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Original Article

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

ISABELLE VALARCHÉ, WIM DE GRAAFF and JACQUELINE DESCHAMPS*

Hubrecht Laboratory, The Netherlands Institute for Developmental Biology, Utrecht, the Netherlands

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 (Carrasco *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 Dollé, 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 (Zeltser *et al.*, 1996). This cluster organization has been strikingly conserved throughout evolution (Duboule and Dollé, 1989; Graham *et al.*, 1989; Kenyon and Wang, 1991; Garcia–Fernàndez 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 (Dollé *et al*, 1991) and the limbs (Dollé *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 Dollé, 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

Abbreviations used in this paper: HOM–C, homeotic cluster; 3'CR, 3' control region; A–P, antero–posterior; PV, prevertebrae; β–gal, béta–galactosidase; PNS, peripheral nervous system; CNS, central nervous system.

***Address for reprints:** Hubrecht Laboratory, The Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, the Netherlands. FAX: 31 302516464, e-mail: jacqueli@niob.knaw.nl.

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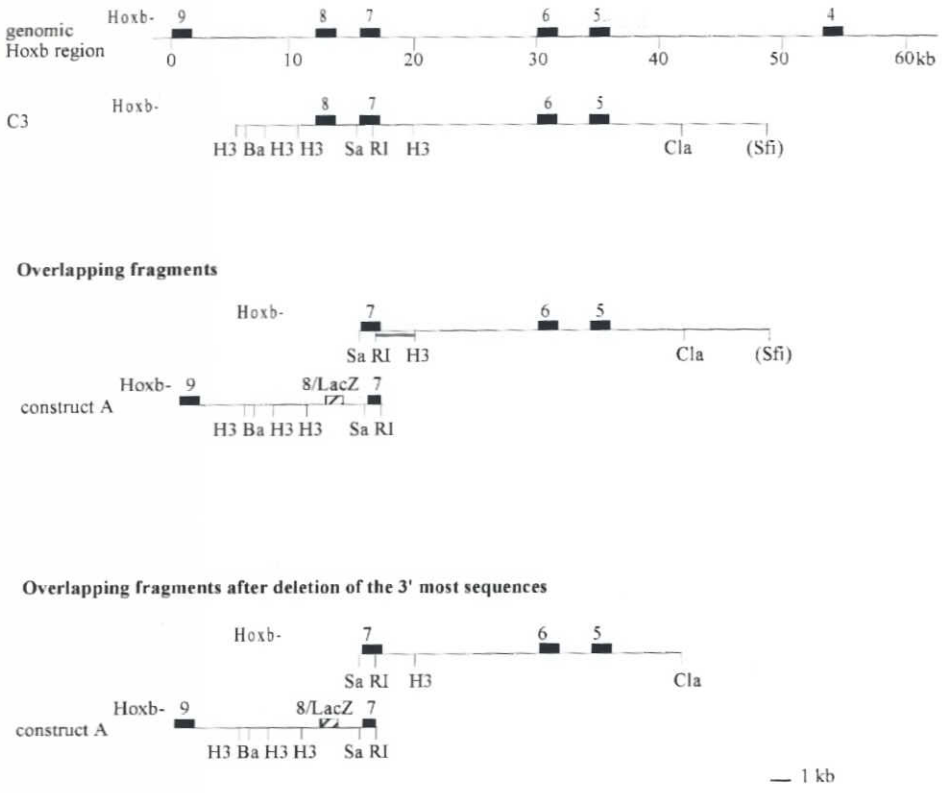

Fig.1. Schematic representation of the initial constructs. *The genomic organization of the relevant part of the Hox b cluster is presented at the top. C3 depicts the Hoxb genomic sequence contained in the cosmid Supercos I. The lower two parts show the overlapping DNA fragments used for zygote injections. The relevant restriction sites are indicated. Genes are represented as black boxes and fusion with LacZ as stripped boxes. The probe used to identify recombination between the co-injected overlapping fragments is represented as a thick line in "Overlapping fragments". Ba: BamHI, Cla: ClaI, RI: EcoRI, H3: HindIII, Sa: SalI, Sfi: SfiI. Restriction sites in between brackets indicate that the site belongs to the vector.*

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 Krumlauf, 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)

