A 3' remote control region is a candidate to modulate Hoxb-8 expression boundaries

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ABSTRACT  Hox genes have been shown to play a key role in the acquisition of positional identity by precursors of embryonic axial, paraxial and limb structures. This function is thought to depend on the sequential, concerted expression of these genes in time and space. However the underlying molecular mechanisms of this collinear expression are still largely unknown. So far we had identified proximal regulatory elements driving expression of Hoxb-8/LacZ transgenes in Hox-like expression patterns with rostral boundaries more posterior than those of the endogenous gene. In this work we have analyzed 30 kb of 3' genomic sequences for Hoxb-8 regulatory activity in transgenic mice. We have identified a control region in the Hoxb-5/b-4 intergenic region that rostrally extends the Hoxb-8/LacZ expression domain into the posterior hindbrain. In combination with the Hoxb-8 minimal promoter, the 3' control region drives transgene expression with boundaries more anterior than those of Hoxb-8 in the neural tube. When combined with a 4.5 kb Hoxb-8 upstream sequence, where essential proximal regulatory sequences are located, the 3' control region drives transgene expression in a domain which seems to correspond to that of the endogenous Hoxb-8. By deletion analysis we have narrowed down to 550bp the regulatory activity interacting with the Hoxb-8 minimal promoter. We discuss the possibility that this remote 3' enhancer, which is the closest regulatory region found in the cluster to rostrally extend Hoxb-8/LacZ expression, could be involved in the regulation of Hoxb-8 and interact with the proximal control elements.

KEY WORDS: Hoxb-8 gene, transcription, antero–posterior axis, transgenic mouse

Introduction

Vertebrate Hox genes have been identified through sequence homology to the homeobox of the Drosophila homeotic cluster (HOM–C) genes (Carrozzo et al., 1984; McGinnis et al., 1984). In addition to this conserved domain, Hox genes also share a similar genomic organization with their Drosophila counterparts (Graham et al., 1988; Duboule and Dolle, 1989). Nevertheless while the fruitfly has one complex containing 8 genes, the vertebrate genome has 4 clusters, Hox–a, b, c and d, localized on different chromosomes (Duboule et al., 1986; Breier et al., 1988) accounting for a total of 39 genes (Zielser et al., 1996). This cluster organization has been strikingly conserved throughout evolution (Duboule and Dolle, 1989; Graham et al., 1989; Kenyon and Wang, 1991; Garcia–Fernández and Holland, 1994).

The phenotypes observed in many gain and loss of function experiments have demonstrated that Hox genes, like HOM–C genes are essential for mediating regional specific development in the embryo (McGinnis and Krumlauf, 1992; Krumlauf, 1994). Among the tissues expressing the Hox genes are the neural tube, the lateral, intermediate, paraxial mesoderm and their derivatives such as the vertebrae, the genitalia (Dolle et al., 1991) and the limbs (Dolle et al., 1989). In these structures, Hox genes are expressed in restricted, antero–posterior overlapping domains. The most 3' genes are transcribed first (Gaunt, 1988; Izpisua–Belmonte et al., 1991) and exhibit more anterior boundaries (Duboule and Dolle, 1989; Graham et al., 1989) than the 5' genes which are also expressed later. This property is referred to as spatio–temporal colinearity of Hox gene expression (McGinnis and Krumlauf, 1992; Duboule, 1994) and would account for the key role of these genes in embryonic development.

