Development 122, 2475-2484 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV2077

Hoxb-13: a new Hox gene in a distant region of the HOXB cluster maintains colinearity

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SUMMARY

The Hox genes are involved in patterning along the A/P axes of animals. The clustered organization of Hox genes is conserved from nematodes to vertebrates. During evolution, the number of Hox genes within the ancestral complex increased, exemplified by the five-fold amplification of the *AbdB*-related genes, leading to a total number of thirteen paralogs. This was followed by successive duplications of the cluster to give rise to the four vertebrate HOX clusters. A specific subset of paralogs was subsequently lost from each cluster, yet the composition of each cluster was likely conserved during tetrapod evolution. While the HOXA, HOXC and HOXD clusters contain four to five *AbdB*-related genes, only one gene (*Hoxb-9*) is found in the HOXB complex. We have identified a new member of paralog group 13 in human and mouse, and shown that it is in fact *Hoxb-13*. A combination of genetic and physical mapping demonstrates that the new gene is found approx. 70 kb upstream of *Hoxb-9* in the same transcriptional orientation as the rest of the cluster. Despite its relatively large distance from the HOX complex, *Hoxb-13* exhibits temporal and spatial colinearity in the main body axis of the mouse embryo. The onset of transcription occurs at E9.0 in the tailbud region. At later stages of development, *Hoxb-13* is expressed in the tailbud and posterior domains in the spinal cord, digestive tract and urogenital system. However, it is not expressed in the secondary axes such as the limbs and genital tubercle. These results indicate that the 5′ end of the HOXB cluster has not been lost and that at least one member exists and is highly conserved among different vertebrate species. Because of its separation from the complex, *Hoxb-13* may provide an important system to dissect the mechanism(s) responsible for the maintenance of colinearity.

Key words: homeobox, HOX cluster, colinearity, development, mouse, A/P axis

INTRODUCTION

Vertebrate Hox genes are clustered in four unlinked complexes in the genome (the HOXA, B, C and D clusters). Each complex spans approx. 200 kb and contains 9-11 genes which are transcribed from the same strand of DNA. Parsimony analysis of homeodomains reveals that the mouse HOX complexes can be aligned with the *Drosophila* complex, suggesting a common phylogenetic origin of the complexes (Duboule and Dolle, 1989; Graham et al., 1989). The four vertebrate clusters arose from an ancestral complex by amplification of some paralogs, followed by large-scale duplications (Kappen et al., 1989). Amplification of the ancestral *AbdB* gene occurred before the large scale duplication of the cluster and may be linked to the evolution of the appendicular system, since the *AbdB*-related Hox genes are coordinately expressed in the limb and genital tubercle (Dolle et al., 1991b, 1989; Yokouchi et al., 1991). One consequence of the large-scale duplications of an ancestral cluster is that specific genes in each of the complexes are evolutionarily related to each other, forming 13 paralogous groups. Some members of the complex have been lost during vertebrate evolution. Each cluster has maintained a different subset of the 13 paralogs, and it appears that the same paralogous subsets are maintained in mice and humans (Krumlauf, 1994). In contrast to the HOXA, C and D clusters, which contain 4-5 *AbdB*-related genes, analysis of the 5′ end of the HOXB locus has revealed the presence of only one *AbdB* homolog, *Hoxb-9*, suggesting that paralogs 10-13 have been lost in all vertebrates (Boncinelli et al., 1991).

Spatial colinearity, or the correlation between the physical order of genes along the chromosome with their expression along the A/P axis of the embryo, is a distinguishing feature of both the *Drosophila* and mammalian HOX complexes (Akam, 1989; Duboule and Dolle, 1989; Gaunt, 1988; Graham et al., 1989). Genes located at the 3′ ends of the complexes (paralog groups 1 and 2) are expressed at anterior positions within the hindbrain, while genes at the 5′ positions (paralog groups 9-13) are expressed in progressively more posterior regions in the main body axis. The posterior *AbdB*-related paralogs exhibit a similar colinearity along secondary axes in the limb and genitalia. Vertebrates also exhibit temporal colinearity, for Hox genes are successively expressed beginning with the 3′ paralogs and ending with the 5′ paralogs (Dolle and Duboule, 1989; Hunt et al., 1991; Izpisua-Belmonte et al., 1991a). While it is possible to analyze the roles of individual Hox genes in embryonic development, the significance of

spatial and temporal colinearity and the mechanism(s) responsible for their maintenance have been difficult to address.