Our previous studies on the transcriptional regulation of *Hoxb-8* led to the identification of five regions, localized in the vicinity of *Hoxb-8* and containing cis acting elements which in combination with the *Hoxb-8* minimal promoter, drive region specific expression of *Hoxb- 8/LacZ* fusion transgenes (Charité *et al.*, 1995). However, all the transgenes tested so far exhibited rostral boundaries more posterior than those of the endogenous gene both in the neural tube and the mesoderm. We therefore assayed genomic sequences extending farther 3' for modulatory activity on *Hoxb-8/LacZ* expression. In the present study we identify a 3' Control Region (3'CR) in the *Hoxb-5/b-4* intergenic region, which has the unique property of endowing the most complete *Hoxb-8/LacZ* transgene hitherto characterized with expression boundaries

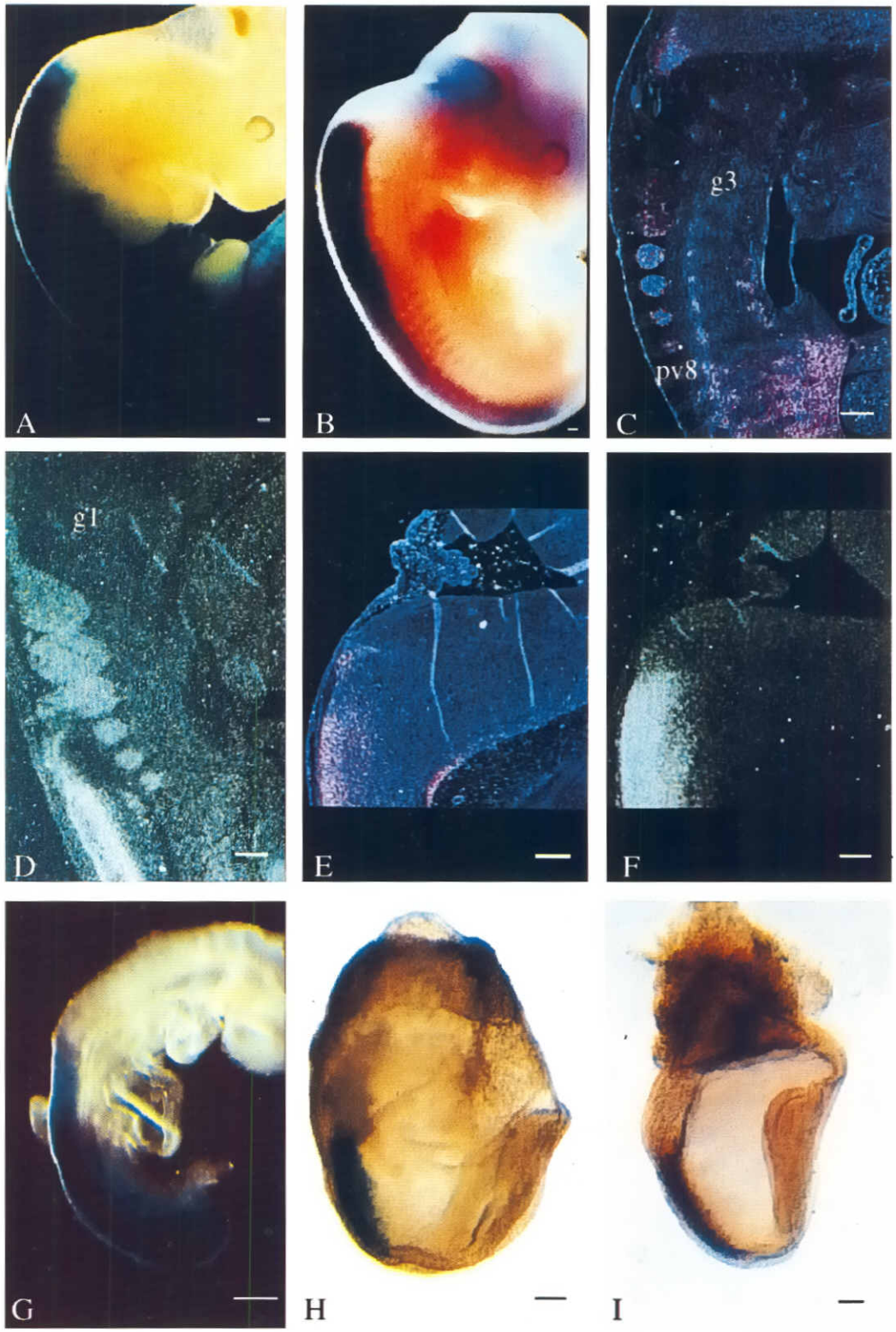

Fig.2. Comparison of the expression patterns of the 48 kb–long *Hoxb–8/LacZ* transgene and the endogenous *Hoxb–8* gene. (A) *X–gal staining pattern of a 11.5–day embryo carrying the long transgene.* **(B)** *Endogenous Hoxb–8 expression pattern at 11.5–day visualized after whole-mount in situ hybridization.