regulation of segmental expression of Hoxb3 in the hindbrain is different from that of Hoxa3, as two separate enhancer elements contribute to expression in r5 and r6. The mesoderm-specific element (Va) directs reporter expression to prevertebra C1 at 12.5 dpc, which is the anterior limit of paraxial mesoderm expression for Hoxb3. When tested in combinations, these cis-elements appear to work as modules in an additive manner to recapitulate the major endogenous expression patterns of Hoxb3 during embryogenesis. Together our study shows that multiple control elements direct reporter gene expression in diverse tissue-, temporal-, and spatially restricted subset of the endogenous Hoxb3 expression domains and work in concert to control the neural and mesodermal patterns of expression. © 2001 Academic Press

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INTRODUCTION

Spatial and temporal regulation of Hox gene expression is an important issue as the restricted expression of these genes is directly linked to their functional roles in development. This is a complex problem as there are a large number of genes organized into clusters, creating the potential for shared elements, inhibitory interactions, local factors, and global control (Mann, 1997; Maconoche et al., 1997; Duboule, 1998; Sharpe et al., 1998; Kondo et al., 1998; Kondo and Duboule, 1999). With the goal of understanding what is required to direct the normal patterns of expression, many groups have investigated cis-acting regulatory elements using lacZ reporter constructs in transgenic mice. Results have varied from an inability to find essential elements to a near reconstruction of the full endogenous patterns. For the Hox genes that are expressed in hindbrain rhombomeres, regulatory elements important for generating rhombomere-specific patterns have been identified for a few genes, including the r2 and r4 elements for Hoxa1 (Frasch et al., 1995), the r3/r5 and r4 elements for Hoxa2 (Kondo et al., 1998; Kondo and Duboule, 1999).
and b2 (Nonchev et al., 1996; Sham et al., 1993), the r4 element for Hoxb1 (Marshall et al., 1994; Studer et al., 1994; Popperl et al., 1995), and the r5 and r5/r6 elements for Hoxb3 and Hoxa3 (Manzanares et al., 1997, 1999a). These rhombomere-specific regulatory regions have been extremely useful in the search for trans-acting factors in the upstream regulatory cascade which can directly interact with these regulatory sites to control gene expression. Using this approach it has been shown that Krox20 regulates Hoxb2 and Hoxa2 expression in r3 and r5 (Sham et al., 1993; Nonchev et al., 1996; Vesque et al., 1996) and that kreisler regulates Hoxb3 in r5 and Hoxa3 in r5/r6 (Manzanares et al., 1997, 1999a,b).

The endogenous expression pattern of the mouse Hoxb3 gene is complex and dynamic. Expression of Hoxb3 gene is first detected at 8.0 dpc at the posterior end of the neural plate (Wilkinson et al., 1989). At 9.25 dpc, Hoxb3 is expressed in the neural tube with an anterior limit at the r4/r5 boundary, but stronger expression is present in the neural tube caudal to the otic vesicle (Gould et al., 1997). By 9.5 dpc, transient high-level expression of Hoxb3 is detected in r5 (Wilkinson et al., 1989; Hunt et al., 1991). At this stage, it is also expressed in the third and posterior branchial arches, surface ectoderm, and mesenchymal tissues. From 10.5 dpc onward expression of Hoxb3 in r5 and r6 is much lower, and by 11 dpc there is a sharp boundary of Hoxb3 expression at the r6/r7 junction (Gould et al., 1997). The multiple types of transcripts for the Hoxb3 gene add another level of complexity to the expression of this gene. In 12.5 dpc embryo sections, there is low-level expression of Hoxb3 in the myencephalon at more anterior regions, with a clear anterior boundary just caudal to the pontine flexure, indicating that at this stage some transcripts are expressed in the anterior region of the neural tube derived from r3 and r4 (Sham et al., 1992). In 12.5 dpc embryos, Hoxb3 is expressed in neural crest derivatives, including mesenchymal tissue of the thyroid gland and thymus, dorsal root ganglia, and the IX/X inferior cranial ganglion complex. Hoxb3 transcripts can be detected in mesodermal and endodermal derivatives, including lung, stomach, pancreas, metanephros and degenerating mesonephric tubules, and in all prevertebrae up to C1 (Sham et al., 1992).

In previous studies we have characterized a 3' flanking region of Hoxb3 that contains a segmental enhancer involved in regulating expression of the adjacent Hoxb2 gene in r3 and r5 (Sham et al., 1992, 1993; Vesque et al., 1996). Similarly, previous analyses of Hoxb4 have identified a 3' neural enhancer (region A) conserved from humans to fish that regulates gene expression up to the r6/r7 boundary in the hindbrain (Aparicio et al., 1995; Gould et al., 1998, 1997; Morrison et al., 1995; Whiting et al., 1991). While the region A enhancer works on Hoxb4 to impose its proper anterior border in the CNS, this element is shared with Hoxb3, as the enhancer also activates the distal Hoxb3 P2 promoter embedded within it, and generates a small subset of Hoxb3 expression (Gould et al., 1997; Sham et al., 1992). Therefore, these two well-characterized enhancers that flank Hoxb3 and regulate the adjacent genes define a 28-kb region in and around Hoxb3 that presumably contains the local cis-elements needed to regulate its expression. The 22-kb intergenic region between Hoxb3 and Hoxb4 is the largest in the Hoxb complex and represents ~20% of the sequences spanning Hoxb1–b9.