Although the concerted regulation of expression of the Hox genes in time and space is assumed to be essential for correct embryonic development, the underlying mechanisms are not yet known. Studying the cis–acting regulatory elements controlling
Hox gene expression is the first step towards elucidating the molecular interactions underlying the coordinated Hox gene expression. The generation of transgenic mice carrying a reporter gene coupled to surrounding genomic sequences has led to the localization of important proximal cis-regulatory elements for several Hox genes. Some of these elements contain binding sites for known DNA-binding proteins such as retinoic acid receptors, Krox-20 (for review Krulmauf, 1994), pbx (Pöpperl et al., 1995), HNF3/forkhead-related proteins (Shashikant et al., 1995) and Cdx (Shashikant et al., 1995; Subramanian et al., 1995). Alterations in Hox gene expression domains have been shown to accompany developmental defects caused by inactivation of Krox–20 (Schneider–Maunoury et al., 1993), Cdx1 (Subramanian et al., 1995) and after the embryos have been exposed to retinoic acid (Kessel and Gruss, 1991; Morrison et al., 1996), suggesting that Krox–20, Cdx1 and retinoic acid signaling could belong to the upstream network setting Hox expression domains. Only in a few cases the expression pattern of the endogenous gene seemed to be fully reproduced by Hox/LacZ transgenes isolated with immediately flanking sequences from the cluster context (Püschel et al., 1991; Whiting et al., 1991; Behringer et al., 1993; Marshall et al., 1994). In most of the other studies aimed at identifying cis–regulatory sequences involved in the transcriptional regulation of Hox genes, the involvement of additional elements or regulatory mechanisms has been hypothesized, since surrounding sequences were not capable of conferring correct expression pattern to Hox/LacZ transgenes (Bieberich et al., 1990; Eid et al., 1993; Gérard et al., 1993; Vogels et al., 1993; Charité et al., 1995, Morrison et al., 1996). Recently a control element has been reported (Bradshaw et al., 1996), located 11 kb downstream of the Hoxc–8 start site, that seems to be responsible for maintaining Hoxc–8/LacZ transgene expression in the most anterior part of the expression domain. However the rostral boundary of the transgene is still more caudal than that of the endogenous Hoxc–8.

Besides gene control through proximal and distal cis–DNA regulatory elements, a higher level of regulation of the whole cluster, possibly affecting chromatin organization, has been proposed to underlie the spatial and temporal concerted expression of Hox genes (Gaunt and Singh, 1990; Duboule, 1994; van der Hoeven et al., 1996). In the models put forward to account for either maintenance (Gaunt and Singh, 1990) or initiation (Duboule, 1994; van der Hoeven et al., 1996) of sequential gene expression, chromatin opening would progress from the 3' to the 5' end of the clusters. Consequently an increasing part of the cluster would be in an open configuration in progressively more caudal regions of the embryo. Hence a progressively increasing number of genes would be expressed from anterior to posterior along the axis. The products of the Polycomb– and trithorax– Group genes are good candidates as trans–acting factors mediating this higher level of regulation. They are thought to be involved in modification of the chromatin organization (Paro, 1990 and 1995). Gain and loss of function mutations in some of these genes lead to homeotic transformations which have been shown to be associated with a shift in the expression boundaries of certain Hox genes (Van der Lugt et al., 1994; 1996; Alkema et al., 1995; Yu et al., 1995, Akasaka et al., 1996; Coré et al., 1997).
Fig. 2. Comparison of the expression patterns of the 48 kb-long \textit{Hoxb–8/LacZ} transgene and the endogenous \textit{Hoxb–8} gene. (A) $\beta$-gal staining pattern of a 11.5-day embryo carrying the long transgene. (B) Endogenous Hoxb–8 expression pattern at 11.5-day visualized after whole-mount in situ hybridization. (C) Parasagittal section of a 11.5-day $\beta$-gal stained embryo from line 74 (carrying the long transgene) showing LacZ anterior expression boundary in PV 8 and in the third cervical ganglion. (D) Sagittal section showing LacZ expression detected by radioactive in situ hybridization in the remnant of the first cervical ganglion and in the most posterior ones. (E and F) Adjacent sagittal sections of an X-gal stained 12.5-day embryo from line 74 showing the anterior boundary of $\beta$-gal activity in the hindbrain (E) and of Hoxb–8 expression detected by radioactive in situ hybridization (F). (G) 8.5-day transgenic embryo stained with X-gal and hybridized as a whole mount with an antisense Hoxb–8 probe. X-gal staining appears blue and Hoxb–8 expression as purple. (H) $\beta$-gal staining pattern of a head fold stage embryo from line 74. (I) Endogenous Hoxb–8 expression pattern visualized by whole mount in situ hybridization on an early fold stage wild type embryo. Orientation: A to G: anterior to the top, dorsal to the left. H, I: anterior to the right. Bars, 0.2 mm in A, B, C, D, E, F, G and 0.75 mm in H and I. g3: third cervical ganglion; g1: Fröhep's ganglion; pv8: eighth prevertebrae.
Results