* **(C)** *Parasagittal section of a 11.5–day X–gal stained embryo from line 74 (carrying the long transgene) showing LacZ anterior expression boundary in PV8 and in the third cervical ganglion.* **(D)** *Sagittal section showing LacZ expression detected by radioactive in situ hybridization in the remnant of the first cervival 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 β–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)*** *X–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 cervival ganglion; g1: Froriep's ganglion; pv8: eighth prevertebrae.*

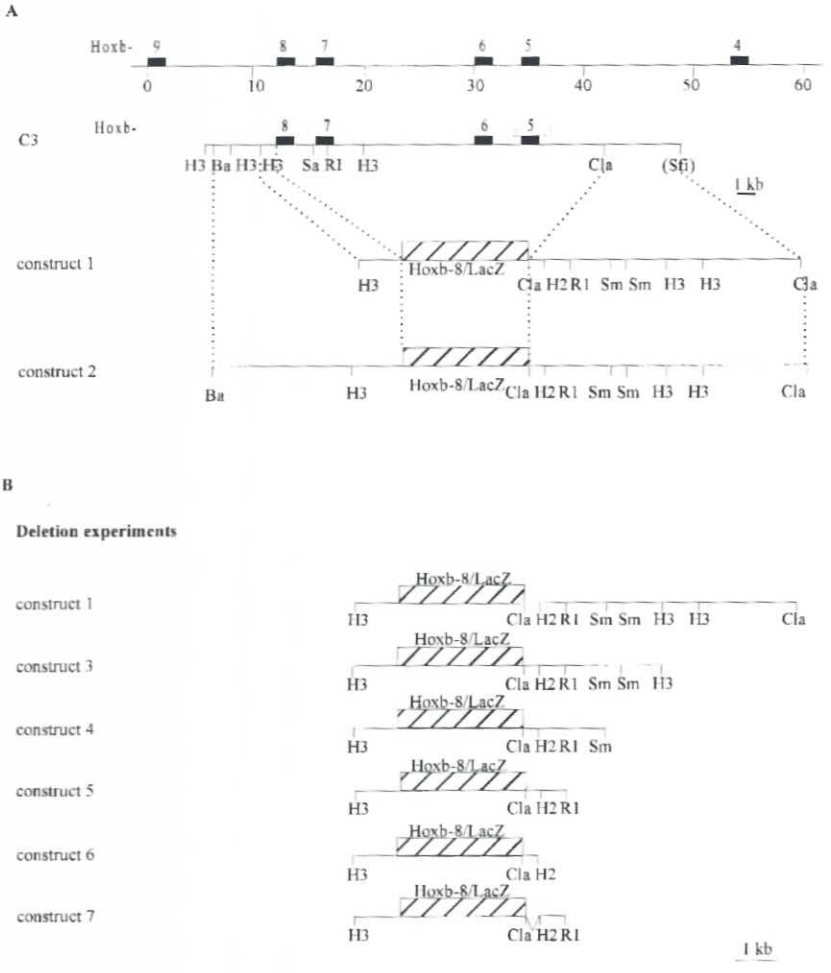

Fig.3. Analysis of the newly identified control region in transgenic mice. (A) *The genomic organization of the relevant part of the Hox b cluster is presented at the top. C3 depicts the Hoxb genomic sequence contained in the cosmid Supercos I. The dotted lines delineated the genomic part used in construct 1 and 2.* **(B)** *Mapping of the regulatory sequences in combination with the Hoxb–8 minimal promoter. The relevant restriction sites are mentioned: H3: HindIII, Cla: ClaI, H2: HincII, R1: EcoRI, Sm: SmaI. The stripped box represents the fusion with LacZ.*