In this study we have used a transgenic approach to investigate the cis-regulatory elements required to control the dynamic temporal and spatial patterns of Hoxb3 expression in mice. By linking a series of DNA fragments from the gene, its 3' flanking regions, and the large 5' intergenic region between Hoxb3 and Hoxb4 to a lacZ reporter vector and assaying for their regulatory activity, we have identified a complex modular arrangement of multiple elements involved in directing Hoxb3 expression. Together with the region A enhancer the activity of these elements recapitulates the majority of endogenous Hoxb3 expression patterns.

**MATERIALS AND METHODS**

**Generation of DNA Constructs**

Transgenic constructs I to VI, named according to the control elements they contain, were made by inserting mouse genomic fragments into a reporter cassette pBG2A that contains 1.4-kb PstI–SalI fragment of the promoter region of Hoxb4 fused in frame to the 3-kb lacZ gene, followed by a 500-bp BclI–BamHI fragment of the SV40 polyadenylation signal (construct 8; Whiting et al., 1991). Six BamHI–BamHI fragments, elements I to VI, were obtained by BamHI digestion of cosmid pCos3.1 (Graham et al., 1988) and cloned into pBG2A in both orientations. In deletion analyses, construct 1a contained a SacI–EcoRI fragment, Ib contained a NcoI–EcoRI fragment, IIa contained a SacI–Stul fragment, IVa contained a BamHI–Stul fragment, IVb contained a Stul–HindIII fragment, VA contained a BamHI–SalI fragment, and construct Vb contained a SalI–SalI fragment. In the coinjection experiments, two separate DNA fragments K2 (a 16-kb KpnI–KpnI fragment; Fig. 1) and K1 (an adjacent KpnI–BamHI fragment, Fig. 1), which was 8 kb in size and had the lacZ sequence inserted in-frame into the Hoxb3 coding region, were coinjected into fertilized oocytes. This K1 fragment was similar to the Hox2.7-lacZ transgene construct described in Sham et al. (1992) except that it did not contain the Hoxb2 enhancer. Construct A (Fig. 4) was a SacI–SacI fragment which contained the functional parts of elements III and IV and included the 1.5-kb BamHI fragment in between elements III and IV. Construct B (Fig. 4) contained the BamHI–Stul fragment derived from IVa and the SacI–Stul fragment from IIIa. In heterologous promoter analysis, the reporter construct contained a SalI–SalI fragment of 80 bp human β-globin promoter, 3 kb lacZ gene, and 500 bp SV40 polyadenylation signal (Yee and Rigby, 1993). Fusion genes were isolated from the vectors for microinjection by either Xhol or NotI digest.
In transient transgenic experiments fertilized oocytes were obtained from either FVB or F1 (CBA × C57BL/10) mice and embryos were harvested at various stages for analysis. To establish transgenic mouse lines, fertilized oocytes from F1 (CBA × C57BL/10) mice were used. No difference in expression patterns was observed between the mouse strains. Transgenic mice were identified by either PCR or Southern blotting of yolk sac or tail DNA. The activity of β-galactosidase was analyzed according to Whiting et al. (1991). Briefly, embryos were fixed in 1% formaldehyde, 0.2% glutaraldehyde, 2 mM MgCl₂, 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 K₄Fe(CN)₆, 5 mM K₃Fe(CN)₆, 2 mM MgCl₂, 0.01% sodium deoxycholate, 0.02% NP40 in PBS at room temperature.

**Histological Sectioning**

For preparation of paraffin sections, lacZ-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 cryosectioning, fresh embryos were rinsed in PBS, embedded in Tissue-tek O.C.T. compound.
FIG. 2. LacZ expression patterns directed by constructs I, Ia, and Ib. The diagram illustrates the constructs used, their relative positions in the gene locus, and the expression rate of each of the constructs in transgenic embryos. Restriction sites: A, AccI; B, BamHI; H, HindIII; N, NcoI; R, EcoRI; S, SalI; Sa, SacI; Sc, Scal; St, Stul; X, XbaI. (A) Lateral and (B) dorsal views of whole-mount lacZ staining of transient transgenic embryo (9.5 dpc) carrying construct I. (C, D) Whole-mount staining of a transient transgenic embryo (9.5 dpc) carrying construct Ia. (E) Whole-mount lacZ staining and (F) eosin-stained paraffin transverse section of a transient transgenic embryo (9.5 dpc) carrying element Ib. Arrows indicate the anterior expression boundary in the CNS of embryos carrying constructs I (at r6/7) and Ia (at r7/8). flb, forelimb bud; lpm, lateral plate mesoderm; me, mesonephros; nt, neural tube.

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<th>Exp. Rate (%)</th>
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<tr>
<td>I</td>
<td>2/2 (100)</td>
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<tr>
<td>Ia</td>
<td>11/16 (68)</td>
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<td>Ib</td>
<td>11/19 (58)</td>
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(Miles), and stored at -80°C. Cryosections (15 μm) were prepared
with a cryostat (Leica CM 3000) on poly-L-lysine (Sigma)-coated
slides. The slides were then air-dried and postfixed in 4% parafor-
maldehyde at 4°C for 10 min. After washing twice in PBS, the
sections were stained overnight in X-gal staining solution (as
above), then counterstained with eosin, dehydrated, and mounted.

RESULTS

Two previously characterized enhancers flank Hoxb3 on
the 3' and 5' sides, regulating Hoxb2 expression in r3/r5 and
the r6/7 limits of Hoxb4, respectively (Sham et al., 1993;
Whiting et al., 1991). These control regions define the two
ends of a 28-kb interval in and around Hoxb3 that is likely
to contain the local cis-elements needed to direct its main
patterns of expression. To investigate this possibility we
have used transgenic analysis with a lacZ reporter gene to
assay the regulatory potential of DNA fragments in this
interval.