Genomic sequences 3' to Hoxb-7 influence Hoxb-8 transcription

Hoxb-8/LacZ transgenes tested up to now were expressed in Hox-like expression domains but exhibited rostral boundaries more posterior to those of Hoxb-8 in the neuroectoderm and the mesoderm (Charité et al., 1995). To test the hypothesis that essential cis-acting elements were missing in the Hoxb-8/LacZ transgenes studied previously, we looked for the presence of additional regulatory sequences in the cluster which would direct Hoxb-8 transgene expression up to anterior boundaries similar to those of the endogenous Hoxb-8 gene. Since the largest fragment previously tested extended 11 kb 5' to Hoxb-8 (including the Hoxb-9 coding sequence), but only 5 kb 3' (down to the EcoRI site, 250 nucleotides 5' to the second exon of Hoxb-7, construct A, Fig. 1), and since control sequences driving more anterior expression were more likely to be found more 3' in the cluster, we analyzed genomic sequences 3' to Hoxb-7. The 11 kb 5' sequences, where Hoxb-8 proximal regulatory elements have been localized (Charité et al., 1995), were retained in the new constructs.

We started with a genomic fragment of about 48 kb extending from the first exon of Hoxb-9 to about 10 kb 3' to Hoxb-5. To circumvent problems of cloning large size DNA pieces, we injected two overlapping fragments in mouse fertilized eggs, since it had been shown that overlapping fragments could recombine when microinjected together in equimolar amount into zygotes (Pieper et al., 1992). One of the fragments was the largest Hoxb-8/LacZ transgene tested in our previous studies (Charité et al., 1995; Fig. 1 construct A). The second fragment was isolated from cosmid C3 by Sall/StI digestion (Fig. 1). It contained Hoxb-7 and extended up to about 10 kb 3' to Hoxb-5. The first exon and 1.9 kb of the intron of Hoxb-7, present in both fragments, represented the overlap which was 2.75 kb long. The rationale was that if elements involved in Hoxb-8 regulation were present in the genomic sequences downstream of Hoxb-7 and if the two fragments had been co-integrated, a dominant change in the β-galactosidase (β-gal) staining pattern was expected, compared to the expression domain of construct A alone. Construct A, the most complete Hoxb-8/LacZ transgene previously characterized, exhibited anterior boundaries at the level of the third spinal ganglion in the neural tube and in prevertebrae (PV) 11 or 12 in the mesoderm (Charité et al., 1995) whereas the endogenous pattern extends to the posterior hindbrain in the neural tube and to PV 8 in the mesoderm with weak expression in PV 7 (Deschamps and Wijgerde, 1993). Among five LacZ expressing 11.5– or 12.5–day embryos obtained after injection of the two overlapping fragments, three showed a β-gal activity pattern (Fig. 2A) similar to the endogenous Hoxb-8 expression domain in the neural tube and the mesoderm (Fig. 2B). Sections of these embryos confirmed that, as is the case for the endogenous Hoxb-8 gene, the transgene expression boundaries were localized in the posterior hindbrain and in PV 8 (data not shown).

Transgenic lines carrying the two co-integrated fragments were established for further study. We identified both the animals in which the two fragments had recombined and those carrying the
two co-integrated fragments by Southern blot analysis. A HindIII restriction fragment recognized both by a LacZ probe and a probe localized downstream from the 3' end of the overlap (Fig. 1) was a diagnostic band since it was present only if the recombination event had occurred or if the two fragments had integrated in a head-to-tail fashion. The discrimination between the two possibilities relied on the size of the band which was respectively 13 and 15.7 kb. Five lines identified as recombined expressed LacZ. However, none of the lines contained a single copy of each injected fragment but several of them, among which two at least had properly recombined. All the 5 lines exhibited the same X-gal staining pattern (Fig. 2A and data not shown).