along the antero–posterior (A–P) axis that are similar to those of the endogenous *Hoxb–8* gene from 7.5–day up to 12.5–day of development (the oldest stage analyzed). In combination with the *Hoxb–8* minimal promoter alone, the 3'CR provides the transgene with an even more anterior expression boundary in the neural tube. When the latter construct also carries a 4.5 kb *Hoxb–8* upstream sequence where previously identified proximal regulatory sequences are located (Charité *et al.*, 1995) the expression domain is more posterior and exhibits boundaries at about the level of that of the endogenous *Hoxb–8* gene.

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 *Eco*RI 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 *Sal*I/*Sfi*I 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. 2 A) 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 *Hin*dIII 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 boundaries in 8.5–day embryos, we combined limited (5 h) X–gal staining and whole-mount *in situ* hybridization with a *Hoxb–8* probe that did not recognize the transgene. β–gal and *Hoxb–8* expression boundaries were very close to each other in the neural tube and the mesoderm (Fig. 2G), *Hoxb–8* transcripts extending slightly more rostrally than b–gal. This might result from weaker X–gal staining in the rostralmost part of the expression domain making the *in situ* signal more obvious there. Anterior expression boundaries were not sharp for either the transgene or endogenous *Hoxb–8* and the exact respective limits were difficult to localize. However if these boundaries differed, it was certainly by less than one somite.

The earliest expression of the transgene containing extended 3' genomic sequences was detectable in head fold stage (7.5–day) embryos (Fig. 2H). The X–gal staining features were similar to those of endogenous *Hoxb–8* expression as revealed by whole-mount *in situ* hybridization at the same developmental stage (Fig. 2I). In both cases expression extended up to the node region. While *Hoxb–8* transcripts are detected by *in situ* hybridization with radioactive probe in the posterior primitive streak region at the neural plate stage (Deschamps and Wijgerde, 1993) β–gal activity was not yet apparent at the neural plate stage. In none of the five lines carrying the two recombined fragments was transgene expression detected earlier than the head fold stage.

The control region (CR) is localized in the Hoxb–5/b–4 intergenic region

To locate the regulatory region that extends the expression domain of *Hoxb–8/LacZ* transgene rostrally, we started a deletion analysis within the 30 kb genomic sequences 3' of *Hoxb–8* . We took advantage of a *Cla*I restriction site to delete the 3' most 7 kb fragment of the Hoxb genomic sequences contained in C3. A *Sal*I/*Cla*I fragment isolated from C3 (Fig. 1) was co–injected with construct A (Fig. 1). The overlap between the two fragments still covered the first exon and most of the intron of *Hoxb–7*. Recombination between the two fragments was identified by Southern blot analysis as previously described. Among 13 β–gal expressing 11.5–day embryos recovered, two carried the two recombined fragments (data not shown). The X–gal staining pattern of these two embryos was similar to that of construct A with anterior boundaries in the neural tube at the level of the third ganglion and in the mesoderm in PV 11 (data not shown). The regulatory activity responsible for the rostral extension of *Hoxb–8/LacZ* expression of the extended construct was therefore likely to be located within the 3' most 7 kb fragment.

The enhancer activity of the 3'CR on the Hoxb–8 promoter is differentially modulated in the presence or in the absence of proximal regulatory elements

The 3' most 7 kb fragment identified above was cloned 3' to a *Hoxb–8/LacZ* transgene containing 1 kb of upstream sequences including the *Hoxb–8* minimal promoter (construct 1, Fig. 3A). The expression pattern of construct 1 was checked in embryos recovered from foster mothers at 11.5–day. While the minimal promoter of *Hoxb–8* coupled to *LacZ* could not drive regionally restricted expression (Charité *et al.*, 1995), 3 out of 5 embryos expressing construct 1 exhibited a *Hox*–like X–gal staining pattern in the neural tube (Fig. 4A) extending up to the posterior hindbrain. One embryo showed ubiquitous *LacZ* expression and the last one only a few stained cells. To localize the anterior boundary in the neural tube we performed radioactive *in situ* hybridization experiments on

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 another 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 neurectoderm 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 boundaries 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 exhibits expression 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*