Reconstruction of the Major Aspects of
Endogenous Hoxb3 Expression

First we focused on the large intergenic region between
Hoxb3 and Hoxb4. The distal Hoxb3 P2 promoter is posi-
tioned in the middle of the Hoxb4 enhancer between
elements that control its early and late expression (Gould
et al., 1998, 1997). P2 lies 17 kb upstream of the proximal P1
sequences (Fig. 1 and Sham et al., 1992). Initially we wanted
to test the domain extending from P1 up to but not
including P2, to avoid the shared Hoxb4 enhancer (region A)
masking other activities. To achieve this we used a 16-kb
KpnI genomic fragment (K2) to stimulate expression. As
a reporter gene we used a construct (K1) that contains the
Hoxb3 P1 promoter and adjacent 3' sequences, spanning the
two coding exons and their intron, with the lacZ reporter
inserted in-frame 3' to the homeodomain (Fig. 1). The two
separate DNA fragments K1 and K2 were coinjected into
fertilized eggs and transgenic founder embryos were assayed
for reporter expression at 8.5, 9.5, and 12.5 dpc. Sixty
percent of the transgenic embryos expressed the reporter
and all expressing embryos had a pattern closely resembling
that of endogenous Hoxb3 at a similar stage.

At 8.5 dpc reporter staining was primarily detected in the
neural tube from the level of the midtrunk up to the caudal
hindbrain with a clear limit at the r4/r5 boundary (Fig. 1A).
At this stage, the level of reporter gene expression in r5 was
clearly higher than that in the rest of the neural tube. At 9.5
dpc transgene expression was upregulated in the neural
tube with a rostral limit still at the r4/r5 boundary and
staining was detected in somites and the third and posterior
branchial arches (Figs. 1B and 1C). The expression in r5
continued to be maintained at high levels. The main
difference between this pattern and the endogenous gene is
the absence of neural expression in posterior regions (Figs.
1B and 1C) as the Hoxb3 expression pattern normally
extends from r5 to the caudal end of the neural tube (Gould
et al., 1997; Sham et al., 1992). At 12.5 dpc transgenic
embryos exhibited similar expression patterns: lacZ stain-
ing was evenly distributed in the neural tube and extended
from the hindbrain to the spinal cord (Figs. 1D and 1E), but
expression in the spinal cord was not detectable posterior to
the hindbrain bud level (Fig. 1E). Reporter expression was
detected in the prevertebral column and dorsal root ganglia,
with a higher level in the anterior cervical regions. At this
stage (12.5 dpc) the main differences between the reporter
and Hoxb3 were the continued lack of posterior neural
expression and an absence/reduction of expression in lateral
plate mesoderm and the forelimb region.

Therefore, in these transgenic embryos the two genomic
fragments K1 and K2 have the ability to mediate reporter
expression in a manner similar to endogenous Hoxb3 ex-
pression patterns with respect to timing and spatial restric-
tions from 8.5 to 12.5 dpc. The absence of lacZ expression
in the posterior neural tube can be accounted for by the fact
that the region A enhancer, not present in these fragments,
is shared by Hoxb3 and directs expression in this domain
(Gould et al., 1998, 1997; Whiting et al., 1991). Hence the
lack of expression in forelimbs and lateral plate mesoderm
are the major patterns missing and are presumably regu-
lated by control elements that lie outside of K1 and K2.

Distal Elements Flanking the Region A Neural
Enhancer Direct Lateral Plate Mesoderm and
Limbbud Expression

To search for cis-elements that control the missing pat-
terns of reporter expression in forelimbs and mesoderm, we
investigated the activities of the remaining 5' flanking
region upstream of Hoxb3 K2 that extended to the very 3'
end of Hoxb4. This BamHI fragment, termed element I,
contained the region A neural enhancer (HindIII-Ncol
region, Fig. 2) and flanking regions and the Hoxb3 P2 pro-
moter. When linked to a heterologous lacZ reporter vector,
it directed the expected patterns of staining in the neural
tube with a boundary at r6/r7 (I; Figs. 2A and 2B). There was
strong staining in posterior parts of the neural tube, consis-
tent with the idea that this enhancer could direct that
aspect of Hoxb3 expression. However, in addition to the
neural expression there was strong staining in lateral plate
mesoderm and forelimb buds at 9.5 dpc (Figs. 2A and 2B). By
generating two 5' deletion variants of this fragment, remov-
ing part (Ia) or all (Ib) of the region A neural activity in
element I, the activity of the limb and lateral plate enhancer
was mapped to an Ncol-EcoRI restriction fragment (Figs.
2C–2F). At 9.5 dpc all transgenic embryos that expressed
the reporter under the control of region Ib showed lacZ
expression in forelimb buds and lateral plate mesoderm
(Fig. 2E). This demonstrates that this DNA fragment (Ib)
contains cis-acting sequences capable of directing gene
expression in forelimb bud and lateral plate mesoderm
independent of the neural enhancer. Whole-mount staining
also revealed low levels of neural expression from the tail bud to an anterior limit level with the forelimb bud in embryos carrying Ib. In sections of two embryos at 9.5 dpc lacZ staining could be detected in the ventral part of the neural tube and the mesonephros (Fig. 2F). These expression patterns represent subsets of endogenous Hoxb3 gene expression patterns in 9.5 dpc embryos (Hunt et al., 1991; Sham et al., 1992). Hence, K1 + K2 in combination with region A and element Ib appear to reconstruct the endogenous patterns of Hoxb3 expression, indicating that cis-elements are spread over the large 24-kb intergenic region between Hoxb3 and Hoxb4.