In the paraxial mesoderm, the most anterior Hoxb–8 transcripts have been detected in PV 8 and weekly in PV 7 (Deschamps and Wijgerde, 1993). Transgenic embryos carrying the extended 3' genomic sequences exhibited β-gal rostral expression boundary in PV 8 (Fig. 2C) or PV 9. This variation of one PV in β-gal activity from one embryo to another was not line related and could be observed between transgenic littermates. Two lines (74 and 83) exhibited a strong X-gal staining in all the prevertebrae posterior to PV 7. However, in three other lines, X-gal staining was stronger caudally to PV 11 or 12 down (data not shown). This particular feature might result from the presence of the two Hoxb–8/LacZ constructs (construct A and the recombined fragments) at the integration site since injected DNA fragments are expected to integrate as a concatenate. Therefore the β-gal activity pattern might result from the superimposition of the expression of construct A on the one hand, and of the 3' extended recombined Hoxb–8/LacZ transgene on the other hand. This might explain why in the three lines discussed above, stronger expression was observed from PV 11 or 12 which was the mesoderm anterior expression boundary described for construct A (Charité et al., 1995). The stability of the X-gal staining might explain the strong expression in the lateral plate mesoderm derivatives while at the same stage Hoxb–8 transcripts are not detected there anymore.

In the peripheral nervous system (PNS), β-gal activity was detected from the posterior half of the 3rd ganglion in some embryos (Fig. 2C) and in the posterior half of the 4th ganglion in others (data not shown), while Hoxb–8 transcripts are detected in all the ganglia. However in in situ hybridization experiments with radiolabeled probe, LacZ transcripts have been detected in all the ganglia (Fig. 2D). We have no explanation so far to account for this observation.

In the central nervous system (CNS) of all the recombined lines, the anterior β-gal activity boundary was localized in the posterior hindbrain. We confirmed that the boundary was similar to that of the endogenous Hoxb–8 gene by comparing the X-gal staining pattern of 12.5–day embryo (line 74) sections (Fig. 2E) with the results of radioactive in situ hybridization experiments performed on adjacent sections with a Hoxb–8 probe that did not recognize the transgene (Fig. 2F). In the three «weak» lines discussed above, X-gal staining was patchy and weaker in the rostralmost part of the expressing neural tissue than caudally to the level of the third cervical ganglion (not shown) which was the anterior neural tube expression boundary described for construct A (Charité et al. 1995).

Transgene expression pattern mimicks Hoxb–8 expression from the head fold stage onwards

Lines carrying the two recombined fragments were used to investigate the early expression of the 3' extended transgene. To compare transgenic and endogenous gene expression bounda-
adjacent sections from X-gal stained embryos with a Hoxb-8 probe. The X-gal staining boundary was obviously more rostral than that of the endogenous Hoxb-8 transcripts (Fig. 4B). Comparison with Hoxb-5 transcription pattern revealed that the anterior expression boundary in the neural tube of construct 1 (Fig. 4C) was at about the same level as that of the Hoxb-5 gene (Fig. 4D). The transgene was expressed more strongly in the dorsal part of the neural tube while Hoxb-5 transcripts were also detected in the ventral neural tube. The X-gal staining, quenched by the silver grains generated in the emulsion by the radioactive signal, was mostly visible where the expression domains did not overlap (Fig. 4B). Since the difference in anterior boundaries between the expression domains of Hoxb-8 and the transgene was larger than the difference between the rostral limit of expression of Hoxb-8, b-7 and b-6 (Graham et al., 1989), that are quite close to each other, we did not perform the comparison with Hoxb-7 and b-6. Expression in the paraxial mesoderm was seen in only one of five embryos and since it was weak the expression boundary was difficult to assess (Fig. 4A). No expression in the lateral plate mesoderm derivatives was seen in any whole-mount embryo. The proportion of embryos exhibiting ectopic (1/5) or weak (2/5) expression suggested that the construct 1 transgene is quite sensitive to influences from the integration site.