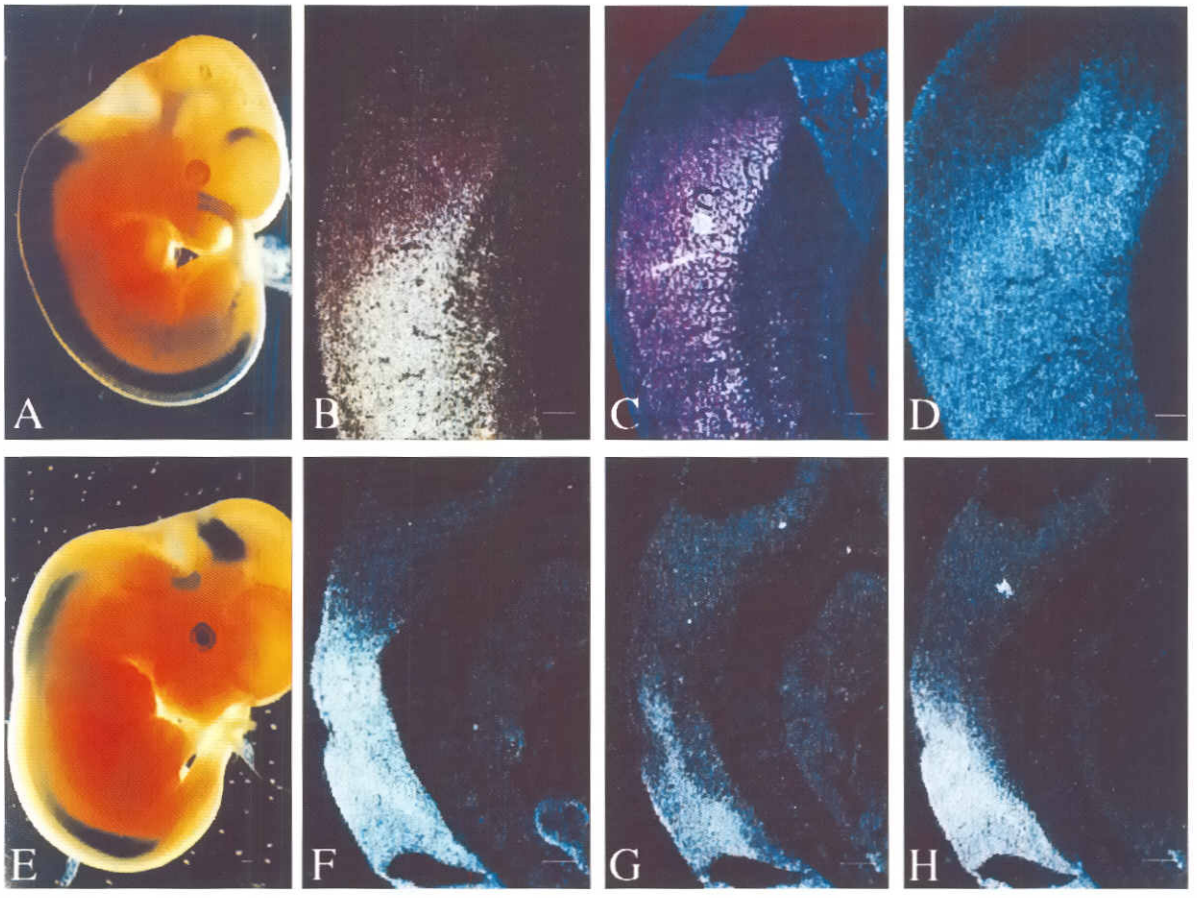

Fig.4. Expression pattern of construct 1 and construct 2 in 11.5–day embryos. (A) *X–gal staining pattern of an embryo carrying the construct 1 transgene.* **(B, C and D)** *Dark field illumination of adjacent sagittal sections of X–gal stained embryo hybridized with a Hoxb–8* **(B)**, *Hoxb–5* **(D)** *radioactive probes. Section C was not submitted to radioactive in situ hybridization. X–gal staining appears pink and the silver grains generated by the radioactive signal on the emulsion white.* **(E)** *X–gal staining of an embryo carrying construct 2.* **(F, G and H)** *Adjacent sagittal sections of X–gal stained embryo hybridized with a Hoxb–5* **(F)**, *LacZ* **(G)** *and Hoxb–**8*** **(H)** *radioactive probes, showing the respective anterior boundaries in the neural tube. Orientation: anterior to the top, dorsal to the left. Bar, 0.2 mm in A, E and 0.1 mm in B, C, D, F, G and H.*

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 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., Charité *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 collinear 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 serie 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 *Sal*I site in the first exon of *Hoxb–9* to the *Eco*RI site in the intron of *Hoxb–7* (Fig. 1; Charité *et al.*, 1995). The 22.5 kb insert was isolated as a *Not*I fragment.