**Element III Mediates Neural and Mesodermal-Specific Expression**

To begin to characterize this large regulatory domain, our subsequent analyses focused on a more detailed examination of cis-acting control elements embedded in the two large DNA fragments K1 and K2. We did this by breaking them into a series of individual BamHI fragments and testing them in transgenic assays on a heterologous lacZ reporter vector that had no activity by itself (pB4ZA, construct 8, Whiting et al., 1991).

We first examined element II, which was a 5-kb BamHI–BamHI fragment located at the 5' end of K2 (Fig. 1). We generated a total of 13 transient transgenic embryos, only 5 of which showed lacZ staining at 9.5 dpc. Of these none had a consistent pattern and they displayed variable and non-specific expression patterns in unexpected parts of the embryos such as the first branchial arch, where Hoxb3 is not expressed (data not shown). This construct therefore behaved as an enhancer/promoter trap, directing ectopic expression in embryonic domains presumably under the influence of the flanking integration site. This suggests that element II has no independent regulatory activity.

We then examined element III, which was the next 3' BamHI–BamHI fragment (4 kb) located in intron 1 (Figs. 1 and 3). This element mediated robust and consistent lacZ expression in neural and mesodermal tissues. With respect to timing of expression, it was not detected at 7.5 dpc, but by 8.5 dpc strong staining was seen in the anterior neural tube with a clear anterior boundary that mapped to the posterior otic sulcus (Figs. 3B and 3H). At 9.5 dpc, the transgene was uniformly expressed from the rostral spinal cord up to the r5/r6 junction in the hindbrain, which was one segment more posterior than endogenous expression (Figs. 3C and 3D). In the spinal cord the posterior expression faded out at the level of the forelimb bud (Fig. 3C). By 10.5 dpc neural expression was downregulated, but higher levels remained in the r6 territory (Fig. 3E). In 12.5 dpc embryos, lacZ expression was lost from the hindbrain and hardly detectable in the spinal cord (Figs. 3F and 3G).

Histological sections of the 9.5 and 10.5 dpc transgenic embryos also showed some positively stained cells in the posterior part of r5 (Figs. 3K and 3M). Therefore, the anterior limit of expression mediated by this element does not coincide strictly with a rhombomeric compartment across the entire D–V extent of r6. In the anterior spinal cord at 10.5 dpc, lacZ staining became less uniform in all cells and was stronger in the peripheral layers of the neuroepithelium (Fig. 3L). This is consistent with the dynamic patterns of Hoxb3 and other Hox gene expression in the developing neural tube, which change in a progressive manner in association with the programs of neuronal differentiation (Davenne et al., 1999; Graham et al., 1991). Element III also directed staining in neural crest derivatives similar to the expression pattern of the endogenous Hoxb3 gene. In 9.5 and 10.5 dpc embryos, the third and posterior branchial arches were positively stained (Figs. 3C, 3E and 3M) and lacZ expression was detected in the IX/X cranial ganglia complex (Fig. 3O). Therefore, element III has the ability to set the proper anterior limits of expression of Hoxb3 in the neural crest derivatives in the branchial region but not in the neural tube.

Element III also directed expression in the mesoderm. In 8.5 dpc embryos, there was transient lacZ expression in the posterior somites extending from the newly condensed somitomeres to the sixth somite (Figs. 3B and 3I). However, no lacZ expression could be found in the somites after this stage (Figs. 3C, 3E, and 3I). By 10.5 dpc, lacZ expression was found in some mesodermal tissues, including the wall of the stomach and the developing kidney (Fig. 3N). At this stage some cells in the pancreas, presumably of endodermal origin, were also stained (Fig. 3N). Thus, element III displays a diverse range of activities. It mediates a temporally specific expression patterns in unexpected parts of the