Previously, cis-acting elements sufficient to drive the expression of a minimal Hoxb-8 promoter/LacZ transgene in a Hox-like pattern had been localized in a 4.5 kb Hoxb-8 upstream fragment (construct 6 in Charité et al., 1995). To test possible interactions between the proximal regulatory elements and the 3'CR, the latter was cloned 3' to a Hoxb-8/LacZ transgene containing these 4.5 kb upstream sequences (construct 2, Fig. 3A). Twelve embryos carrying construct 2 were recovered at 11.5 day of gestation (Fig. 4E). Neural tube expression of LacZ was seen in 10 embryos. The level of expression varied from one embryo to the other from very weak to very strong. We localized the transgene anterior boundary with respect to the expression boundary of other Hoxb genes by comparing the results of radioactive in situ hybridization experiments performed on adjacent sections with LacZ, Hoxb-5 and Hoxb-8 probes. The Hoxb-5 boundary in the neural tube (Fig. 4F) was obviously more anterior than that of LacZ (Fig. 4G) which was at a level similar to that of the endogenous Hoxb-8 gene (Fig. 4H). 7 embryos exhibited paraxial mesodermal expression of LacZ with a rostral expression boundary around the level of the posterior part of the forelimb bud (data not shown). The level of expression varied from one embryo to another and was weaker in the two embryos which had the weakest neural tube expression. All 7 embryos exhibited lateral plate mesoderm expression.

Deletion analysis delineates a 550 bp regulatory fragment

To narrow down the regulatory sequences present on the 7 kb genomic fragment, we started nested deletions of construct 1 (Fig. 3B), which does not contain the Hoxb-8 proximal elements. 4 β-gal expressing embryos carrying construct 3 were recovered. They all exhibited strong neural tube expression with a clear boundary in the hindbrain which looked more anterior than that of endogenous Hoxb-8. Two embryos did not show mesoderm expression. There was mesoderm expression in the other two embryos but the boundaries were difficult to locate because the expression was weak in one of them and obscured by ectopic expression in the other. 8 β-gal expressing embryos were recovered from injection of construct 4. Among them three embryos were useless to draw any conclusion since one developed abnormally, a second one exhibited ubiquitous expression and the expression in the third one was too weak to allow localization of the boundaries. None of the other 5 embryos exhibited mesoderm expression. In one embryo the expression in the neural tube was weak and patchy, interrupted at the level between the fore and hindlimbs, and it was difficult to determine the boundary with certainty. The X-gal staining in the neural tube of the other 4 embryos was rostrally and caudally stronger than at the inter-limb level. The anterior expression boundary in the neural tube in these 4 embryos was reproducible and similar to that of construct 1. 11 β-gal expressing embryos which carried construct 5 were obtained. One expressed LacZ ubiquitously. In only one embryo sclerotome expression was seen which was not A-P restricted. The X-gal staining in the neural tube of 2 embryos was too weak to allow determination of the boundary. The other 8 embryos all exhibited a clear rostral expression boundary in the spinal cord, similar to that of construct 1 and among them 2 showed a weaker expression at the level of the inter-limb region. The 5 β-gal expressing embryos obtained with construct 6 showed non reproducible A-P restricted X-gal expression (data not shown). 7 embryos from construct 7 that combined the Hoxb-8 minimal promoter with a 550bp fragment deleted in the previous construct were recovered. One of them was completely blue. The other ones exhibited an anterior boundary in the neural tube similar to that of construct 1. Mesodermal expression with a localized rostral boundary was seen in 3 embryos (data not shown). The transgene was always expressed at a higher level in the neur ectoderm than in the mesoderm, where the boundary was difficult to map. These results demonstrated that this 550bp fragment contained the regulatory sequences of the 3' control region interacting with the Hoxb-8 promoter. It will be important to assay the 550bp element in the context of construct 2 (Fig. 3A) to document its activity in combination with the 5' proximal elements.