The role of clustering in the regulation of Hox gene expression remains unclear, in a large part because of functional overlap and redundancy among Hox genes and because of extensive cross- and autoregulation. The properties of colinearity, the presence of multiple transcripts, differential splicing, shared promoters, interspersed regulatory regions and evolutionary conservation of the chromosomal complexes seem to suggest that the organization of Hox genes is integral to their proper regulation (Krumlauf, 1994). However, the ability of relatively small promoter regions of the complexes to direct proper A/P expression boundaries, tissue-specific expression, lineage restriction and timing of activation in transgenic mice seemed to contradict a simple 'locus control region'-based model of Hox gene regulation (Behringer et al., 1993; Gerard et al., 1993; Marshall et al., 1992; Puschel et al., 1990, 1991; Sham et al., 1992; Whiting et al., 1991). Since the transgenic experiments were performed in a wild-type genetic background, where the endogenous Hox genes are properly distributed, it is possible that the correct expression of the transgenes may simply reflect their ability to respond to cross- and auto-regulatory signals from other Hox genes, and not the ability to fully establish proper expression patterns themselves.

An analysis of the relationship between the expression patterns of *Evx* genes and their physical linkage to HOX clusters provides some insight into the role of clustering in Hox gene regulation. *Evx-2*, which is found within 8 kb of *Hoxd-13*, exhibits spatial and temporal expression patterns typical of Hox-13 genes together with the loss of some features typical of *Evx* genes, such as the early phase of expression during gastrulation (Bastian et al., 1992; Dolle et al., 1994). In contrast, *Evx-1*, which resides approx. 45 kb upstream of *Hoxa-13*, is expressed at early stages of gastrulation and not in a posterior Hox-like pattern (Bastian and Gruss, 1990; Dush and Martin, 1992; Faiella et al., 1991). These results suggest that there may be a global regulatory mechanism coordinating and regulating gene expression in the complex, that is impaired at a distance from the cluster (Duboule, 1994).

In this paper we report the discovery of the mammalian *Hoxb-13* gene. Genetic and physical mapping studies in mice reveal that this gene has remained associated with the HOXB cluster, although it is separated from *Hoxb-9* by approx. 70 kb. It is transcribed in the same orientation as the other HOXB genes, suggesting that the additional genomic DNA between the 9 and 13 group paralogs results from an insertion or expansion, rather than inversion of the 5′ end of the cluster. Despite the physical separation from the cluster, *Hoxb-13* exhibits both spatial and temporal colinearity within the main body axis. However, it is not expressed in secondary axes such as the limb and the genital tubercle. These data support the idea that proper expression of Hox genes in the main body axis can be achieved by cross-regulatory mechanisms, whereas expression in the secondary axes may require *cis* regulation within the cluster. Furthermore, the existence of a new paralog must be considered when assessing the functions of other *Hox-13* genes in knockout experiments.

MATERIALS AND METHODS

Isolation of the human *HOXB13* gene

A HeLa cDNA expression library in λgt11 was screened with the DNA binding site for the putative replication protein, RIP60 (Dailey et al., 1990), as described by Singh et al. (1988). The double-stranded binding site oligonucleotide containing a $(TAA)_{15}$ repeat was used to screen 1×10^6 phage clones induced with 10 mM IPTG. The protein filters were denatured in 6 M guanidine HCl, gradually renatured in Binding Buffer (200 mM Hepes pH 7.9; 30 mM $MgCl_2$; 400 mM KCl; 1 mM DTT), and blocked in 5% dry milk in Binding Buffer. 10^6 cpm/ml of the oligo was used to probe the filters overnight at 4°C followed by washing with 0.25% milk in Binding Buffer. The screen yielded two identical clones that encoded the human *HOXB13* cDNA. The approx. 1.1 kb *Eco*RI insert was subcloned into the pks+ vector. Digestion with *Not*I releases the homeobox from the N-terminal portion of the gene.

Isolation of the mouse *Hoxb-13* gene

A genomic clone of the mouse *Hoxb-13* gene was isolated by screening a 129 SV library in λFixII with the approx. 0.7 kb *Eco*RI/*Not*I non-homeobox fragment of the human cDNA clone. approx. 2×10^6 clones were screened under high stringency conditions (50% dextran sulfate; 1 M NaCl; 1% SDS; 0.1 mg/ml salmon sperm DNA at 65°C) and washed in 0.1× SSC; 0.1% SDS, yielding 1 clone containing the mouse *Hoxb-13* gene. Various fragments were subcloned into Bluescript.