![FIG. 3. The lacZ expression patterns of transgenic embryos carrying constructs III and IIIa. Whole-mount lacZ staining of (A) 7.5 dpc, (B) 8.5 dpc, (C–D) 9.5 dpc, (E) 10.5 dpc, and (F–G) 12.5 dpc transgenic embryos carrying construct III. The position of the posterior otic sulcus in the 8.5 dpc embryo (B) is marked by an arrow. The anterior lacZ expression boundary (r5/r6) at 9.5 dpc is indicated by an arrow (D). Note that lacZ began to be expressed at around 8.5 dpc and started to fade at around 12.5 dpc in the neural tube. The position of the otic vesicle (ov) is used to mark the location of r5 and r6. At 9.5 dpc, lacZ is expressed in the third (ba3) and posterior branchial arches (C, E). (H–O) Paraffin sections of embryos at stages 8.5 dpc (H–I), 9.5 dpc (J–K), and 10.5 dpc (L–O). The position of the posterior otic sulcus in the 8.5 dpc embryo (H) is marked by an arrow. (P–Q) Whole-mount lacZ staining of 9.5 dpc embryos carrying construct IIIa. Note that the expression level is upregulated in r6 (bracketed). 1, the first branchial arch; 2, the second branchial arch; ba3, the third branchial arch; gIX/X, inferior glossopharyngeal and vagal ganglia complex; h, heart; lI, liver; me, mesonephros; nc, neural crest; nt, neural tube; ov, otic vesicle; p, pancreas; r5/r6, junction of rhombomeres 5 and 6; r6, rhombomere 6; s6, the sixth somite; so, somite; st, stomach.](https://example.com/figure3.jpg)
FIG. 4. Transgenic analysis of expression patterns directed by constructs IV as well as deletion and combinatorial analyses of elements III and IV. (A–C) Whole-mount lacZ staining of (A) 9.5 dpc, (B) 10.5 dpc, and (C) 12.5 dpc transgenic embryos carrying construct IV. (D–I) Paraffin sections of transgenic embryos at stage (D) 8.5 dpc and (E–I) 9.5 dpc. (J–K) lacZ staining patterns of 12.5 dpc transgenic embryos shown in sagittal cryosections. c1, the first prevertebra; c2, the second prevertebra; c3, the third prevertebra; flb, forelimb bud; fp, floor plate; hb, hindbrain; pr5, presumptive r5; r5, rhombomere 5; r6, rhombomere 6; s6, the sixth somite; so, somite; st, stomach; v, ventral. (L–M) Whole-mount lacZ staining of (L) 9.5 dpc and (M) 10.5 dpc transgenic embryos carrying construct IVa. Identical patterns of lacZ staining were found in transgenic embryos generated with construct β-IVa in which two copies of element IVa were linked in a 3’ to 5’ orientation to a β-globin promoter/lacZ expression vector. (N) Whole-mount lacZ staining of 10.5 dpc transgenic embryos carrying construct IVb. (O–Q) Whole-mount lacZ staining of transgenic embryos carrying construct A at (O) 8.5 dpc and (P, Q) 9.5 dpc. Identical patterns of lacZ staining were observed in transgenic embryos carrying construct B. High level of lacZ expression was found in the posterior portion of the developing hindbrain of the 8.5 dpc embryo. Upregulated lacZ expression was observed in both r5 and r6 of the 9.5 dpc embryos. lacZ expression was observed in the third (indicated by arrowhead) and posterior branchial arches and in somites, but not in the posterior neural tube (P).
dynamic pattern in both neural and mesodermal tissues (somites) and contains sequences that mediate expression in cranial neural crest and a variety of mesodermal tissues characteristic of Hoxb3 expression.

Further deletion analysis had narrowed the activities within this fragment to a 1.3-kb element IIIa, which was sufficient to direct lacZ expression in the neural and mesodermal tissues in exactly the same domains as those directed by the 4-kb element III (Figs. 3P and 3Q and data not shown). However, the expression rate of element IIIa was much lower (33%) than that of element III (71%) (Fig. 3).

**Element IV Defines the r5 Expression Domain of Hoxb3**

Element IV covers a 2.6-kb genomic fragment (Figs. 1 and 4) which contains exon 2 and flanking intronic sequences. Expression was first detected in 8.5 dpc transgenic embryos; histological analysis illustrated that lacZ was expressed as a single band around the posterior rhombomeric sulcus of the developing hindbrain (Fig. 4D). In 9.5 dpc embryos, lacZ expression was clearly restricted within a single rhombomere (r5) in the neural tube (Figs. 4A and 4G). Coronal and transverse sections of 9.5 dpc embryos showed lacZ staining in almost the whole of r5 with the exception of the floorplate (Figs. 4E and 4F). The r5-specific expression was maintained in 10.5 and 12.5 dpc transgenic embryos, though the levels of lacZ staining in the hindbrain were much reduced (Figs. 4B and 4C). Within the developing hindbrain of 12.5 dpc embryos, lacZ staining was not uniform but was much weaker in the ventral cells (Fig. 4J). This dynamic change of neural expression pattern in r5 is similar to that of endogenous Hoxb3 (Graham et al., 1991). This is not surprising as at the 5' end of this region is a 650-bp BamHI–StuI fragment (IVa) that has two short regions of homology (19 + 45 bp) with the chick Hoxb3 gene and functions as an r5 specific enhancer as we have previously shown (Manzanares et al., 1997). The conserved motifs correspond to binding sites for kreasler, a Maf-bZip transcription factor important in controlling hindbrain segmentation (Cordes and Barsh, 1994; Manzanares et al., 1999b). Hence the anterior domain of Hoxb3 in r5 is directed by this short segmental enhancer that works in an orientation-independent manner (Figs. 4L and 4M).

However, element IV also directed reporter staining in craniofacial structures, somites, and other mesodermal derivatives. In the branchial region of the head, a few cells in the fourth and posterior branchial arches were positively stained (Fig. 4G). Somitic expression became detectable in late 8.5 dpc embryos (11-somite stage); sagittal sections of an 8.5 dpc embryo demonstrated lacZ expression in the sixth and posterior somites (Fig. 4D). At 9.5 dpc, expression was continuous from the sixth somite to the tail-bud region (Fig. 4H); transverse sections showed lacZ expression in the mesonephros (Fig. 4I). In 10.5 dpc embryos, the whole forelimb buds were stained (Fig. 4B), but by 12.5 dpc, lacZ expression became restricted to the posterior half of the forelimb buds (Fig. 4C). Histological studies of 12.5 dpc embryos demonstrated that lacZ was expressed throughout the prevertebral column with an anterior boundary in C3 (Fig. 4K), in the mesonephros, and in the metanephric tubules within the metanephros (data not shown). When the activity of element IVb alone was examined in 10.5 dpc transgenic embryos, lacZ expression was detected in posterior somites from the tail-bud region through the trunk with an anterior limit at the level of the forelimb bud (Fig. 4N). The anterior lacZ expression boundary in the somites and forelimb buds was the same as that of element IV at a similar developmental stage except that the relative efficiency of expression was lower, 23 vs 56% (compare Fig. 4N with Fig. 4B). Hence, elements in the neural enhancer (IVA) may aid element IVb. These results showed that, in addition to the r5 enhancer, element IV contains regulatory sequences for important aspects of mesodermal Hoxb3 expression.