Discussion

Characterization of a new regulatory region in the Hoxb cluster

Cis-acting control sequences mediating Hoxb-8 expression that have been so far localized in the proximal 5' flanking region were not able to generate a LacZ pattern with expression boundary as anterior as those of Hoxb-8 (Charité et al., 1995). In this work we describe a new regulatory region (3'CR) in the Hoxb cluster capable of rostrally shifting the expression boundary of Hoxb-8/LacZ constructs generated until now. A Hoxb-8/LacZ transgene associating the Hoxb-8 promoter and proximal elements (Charité et al., 1995) and the 3’CR express features similar to that of endogenous Hoxb-8 in the neuroectoderm and probably also in the paraxial and lateral plate mesoderm. The lower level of expression in the mesoderm than in the neuroectoderm possibly reflects either endogenous features or a higher sensitivity in the mesoderm than in the neuroectoderm to influences from the integration site. The 3’CR does not drive transgene expression in the lateral plate mesoderm derivatives. The 5’ proximal elements have been shown to drive lateral plate mesoderm expression (Charité et al, 1995). A regulatory element has also been reported, 5' to Hoxb-6, which directs spatially restricted Hoxb-6/LacZ expression in the limb/lateral plate mesoderm (Eid et
al, 1993). These elements are present in the largest transgene we have generated which carries all the sequences between Hoxb–8 and the 3’CR and which is strongly expressed in the lateral plate mesoderm derivatives with a rostral boundary at the level of that of endogenous Hoxb–8. It is a reasonable hypothesis to propose that they both might contribute to the expression of Hoxb–8 in the lateral plate mesoderm.

The 3’CR we report about in this work, is located in the Hoxb–5/b–4 intergenic region. It is likely that this element is involved in the regulation of one or both of these flanking genes. Enhancers which mediate the correct boundaries of expression of Hoxb–5 in the paraxial mesoderm and the neural tube (Sharpe, Nonchev, Gould, Whiting and Krumlauf, submitted) have been reported in this region as well as a Hoxb–4 regulatory element (Gutman et al, 1994; Morrison et al, 1995) which is located 3’ to the 3’CR. The influence of the 3’CR cloned in the vicinity of Hoxb–8 promoter could be expected since several Hox enhancers have been shown to work in combination with heterologous promoters (Whiting et al, 1991; Gérard et al, 1993; Knittel et al, 1995; Becker et al., 1996). However the possibility that the 3’CR modulates Hoxb–8 at a distance spanning three Hox genes in the cluster is not unlikely since no other sequence between Hoxb–9 and the Hoxb–5/b–4 intergenic region is capable of providing Hoxb–8/LacZ transgene with expression boundaries at the A–P level of those of the endogenous Hoxb–8. This possibility is particularly interesting since it would mean that this remote enhancer contributes, together with the proximal regulatory elements, to the generation of the endogenous Hoxb–8 expression pattern with correct A–P boundaries, and that long range interactions can occur in the Hox clusters.

Building up Hox expression domains

It has become clear that combinations of cis–regulatory elements together with Hox minimal promoters often generate discrete A–P restricted expression patterns with rostral boundaries which will depend on the elements present and their interaction/ cooperation (Vogels et al, 1993., Charié et al, 1995). Therefore it is not surprising that the Hoxb–8 minimal promoter drives gene expression with boundaries differing depending on whether the 3 CR is the only cis–acting element present or whether the proximal regulatory elements are included in the transgene. Of course, this difference might also result from an increased distance between the 3’CR and the promoter in the downstream copy of the
tandemly integrated transgenes when the 5' proximal region is present. A longer distance might be less favorable to the establishment of interactions between the 3'CR and the minimal promoter and might explain the difference in the rostral boundaries observed between the two transgenes. However, the extent of the effect suggests that the 3'CR can be modulated by the Hoxb-8 proximal elements at least in a transgenic context. This observation raises several questions. Would the interaction between the 3'CR and the Hoxb-8 promoter and proximal regulatory elements also occur in the cluster context which means over a distance spanning several genes? If it turns out to be so, one can wonder whether the modulation described is specific to Hoxb-8. Indeed, the 3'CR might as well influence the transcription of Hoxb-7, b-6 and b-5 located within its range of action. Recent work in the HoxD cluster (van der Hoeven et al., 1996) has suggested that the regulatory network governing Hox gene expression might be organized at three hierarchical levels. The primary control would take place at the level of the whole cluster possibly through an opening of the chromatin structure for transcription from 3' to 5' (Gaunt and Singh, 1990; Duboule, 1994; van der Hoeven et al., 1996). This higher order mechanism would control the consecutive initiation of Hox gene transcription from 3' to 5' and would therefore account for the temporal collateral expression of the Hox genes. Regulatory interactions between discrete proximal control elements and individual Hox promoters would subsequently modulate Hox gene expression patterns. An intermediate level of gene control might be operated by enhancers that control several genes simultaneously. If further work confirms the simultaneous influence of the 3'CR on a series of Hox promoters in the Hoxb cluster context, this element might well belong to this intermediate type of regulatory effector.