Sequencing and homology searches

DNA sequencing was performed on either ABI 370A or 373A automated sequencers (Applied Biosystems, Inc.) using plasmids, PCR and RT-PCR products and phage as templates. Sequence homology searches were performed using the National Center for Biotechnology (NCBI) data base.

Zoo blot

Genomic blots containing DNA (20 μg) from several vertebrate species, digested with *Bam*HI, were probed with the non-homeobox probe from the human cDNA under standard high stringency conditions.

Genomic mapping

A 2.5 kb *Eco*RI probe derived from the genomic phage clone could detect a polymorphism between *Mus musculus* and *Mus spretus*. The polymorphism was used to type the interspecific backcross (C57BL/6JEi × SPRET/Ei) × SPRET/Ei (The Jackson Laboratory DNA BSS Panel) by Southern hybridization under standard conditions. Data are available on the World Wide Web at http://www.jax.org/resources/documents/cmdata.

Pulsed field gel electrophoresis mapping

Mouse spleens were homogenized and embedded in low melting point agarose (approx. 5×10^6 cells/block). The genomic agarose blocks were then treated with proteinase K and N-lauroylsarcosine (Zuo et al., 1992), digested with different enzymes (New England Biolabs), and separated by pulsed field gel electrophoresis under conditions that fractionate DNA from 50 to 1500 kb. Samples were loaded on a 1% agarose gel, and run at 6 V/cm for 24 hours with a 50-90 second switch time in 0.5× TBE at 14°C in the CHEF-DR II system (Bio-Rad). Gels were then acid-nicked and transferred to membranes. Blots were successively hybridized with probes from *Hoxb-1*, *Hoxb-9* and *Hoxb-13* under standard conditions. The *Hoxb-1* probe was a 316 bp PCR product generated with the following primers: 5′GGTTGCCCAAAGGAAGCCGC3′ (forward) and 5′TTGAGTGTTCCCCAGGTCCC3′ (reverse). The *Hoxb-9* probe was a 247 bp PCR product generated with the following primers: 5′GATCATAAGT-

CACGAGAGCG3′ (forward) and 5′TGCGGAGGTACCTGCTTTCG3′ (reverse). PCR conditions were as follows: 94°C, 30 seconds; 55°C, 30 seconds; 72°C, 30 seconds for 35 cycles. The *Hoxb-13* probe was a 3.3 kb *Xho*I/*Xba*I fragment from the mouse genomic phage clone that contained the non-homeobox portion of the gene.

Isolation and analysis of a BAC clone

A 129 SV bacterial artificial chromosome (BAC) library (Research Genetics) was screened in a PCR-based assay with primers from *Hoxb-9* and *Hoxb-13* as described by Shizuya et al. (1992). The *Hoxb-9* primer pair was described in the previous section. The *Hoxb-13* primers were the following: 5′GACGGGGCCAAGGATATCGA3′ (forward) and 5′CCGCCTCCAAAGTAGCCATA3′ (reverse). 1-2 μg BAC DNA, prepared according to a standard plasmid preparation protocol, was digested with various restriction enzymes and run under pulsed field gel electrophoresis conditions that separate 50-1000 kb fragments. BAC digests were run in 1.5% agarose/0.5× TBE at 200 V with 1-10 second switch time for 14 hours at 14°C.

Whole-mount in situ hybridization

Whole-mount in situ hybridization on E7.5-9.5 mouse embryos was performed according to the method of Conlon and Rossant (1992). Digoxigenin-labeled probes were prepared as described by Wilkinson and Nieto (1993). Human and mouse probes gave identical results, but the 1.1 kb human cDNA gave the strongest signal-to-noise ratio.

In situ hybridization

The protocol used for in situ hybridization was adapted from the method of Schaeren-Wiemers and Gerfin-Moser (1993). E12.5 mouse embryos were rinsed in PBS and directly frozen in OCT embedding solution, and the resulting cryostat sections were fixed and dehydrated before storage. The probe used in these experiments was a 398 bp *Bgl*II/*Xba*I fragment that extends from K-33 of the homeobox through the 3′ untranslated region.