**Elements III and IV Act in an Additive Manner to Generate a Major Part of the Pattern**

Once the activities of different fragments of K2 were tested, it appeared that element IIIa and the two enhancers in element IV represented the major aspect of the K2 activity. To verify this and see if there is any cooperation between the enhancers, we constructed two variant combinations of regions from elements III and IV. The first was a genomic fragment extending from the 5' end of element IIIa into the middle of element IV (construct A, Fig. 4). Transgenic analysis showed that in 8.5 dpc embryos construct A was able to direct lacZ expression in the neural tube from an anterior level of the posterior otic sulcus into the anterior spinal cord region, fading out at around the midtrunk level (Fig. 4O). This pattern was similar to the neural expression domains seen with K1 + K2 (Fig. 1A). At 9.5 dpc, whole-mount staining showed lacZ expression in r5, r6, r7 and the anterior spinal cord (Figs. 4P and 4Q) and as with K1 + K2 neural expression could not be detected posterior to the forelimb bud level (Fig. 4P). However, unlike with K1 + K2, which showed an upregulation of lacZ expression distinctly in r5, embryos generated with construct A had high levels of expression in both r5 and r6 (Figs. 4P and 4Q). Construct A also mediated lacZ expression in the third and posterior branchial arches and in somites in the posterior region from around the forelimb bud level to the tail bud (Fig. 4P and data not shown). As this construct truncates element IV at an internal Sall site, it further maps the mesodermal enhancer in IVb to a small Stu–SacI fragment adjacent to the r5 enhancer (Fig. 4).

The enhancer activities of elements III and IV were also tested in a different configuration, where element IV was placed 5' of element III, and the orientation of both elements was maintained at 5' to 3' (construct B). In 8.5 and
9.5 dpc transgenic embryos, the patterns and level of lacZ expression were identical to those observed for construct A above (Figs. 4O-4Q). Interestingly, when placed in this configuration, the expression rate of this construct was 100%; all five transgenic embryos expressed the reporter gene. Therefore, the neural and mesodermal expression domains of constructs A and B overlapped exactly with that mediated singly by elements III and IV, showing that they could work in an additive manner. However, the coordination of enhancer activities in adjusting levels of gene expression in the rhombomeres could not be achieved by these two elements alone and genomic regions outside of these elements are required.

**Element V Directs Expression in Mesodermal Derivatives**

In examining the patterns obtained with injections of K1 + K2 versus the regulatory activity of K2 and its individual components, we noted that there were aspects of the mesodermal pattern, such as somites, that were not present. This suggested that elements within K1 were important and we analyzed its potential in more detail. Element V is a 4.5-kb BamHI fragment that starts from the second intron of Hoxb3 and contains the proximal promoter P1 region (Sham et al., 1992), exons 3 and 4 including the coding sequence for the homeodomain (Figs. 1 and 5). When a construct containing this element was used in transient transgenic experiments, 59% of transgenic embryos showed lacZ expression in mesoderm derivatives. Two transgenic mouse lines were established and analysis of transgenic embryos showed that they all exhibited similar expression patterns. At 8.5 dpc element V directed expression strongly in all somites and in the mesenchymal wall of the developing gut (Figs. 5A, 5G, and 5H). At 9.5 dpc, lacZ expression persisted in somites (Fig. 5B) and histological sections revealed that all except the first pair of somites were stained with lacZ, as indicated by staining of the sclerotome (Fig. 5J). More detailed studies detected reporter staining in mesenchyme, limb bud, and also the nephrogenic cord tissues (Fig. 5I). No expression was ever detected in the neural tube. Expression of element V was maintained in 12.5 dpc transgenic embryos, evidenced by staining in the whole forelimb bud and in the cartilaginous structure of the hindlimb buds (Fig. 5C). Furthermore, lacZ staining was found in the dorsal epidermal layer extending from the tail-bud region to the cervical level (Figs. 5C and 5D). Cryosections of 14.5 dpc embryos showed a high level of lacZ expression in the adrenal gland (Fig. 5L) and in most of the prevertebrae. The most anterior expression boundary in the prevertebral column was mapped behind the first cervical prevertebra (C1), at the body of the second cervical prevertebra (C2) (Fig. 5K). These findings showed that there are mesodermal regulatory sequences within element V which are able to direct the paraxial mesodermal expression patterns of Hoxb3.

Since the element V fragment was composed of many functional components, such as promoter and coding regions, we generated two subfragments (Va and Vb) to locate the minimal control region required for mesodermal expression. Element Va was a 2.4-kb BamHI-Smal fragment containing the P1 promoter region (Fig. 5). Though the expression rate of element Va was 29%, which was lower than that of element V, it was sufficient to display all the spatial-specific activity of element V. In 9.5 dpc transgenic embryos, lacZ expression was detected in the mesoderm, in somites, and in the forelimb buds (Fig. 5E). Whole-mount lacZ-stained 12.5 dpc transgenic embryos showed expression in the dorsal epidermal layer with a rostral expression limit similar to that directed by element V (compare Figs. 5C and 5F). In contrast, element Vb, which spanned exons 3, 4, and the intron in between, did not appear to have any spatial-specific activity. Only one of the eight transgenic embryos showed nonspecific lacZ staining in the forelimb bud, midbrain, and optic vesicle (data not shown). Therefore, element Va contains the sequences necessary for mediating mesodermal-specific expression of the Hoxb3 gene.