Materials and Methods

Constructs

Construct A contains the LacZ gene fused in frame to the first exon of Hoxb-8 and extends from the SalI site in the first exon of Hoxb-9 to the EcoRI site in the intron of Hoxb-7 (Fig. 1). The 22.5 kb insert was isolated as a Not fragment.

C3 is a Supercopecosmid vector containing about 39 kb genomic sequences of the mouse Hoxb cluster extending from about 6 kb 5' to the first exon of Hoxb-8 to about 10 kb 3' to the second exon of Hoxb-5 gene (Fig. 1). The vector CsaI site was replaced by a Sfi linker of 14 mers (Biolabs).

To obtain construct 1 (Fig. 2), a 7 kb ClaI/ClaI fragment from C3 was cloned blunt end into the SpeI poly linker site of the Bluescript KS' vector containing the minimal promoter Hoxb-8/LacZ 4.4 kb HindIII/BamHI 1 fragment. The 11 kb insert was isolated as a Salf/Not fragment.

Construct 2 was obtained by replacing the 1.8 kb SalI/ClaI fragment of construct 1 by a 6.3 kb SalI/ClaI fragment containing 4.5 kb 5' to the minimal promoter of Hoxb-8.

A 7.5 kb HindIII fragment from construct 1, cloned in Bluescript KS' gave construct 3. Construct 4, 5 and 6 were derived from construct 3 by HindIII and Smal, EcoRI, HindIII digestion respectively.

To obtain construct 7 a 550 bp HindIII/EcoRI fragment was cloned in Bluescript KS' cut HindIII and EcoRI. A 6 mers BgII phosphorylated linker from Biolabs is introduced in the HindIII restriction site. A BgII/ Not fragment is isolated and cloned in the BamHI Not poly linker sites of the Bluescript KS' vector containing the minimal promoter Hoxb-8/LacZ 4.4 kb HindIII/BamHI 1 fragment.

Generation of transgenic mice

Electropurified DNA (Vogels et al., 1993) was injected into the male pronucleus of fertilized C57 BI 6XCBA F2 eggs. Surviving 2 cell-embryos were transferred into the oviduct of pseudopregnant C57 BI 6 X CBA foster mice.

For coinjection experiments, construct A (Fig. 1) was mixed in equimolar amount either with a 29 kb genomic fragment extending from a SalI site 5' to Hoxb-7 to a BamHI site 3' to Hoxb-5 or with a 22 kb fragment extending from a SalI site 5' to Hoxb-7 to a ClaI site 3' to Hoxb-5 (Fig. 1) which were both isolated from cosmid C3 as a Salf/Stu and a Salf/ClaI fragments respectively. In both cases, the coinjected fragments overlapped by the SalI/EcoRI 2.75 kb fragment in Hoxb-7 gene (Fig. 1).

Southern blot analysis

The co-integration of the two overlapping fragments injected was checked by Southern blot analysis. The two probes used for radioactive hybridizations were: a 2.18 kb ClaI/EcoRI LacZDNA fragment and a EcoRI/HindIII genomic fragment extending 250 nucleotides 5' and 1.25 kb 3' to the second exon of Hoxb-7 (Fig. 1). The probes were labeled by random priming using the Gibo BRL RadPrime DNA labeling System, according to manufacturers instructions. 10 µg of placenta or tail DNA were digested with HindIII, electrophoresed on a 0.6% agarose gel, transferred by capillarity (using the Schleicher & Schuell Turboblotter according to instructions) on Schleicher & Schuell BA-85 reinforced membrane and hybridized according to standard procedures (Sambrook et al., 1989). Dehybridization of the first probe before using the second one was checked by a 2 days exposure on a phosphomager screen. For exposure, Kodak X-OMAT—AR films were used.