RT-PCR

RT-PCR was performed using a protocol adapted from that of Wilson and Melton (1994). In short, RNA was extracted from head, trunk and limb regions of E9-14 mouse and subjected to reverse transcription from an oligo d(T) primer. 1-2 μl of each reverse-transcribed sample was used as the template for a radioactive PCR reaction with [^{32}P]dATP. PCR was performed in a DNA Engine (M. J. Research) with the following profile: 94°C for 30 seconds; 55°C for 30 seconds; and 72°C for 30 seconds for 25-30 cycles (determined empirically to be in the linear range according to Rupp and Weintraub, 1991). One-quarter of the sample was electrophoresed on 6% polyacrylamide gels, and the resulting PCR bands were visualized by autoradiography. The ubiquitous message HPRT was used to monitor RNA recovery. PCR on the E10 trunk sample that had not been reverse-transcribed was a control for RT-PCR contamination (first lane). PCR oligonucleotide sequences were: *Hoxb-13* forward, 5′CTGGAACAGCCAGATGTGTT3′; reverse, 5′CCTGCTAAAGGTGTCATCTC3′; *Hoxd-12* forward, 5′AGTATGACTACGCGGGTGT3′, reverse, 5′AAAAGGGCAGGCTTGGCAA3′; *HPRT* forward 5′CCTGCTGGATTACATTAAAGCACTG3′, reverse 5′GTCAAGGGCATATCCAACAACAAAC3′.

RESULTS

Cloning human and mouse *Hoxb-13*

Southwestern screening of a HeLa cDNA expression library with a $(TAA)_n$ binding site oligomer yielded a novel homeobox-containing gene. Homology searches revealed that the gene is most closely related to the *Abdominal-B* (*AbdB*) family of Hox genes. This result was surprising, because it was believed that all members of the vertebrate HOX complexes had been identified (Scott, 1992). Genomic mapping of the regions spanning all four HOX clusters (Dolle et al., 1991b; Goto et al., 1993; Haack and Gruss, 1993; Peterson et al., 1994; Rubock et al., 1990) had shown that particular genes are deleted from each cluster. Whereas one *AbdB* family member is missing from the HOXA cluster, the four posterior-most paralogs have not been indentified in HOXB.

Amino acid comparison in the homeodomain of the novel gene demonstrated 78-83% identity with Hox proteins in paralog group 13 (Hox-13) and less than 60% identity to other *AbdB*-related genes in paralogous groups 9-12 (Fig. 1). Furthermore, it possessed virtually all of the hallmark amino acids in the homeodomain that characterize the 13 group paralogs. The novel homeodomain differed from the other Hox-13 paralogs at six positions (6, 9, 14, 33, 39 and 45), but all of the changes were conservative. Thus, this gene was likely a member of Hox-13 that diverged slightly from the other

	10 20 30 40 50 60	
consensus	RRRKRTAYTRYQLLELEKEFHFNRYLTRRRRIELAHSLNLTERQVKIWFQNRRMKWKKEN	Homology
Abd-B	V.K..KP.SKF.T.....L..A.VSKQK.W...RN.Q.....N..NS	51.7
Hoxb-13	**G.K..IP.SKG..R...R.YAA.KFI.KDK.RKISAATS.S...IT.....V.E..VL**	100.0
Hoxa-13	G.K..VP..KV..K...R.YAT.KFI.KDK.RRISATT..S....T.....V.E..VI	83.0
Hoxc-13	G.K..VP..KV..K.....YAASKFI.KEK.RRISATT..S....T.....V.E..VV	80.0
Hoxd-13	G.K..VP..KL..K...N.YAI.KFINKDK.RRISAAT..S....T.....V.D..IV	78.3
Hoxc-12	S.K..KP.SKL..A...G..LV.EFI..Q..R..SDR...SDQ.....K.RLL	56.7
Hoxd-12	A.K..KP..KQ.IA...N..LV.EFIN.QK.K..SNR...SDQ.....K.RVV	51.7
Hoxa-11	T.K..CP..K..IR...R..F.SV.INKEK.LQ.SRM....D.....E..I.	55.0
Hoxc-11	T.K..CP.SKF.IR...R..F..V.INKEK.LQ.SRM....D.....E..LS	58.3
Hoxd-11	S.K..CP..K..IR...R..F..V.INKEK.LQ.SRM....D.....E..L.	56.7
Hoxa-10	G.K..CP..KH.T.....L..M....E..L.ISR.VH..D.....L..M.	51.7
Hoxc-10	G.K..CP..KH.T.....L..M....E..L.ISKTI...D.....L..M.	51.7
Hoxd-10	G.E..CP..KH.T.....L..M....E..L.ISK.V...D.....L..MS	50.0
Hoxa-9	T.K..CP..KH.T.....L..M....D..Y.V.RL.....M..I.	50.0
Hoxb-9	S.K..CP..K..T.....L..M....D..H.V.RL...S.....M..M.	51.7
Hoxc-9	T.K..CP..K..T.....L..M....D..Y.V.RV.....M..M.	50.0
Hoxd-9	T.K..CP..K..T.....L..M....D..Y.V.RI.....M..MS	50.0