Element VI contains the most 3' sequence of the Hoxb3 gene locus; it is located immediately upstream of a Hoxb2 enhancer element previously identified (Sham et al., 1992, 1993). Transient transgenic analysis showed that element VI had a low expression rate (7%); only 2 out of 26 transgenic embryos (9.5 dpc) showed ectopic lacZ expression and no spatially specific expression was detected (data not shown).

Taken together our studies have uncovered a complex array of enhancers that can work in an additive manner to regulate the major aspects of Hoxb3 expression. There is no single neural or mesodermal element, but rather spread over a 22-kb region is a diverse array of elements that direct different spatial and temporal aspects of the expression pattern. Multiple elements work in concert to control the neural and mesodermal domains of Hoxb3 expression.

**DISCUSSION**

In this study we have performed a transgenic regulatory analysis to investigate the cis-acting components that direct the dynamic pattern of Hoxb3 expression in mouse embryos. By assaying the regulatory potential of fragments spanning a 28-kb region of the locus, including the Hoxb3 gene itself and flanking intergenic sequences, we have identified four new cis-acting regulatory regions (elements Ia, IIa, IVb, and Va). These control elements act as enhancers to direct reporter gene expression in diverse tissue-, temporal-, and spatially restricted subsets of the endogenous Hoxb3 expression domains. All of these elements are located upstream of the first coding exon (exon 3) spread over the large 22-kb intergenic region between Hoxb3 and Hoxb4, while the region 3' of Hoxb3 does not appear to
contain any regulatory activity. In combination with two well-characterized neural enhancers also located in this large intergenic region [region A (element I) and an r5-restricted element (IVa)], these cis-elements appear to work as modules in an additive manner to recapitulate the major patterns of Hoxb3 expression (summarized in Fig. 6). The organization and properties of these multiple control regions have provided insight into the complex nature of Hoxb3 regulation and raised interesting issues with respect to its paralogs and other Hox genes.

**Multiple Elements Are Responsible for the Dynamic Expression of Hoxb3 in the Neural Tube**

Hox genes play important roles in patterning the hindbrain and spinal cord (Lumsden and Krumlauf, 1996; Tiret...
et al., 1998; Trainor et al., 2000) and our results have illustrated the regulatory complexity needed to generate the temporal and spatial patterns associated with these roles. Endogenous Hoxb3 expression is dynamically modulated at different stages of neural development (Gould et al., 1997, 1998; Hunt et al., 1991; Sham et al., 1992; Wilkinson et al., 1989). Our analysis illustrates that for Hoxb3 it is not a simple case of a rhombomere-specific control element, such as the r5 enhancer (element IVa), being added to a single element that governs the remaining global neural expression along the axis. Rather it is a combination of enhancers with coordinated early and late as well as anterior and posterior activities (elements I, III, and IV) that creates the final pattern. All three neural elements (I, IIIa, and IVa) identified in the Hoxb3 locus are capable of directing expression in the CNS by 8.5 dpc of development. Elements IIIa, IVa, and a retinoic acid-dependent early neural enhancer (ENE) in element I (Gould et al., 1998) act in specifying the initial wave and the anterior domains of Hoxb3 expression by 9.5 dpc. The activity of elements IIIa and IVa is transient and reporter expression is downregulated over the next 2 days of development (Figs. 3 and 4). Like the endogenous gene posterior reporter expression is very weak at early stages, but becomes upregulated around 9.5 dpc and stays on throughout later stages. Only element I shared between Hoxb3 and Hoxb4 has the ability to stimulate this expression in posterior regions and at later stages, and this is mediated through a separate late neural enhancer (LNE) that functions as an autoregulatory element (Gould et al., 1997, 1998).

In addition to the neural enhancers spread over a large region, there is added complexity in that multiple transcripts are initiated from different promoters spaced 16 kb apart (Sham et al., 1992). Transcripts from the two promoters are differentially expressed and display offset anterior boundaries of neural expression. This implies that the widely spaced neural enhancers we have identified do not work equally on each promoter. The enhancers in element

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**FIG. 6.** Genomic organization of the regulatory elements within the intergenic regions of Hoxb3 and Hoxb4, Hoxa3, and Hoxa4. The position and specificity of the regulatory elements identified in this study are illustrated in blue (neural-specific enhancer), yellow (mesodermal-specific enhancer), and red (enhancer with combined neural and mesodermal activities). For the control of Hoxb3 in neural tissues, three enhancer elements, I, III, and IV, are involved to direct gene expression to anterior boundaries at r6/7, r5/6, and to r5: for mesodermal control in a diversified range of tissues, elements I, III, IV, and V are required. The enhancer elements for regulating Hoxa3 expression are also illustrated using the same color scheme for comparison. flb, forelimb bud; hb/sc, neural tube expression up to bindbrain and spinal cord junction; lb, limb bud; lpm, lateral plate mesoderm; meso, mesodermal expression; r5, rhombomere 5; r5 + r6, rhombomeres 5 and 6; r5/6, neural tube expression up to r5/6 boundary; r6/7, neural tube expression up to r6/7 boundary; s4/5, somitic expression up to the fourth somite; sc, spinal cord.
I act on P2 to set an anterior limit at r6/r7 (Gould et al., 1997). While elements IIIa and IVa appear to stimulate the proximal P1 promoter regulating the more anterior domains in r5 and r6 characteristic of Hoxb3, by 5' RACE experiments we have identified a new 5' noncoding exon among the Hoxb3 transcripts, indicating the presence of another promoter for this gene (M. L. Kahlmeier-Gabbe and M. H. Sham, unpublished data). Therefore, at present it is still unclear with which specific promoters the enhancer elements IIIa and IVa may interact to define the early neural expression boundary of Hoxb3.