Embryo day

The day when the injected 2-cell embryos were transferred into a foster mother was considered as embryonic day 0.5. To establish lines, transgenic males containing the two co-integrated fragments were mated with non transgenic C57 BI 6XCBA F1 females. The day of detection of the vaginal plug was considered as embryonic day 0.5. The youngest embryos were staged according to a modification of the Downs and Davis system, 1993 (Lawson, unpublished).

Genotyping was performed on placenta, tail or yolk sac/amnion DNA either by dot blot using a LacZ specific probe (Clal/EcoRI fragment from a LacZ expression vector) or by hot start PCR using two LacZ specific primers, primer I: 5'GTCGTTTTACAACGTCGTGACT3' (nucleotides 9 to 30 of the LacZ cDNA), primer II: 5'GATGGGCGCATCGTAACCGTGCA3' (nucleotides 258 to 281 of LacZ cDNA). The PCR reaction was performed in 20 µl: 3 µl DNA, 1.5 mM MgCl2, 100 mM dNTPs, 0.5 mM each primer, 1 X PCR reaction buffer (Goldstar, Eurogentec), 0.4 units Goldstar polymerase (Eurogentec). The cycling conditions were: 5 min 96 C, 5 min 92 C, 28 cycles 45 sec 96 C, 1 minute 55 C, 2 min 72 C, 10 min 72 C, hold 4 C (PCR protocol and primers were given by Dr. C. Biben). The expected fragment length is 272 bp.

To assess β-gal activity, embryos were fixed in 1% formaldehyde, 0.2% glutaraldehyde, 0.02% NP40 in PBS for 30 min at 4 C, washed twice 20 min in PBS at room temperature and stained overnight either at 30 C or 37 C in 1 mg/ml X-gal, 5mM K3Fe(CN)6, 5mM K3Fe(CN)6, 2mM MgCl2 in PBS. Stained embryos were washed once in PBS, post-fixed in 4% paraformaldehyde overnight at 4 C before to be embedded in paraffin and sectioned at 6 µm.

To combine X-gal staining with either radioactive or whole-mount in situ hybridization (Tajbaksh and Houzellestein, 1995), embryos were fixed for 2 h in 4% paraformaldehyde at 4 C. They were stained for β-galactosidase as described above, overnight when radioactive in situ hybridization experiments were planned and 5 h for whole-mount in situ hybridization experiments. Stained embryos were washed once in PBS and post-fixed in 4% paraformaldehyde at 4 C for 14 h. For radioactive in situ hybridization they were dehydrated, embedded in paraffin and sectioned at 6 µm. Sections were kept under desiccant at 4 C until used. For whole-mount in situ hybridization, embryos were fixed in 4% paraformaldehyde at 4 C. They were then dehydrated in methanol and stored at -20 C until use.
Radioactive in situ hybridization

Radioactive in situ hybridization on 6 mm paraffin sections was performed as described in Deschamps and Wijgerde (1993). The Hoxb-8 antisense probe was transcribed with Sp6 RNA polymerase (Biolabs) from a 420bp SacI/SacI fragment in the first exon of the gene. The Hoxb-8 antisense probe was transcribed from a 800 bp EcoRI/EcoRI fragment with T7 RNA polymerase (Biolabs). The LacZ antisense probe was transcribed with T3 RNA polymerase (Biolabs) from a 700bp PstI/Rsal fragment. The exposure time varied between 10 and 16 days.

Whole-mount in situ hybridization

Whole-mount RNA in situ hybridizations using digoxigenin-labelled RNA probes were performed as described in Wilkinson (1992) with the following modifications: the pre-absorption of the antibody with embryo powder was performed for at least 4 h and the post-antibody washes were done over the weekend with daily buffer change. The Hoxb-8 antisense probe was transcribed with Sp6 RNA polymerase (Biolabs) from a 420bp SacI/SacI fragment in the first exon of the gene.

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