Fig. 1. Alignment of the novel homeodomain with other *AbdB*-related genes. All known murine *AbdB*-related homeodomain sequences are shown in their single letter amino acid code in comparison to the *AbdB* sequence from *Drosophila*. The novel homeodomain, *Hoxb-13*, is depicted in boldface type with the percentage amino acid homology to the other Hox9-13 paralogs. The novel gene shares a high level of homology with the Hox-13 homeodomains.

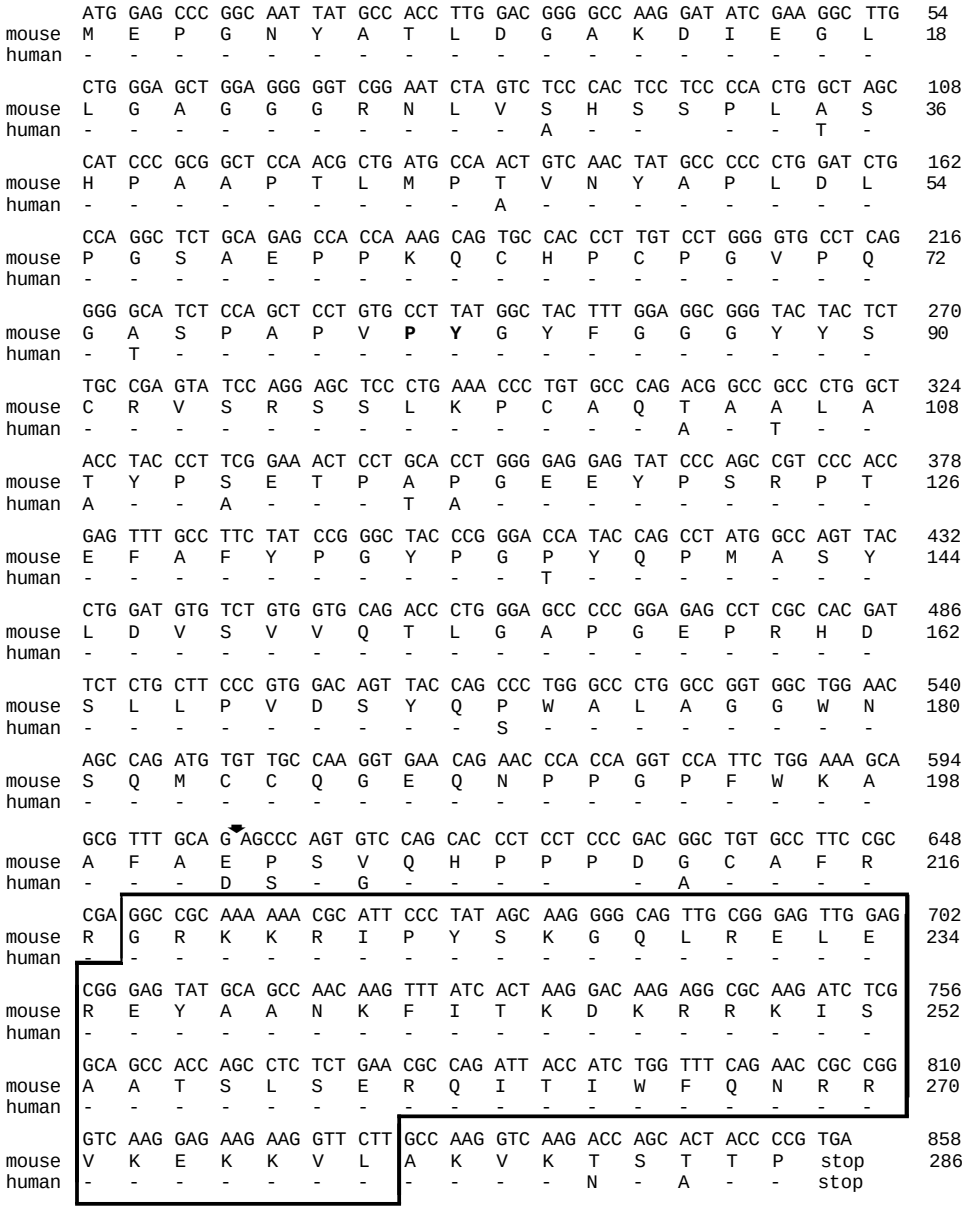

Fig. 2. DNA and protein sequences of mouse genomic and human cDNA *Hoxb-13* clones. The initiation codon was assigned based on the divergence of the sequence between the mouse and human clones upstream of this point. The position of the intron is in residue 202, based on the analysis of mouse genomic DNA and RT-PCR products (see arrow). The homeodomain sequences are boxed and the bold-face type indicates the proline/tyrosine *AbdB* consensus doublet (Izpisua-Belmonte et al., 1991a). GenBank accession numbers are U57051 and U57052.

paralogs, which share 87-92% homology in the homeodomain. Furthermore, as in the case of the other Hox-13 paralogs, the new gene contained neither a hexapeptide sequence nor a tryptophan at position −6 or −7 relative to the homeodomain. The proline and tyrosine doublet at positions 80 and 81 in the Hoxb-13 protein is found in the central regions of all *AbdB*-related genes (Izpisua-Belmonte et al., 1991a), suggesting that they arose by successive duplications of an ancestral *AbdB*-like gene. Since Hox-13 members have been identified in the HOXA, C, and D clusters, the novel homeobox-containing gene was likely to represent the missing *HOXB-13*. We hereafter refer to this gene as *Hoxb-13*.

We cloned the murine homolog from a 129 SV genomic library in λFixII by hybridization to the non-homeobox portion of the human cDNA clone under high stringency. The mouse and human homologs share 100% amino acid identity in the homeodomain and approx. 91% in the remainder of the gene (Fig. 2). The mouse gene was two amino acids longer than its human counterpart, due to the duplication of single residues (serine-31/32 and proline-208/209/210). We deduced the position of the initiation codon based on the marked drop in homology between the mouse and human clones upstream of this position. This size was also consistent with the sizes of other Hox genes. Comparison between cDNA and