C3 is a Supercos I cosmid 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 *Cla*I site was replaced by a *Sfi*I linker of 14 mers (Biolabs).

To obtain construct 1 (Fig. 2), a 7 kb *Cla*I/*Cla*I fragment from C3 was cloned blunt end in the *Spe*I polylinker site of the Bluescript KS$^-$ vector containing the minimal promoter *Hoxb– 8*/*LacZ* 4 kb *Hin*dIII/*Bam*H I fragment. The 11 kb insert was isolated as a *Sal*I/*Not*I fragment.

Construct 2 was obtained by replacing the 1.8 kb *Sal*I/*Cla*I fragment of construct 1 by a 6.3 kb *Sal*I/*Cla*I fragment containing 4.5 kb 5' to the minimal promoter of *Hoxb–8*.

A 7.5 kb *Hin*dIII fragment from construct 1, cloned in Bluescript KS$^-$ gave construct 3. Construct 4, 5 and 6 were derived from construct 3 by *Hin*dIII and *Sma*I, *Eco*RI, *Hin*cII digestion respectively.

To obtain construct 7 a 550 bp *Hin*cII/ *Eco*RI fragment was cloned in Bluescript KS$^-$ cut *Hin*cII and *Eco*RI. A 8 mers *Bgl*I phosphorylated linker from Biolabs is introduced in the *Hin*cII restriction site. A *Bgl*I/ *Not*I fragment is isolated and cloned in the *BamH*I/ *Not*I polylinker sites of the Bluescript KS$^-$ vector containing the minimal promoter *Hoxb–8*/*LacZ* 4 kb *Hin*dIII/ *Bam*H I fragment.

Generation of transgenic mice

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

For coinjection experiments, construct A (Fig. 1) was mixed in equimolar amount either with a 29 kb genomic fragment extending from a *Sal*I site 5' to *Hoxb–7* to a *Bam*HI site 3' to *Hoxb–5* or with a 22 kb fragment extending from a *Sal*I site 5' to *Hoxb–7* to a *Cla*I site 3' to *Hoxb– 5* (Fig. 1) which were both isolated from cosmid C3 as a *Sal*I/*Sfi*I and a *Sal*I/*Cla*I fragments respectively. In both cases, the coinjected fragments overlapped by the *Sal*I/*Eco*RI 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 *Cla*I/*Eco*RI *LacZ* DNA fragment and a *Eco*RI/*Hin*dIII 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 GibcoBRL RadPrime DNA labeling System, according to manufacturers instructions. 10 µg of placenta or tail DNA were digested with *Hin*dIII, electrophoresed on a 0.6% agarose gel, transferred by capillarity (using the Schleicher & Schuell Turboblotter according to instructions) on Schleicher & Schuell BA–S 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 phosphoimager screen. For exposure, Kodak X–OMAT–AR films were used.

Embryo analysis

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 Bl 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 (*Cla*I/*Eco*RI 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 $MgCl_2$, 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 272bp.

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 1mg/ml X–gal, 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, 2mM $MgCl_2$ 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 (Tajbakhsh and Houzelstein, 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 8 to 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 *Sac*I/*Sac*I fragment in the first exon of the gene. The *Hoxb–5* antisense probe was transcribed from a 800 bp *Eco*RI/*Eco*RI fragment with T7 RNA polymerase (Biolabs). The *LacZ* antisense probe was transcribed with T3 RNA polymerase (Biolabs) from a 700bp *Pst*I/*Rsa*I fragment. The exposure time varied between 10 and 16 days.

Whole-mount in situ hybridization

Whole-mount RNA *in situ* hybridizations using digoxygenin–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 *Sac*I/*Sac*I fragment in the first exon of the gene.

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