Elements Involved in Mesodermal Expression Domains

An even greater degree of complexity is also seen in the array of elements we have identified (Ib, IIIa, IVb, and Va) that mediate expression in mesodermal and branchial arch derivatives (summarized in Fig. 6, elements shown in yellow and red). The major mesoderm-specific element is Va, which is closely associated with the proximal promoter P1 in terms of its position (Fig. 5). Element Va directs expression in all but the first pair of somites and then the boundary regresses posteriorly to C1 at 12.5 dpc, which corresponds to the endogenous expression boundary of Hoxb3 in the vertebral column at this stage (Sham et al., 1992). Other than element Va, elements IIIa and IVb can also direct different patterns in paraxial mesoderm (somites) starting from 8.5 dpc. Element IIIa transiently stimulates expression from the newly condensed somitomere up to the last six pairs of somites at 8.5 dpc, but expression is rapidly downregulated and never reappears. At this same stage element IVb reporters are expressed up to the level of somite 6 and remain on at later stages (12.5 dpc) with a boundary at C3. Therefore, there is a mesoderm-specific element (Va) responsible for setting the anterior boundary of paraxial mesoderm expression for Hoxb-3, but two other elements (Iib and IVb) also have partial contributions in paraxial mesoderm and their activities would have to be coordinated during embryogenesis.

In the limb buds elements Ib, IVb, and Va all contribute to expression and the individual patterns are stage dependent. For example in an early stage (10.5 dpc) IVb directs expression throughout the forelimb bud but only in the posterior half later (12.5 dpc). Individually the activity of either element IVb or Va is strong in the forelimb buds but when combined and in the context of the endogenous Hoxb-3 promoter the level of reporter expression is attenuated, as seen in the embryos generated with K1 + K2 (Figs. 1C–1E). Hence the activity of element Ib remains important for expression in the forelimb buds. The main activity capable of regulating gene expression in the lateral plate mesoderm also resides in element Ib. In addition, Ib, IIIa, IVb, and Va have other regulatory activities that can mediate expression in a diverse range of mesodermal, branchial arch, and mesenchymal tissues: mesonephros, metanephric tubules, kidney, stomach and gut wall, third and posterior branchial arches, and IX/X cranial ganglia complex; all of these are sites of endogenous Hoxb3 expression. The arrangement of these dispersed mesodermal activities is illustrated in Fig. 6.

Comparison of Organization and Regulatory Mechanisms for Hoxa3 and Hoxb3

It is interesting that the intergenic region between Hoxb3 and Hoxb4 (22 kb) is the largest between any genes in the Hoxb complex, with the exception of the position of Hoxb13 at the end of the cluster. This interval contains all the Hoxb3 regulatory elements found to date and is almost like a minicluster, as it has multiple promoters, a complex array of dispersed enhancers that direct different anterior boundaries of expression at different times of development, and shared elements with the adjacent Hoxb4 gene. Hence there is an opportunity for mechanisms such as enhancer sharing, promoter competition, selectivity, and long-range interactions to participate in controlling its patterns of expression. The complex regulatory mechanism involved suggests that it may be difficult to alter or change this region and properly maintain the correct patterns of expression for Hoxb3 and the neighboring genes in the complex.

In other complexes the intergenic regions between the group 3 and 4 paralogs is also very large and regulatory analysis aimed at identifying segmental enhancers for Hoxa3 uncovered the presence of several additional enhancer elements between Hoxa3 and Hoxa4 (Manzano et al., 1999a). These are summarized in and compared with Hoxb3 in Fig. 6. Although the expression patterns of Hoxb3 and Hoxa3 are similar, there are many detailed differences in the overall arrangements of cis-acting regulatory elements for their neural and mesodermal specific regulations (Fig. 6). The anterior rhombomere boundaries of expression for the paralogous genes Hoxa3 and Hoxb3 at 9.5 dpc are the same, but there are subtle differences in the extent of segmental expression. Hoxb3 is upregulated by kreisler only in r5, while Hoxa3 is upregulated by the same trans-acting factor in both r5 and r6 (Manzano et al., 1997, 1999a,b). A separate element (IIa) controls the expression of Hoxb3 in anterior spinal cord and hindbrain up to r6 (Fig. 3), so for Hoxb3 the expression in r5 and r6 are regulated by separate elements located more than 4 kb apart, while for Hoxa3 r5 and r6 expression is regulated by the same kreisler-dependent element. Other than these segmental activities, there is almost nothing similar between the organization of these control elements other than the fact that there are multiple elements spread throughout the region. Therefore, the regulation of mesodermal patterns of expression may involve enhancers that are less constrained with respect to position and large blocks of conserved identity. Interestingly, analysis in mice has recently examined the ability of two group 3 Hox genes (Hoxa3 and Hoxd3) to functionally replace each other when targeted
into each other’s loci (Greer et al., 2000). Each of the genes can compensate for the other, suggesting that both their function and their regulation are similar. Therefore, further detailed comparisons of the individual elements regulating group 3 genes in the Hoxa, Hoxb, and Hoxd clusters will help to unravel the extent that key elements have been maintained following duplication and divergence from the ancestral cluster.

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REFERENCES


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