genomic sequences revealed that the structure of the gene is the same as the other *AbdB*-related genes. *Hoxb-13* is composed of two exons separated by a small intron of less than 1 kb. The homeodomain is in the second exon, and the protein terminates soon after the C-terminal end of the homeobox. The non-homeobox region of the human cDNA clone hybridized under high stringency conditions to DNA from several vertebrate species including chicken and *Xenopus*, suggesting that the gene is likely conserved in those species (Fig. 3).

Mapping *Hoxb-13* relative to the HOXB cluster

A combination of genetic and physical mapping approaches

were employed to determine the precise physical relationship between *Hoxb-13* and the HOXB cluster. A panel of mouse-hamster somatic cell hybrid cell lines was used to map *Hoxb-13* to mouse chromosome 11, which also contains the HOXB cluster (data not shown). Using the mouse genomic clone, we generated a probe that could detect an *Eco*RI restriction length polymorphism to distinguish between C57/B6 and Spretus mouse strains (Fig. 4A). Typing animals in the Jackson Laboratory Spretus Backcross demonstrated that *Hoxb-13* is non-recombinant with the HOXB cluster in 100 animals, yielding a genetic distance of less than 1 centiMorgan (Fig. 4B). This suggested that *Hoxb-13* is within a few megabases of the HOXB cluster.

Genomic pulsed field gel electrophoresis (PFGE) was used to obtain a more precise estimate of the physical distance (Fig. 5). Genomic DNA was digested with rare-cutting enzymes, run under PFGE conditions that separate fragments 50-2000 kb in size, and transferred onto a nylon membrane. The genomic blot was probed sequentially with *Hoxb-1*, *Hoxb-9* and *Hoxb-13* probes. Previous genomic mapping using YACs (Rubock et al., 1990) indicated the presence of two *Not*I sites in the region, one between *Hoxb-1* and *Hoxb-2*, and a second distal to *Hoxb-9*. The *Hoxb-1*, *Hoxb-9* and *Hoxb-13* probes each hybridized to a different band in the *Not*I digest, as predicted by the YAC data. All three probes hybridized to a approx. 800 kb *Mlu*I fragment. *Hoxb-9* and *Hoxb-13*, but not *Hoxb-1*, shared the same *Sal*I and *Sac*II bands, narrowing the distance to less than 100 kb (Fig. 5). Chromosome walking in the mouse HOXB cluster has not uncovered any Hox genes 30-35 kb upstream of *Hoxb-9* (R. Krumlauf, personal communication).

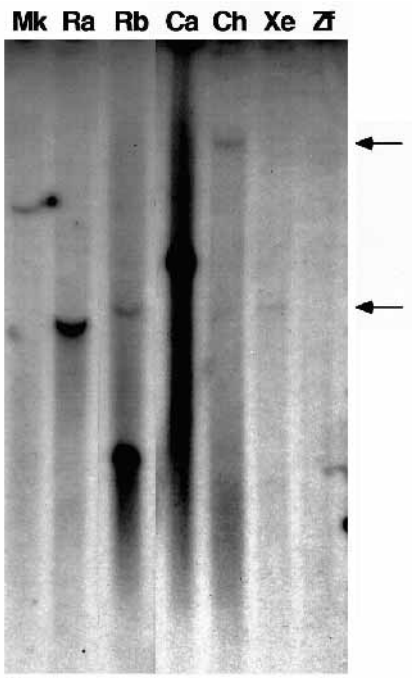

Fig. 3. Zoo blot hybridized with the human cDNA non-homeobox probe. A genomic Southern blot of *Bam*HI digests from different vertebrate species probed with the non-homeobox human cDNA fragment. The species analyzed are monkey (Mk), rat (Ra), rabbit (Rb), cat (Ca), chicken (Ch), *Xenopus* (Xe), and zebrafish (Zf). The positions of the chicken and *Xenopus* bands are indicated by arrows.

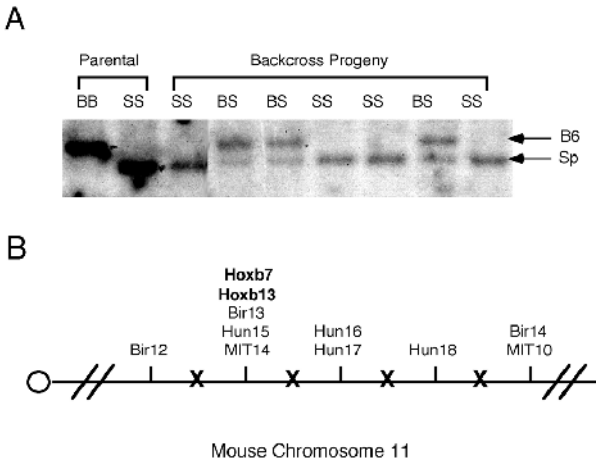

Fig. 4. Genomic mapping of *Hoxb-13* on the Jackson Laboratory Backcross. **(**A) Analysis of parental strains and backcross progeny with a probe from the *Hoxb-13* genomic phage clone. The first two lanes show the sizes of fragments detected in the C57/B6 and Spretus parental strains. The next seven lanes are examples of RFLP analysis of homozygous (SS) and heterozygous (BS) progeny. (B) The resulting genomic map of the relevant region on mouse chromosome 11. Crosses indicate genetic crossovers. *Hoxb-7* and *Hoxb-13*, which are non-recombinant, are shown in bold. The other markers are derived from three different sources, information regarding them is available from the Jackson Laboratory.

Since *Hoxb-13* was 30-100 kb from the 5′ end of the cluster, we screened a mouse bacterial artificial chromosome (BAC) library (Research Genetics) with both *Hoxb-9* and *Hoxb-13*, to yield one clone that contained both genes. The restriction map of the BAC indicated that *Hoxb-13* is approx. 70 kb away from *Hoxb-9*, whereas the other Hox-13 genes are no more than 10.5 kb upstream of the nearest Hox gene (Fig. 6). The *Sac*II site within the *Hoxb-13* gene was used to ascertain that *Hoxb-13*

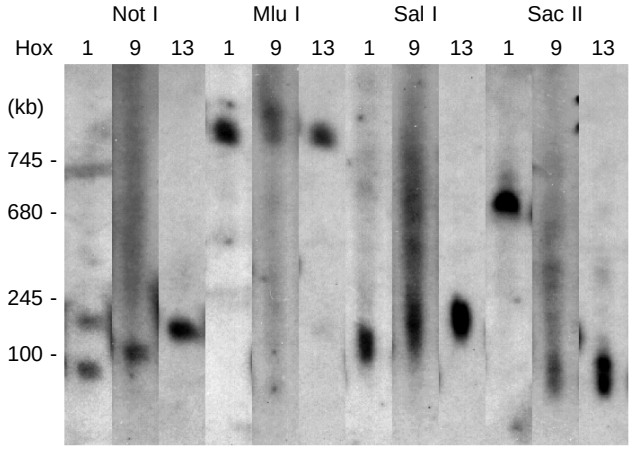

Fig. 5. Pulsed field gel mapping of the region spanning the HOXB cluster and *Hoxb-13*. Mouse DNA digested with rare-cutting enzymes was run under PFGE conditions to separate large genomic fragments from 50-2000 kb in size. The blot prepared from this gel was sequentially hybridized with probes from *Hoxb-1, Hoxb-9* and *Hoxb-13*. The *Not*I digest served as a positive control, because the size of the *Hoxb-9* fragment (110 kb) was known (Rubock et al., 1990). Marker sizes are given in kb.

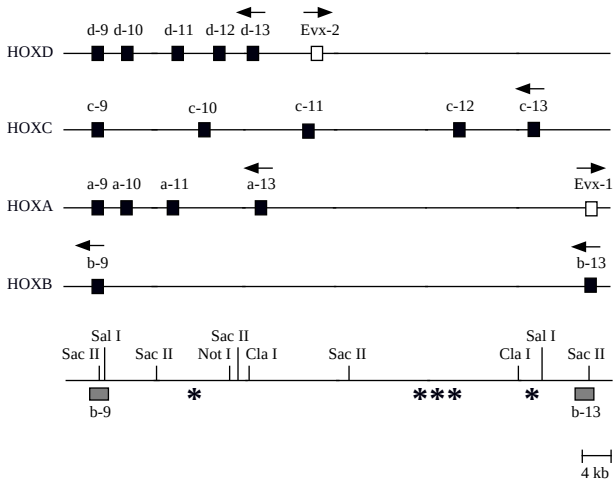

Fig. 6. Structural relationships between the four murine HOX clusters. A mouse BAC library was screened with PCR primers from *Hoxb-9* and *Hoxb-13*, and one clone was isolated that contained both genes. The *AbdB*-related paralogs (*Hox9-13*) are depicted as black boxes. *Evx* genes are shown as open boxes. The distances are drawn to scale. Note that *Hoxb-13* is transcribed in the same direction as the rest of the HOXB cluster, while the *Evx* genes lie on the opposite strand of DNA. The bottom line shows the restriction map of the BAC that spans the region. The *Hoxb-9* and *Hoxb-13* genes are depicted as gray boxes. Each repetitive sequence element identified is depicted with an asterisk. The repetitive elements include B1-type repeats and $(GA)_n$ and $(CA)_n$ dinucleotide repeats. The list of repeats is not complete. Data on HOXA, C and D clusters from Bastian et al. (1992); Dolle et al. (1994, 1991b); Faiella et al. (1991); Haack and Gruss (1993); Peterson et al. (1994).

is in the same transcriptional orientation as the rest of the cluster (Fig. 6).

Hoxb-13 expression

The ability of HOX cluster regulatory control mechanisms to influence neighboring genes may be impaired at a distance somewhere between 8-45 kb, as shown by the expression pattern of *Evx* genes (see above). This may result from either the physical separation itself, or the existence of a block in the propagation of the regulatory mechanism along the chromosome in the HOXA cluster. Since the position of *Hoxb-13* relative to the HOXB cluster is similar to that of *Evx-1* to the HOXA cluster (Fig. 6), and since *Evx-1* does not exhibit colinearity of expression, it was critical to determine whether the regulation of *Hoxb-13* expression is that of a paralogous group 13 gene.

Using whole-mount in situ hybridization on mouse embryos, *Hoxb-13* expression was first detected at approx. E9.0 in the caudal extent of the hindgut diverticulum (Fig. 7). *Hoxb-13* was not observed in embryos just prior to this stage, which had almost completed the process of turning, but whose neural tubes were still open in the forebrain region (Fig. 7A). The staining appeared at approx. E9.0, when the cephalic neural folds overlying the future forebrain and midbrain are fused, and the hindgut diverticulum was dilated (Fig. 7B,C). Both the onset and posterior localization of the initial *Hoxb-13* expression pattern was similar to that reported for *Hoxd-13* (Dolle et al., 1994, 1991a). Like its paralogs in the HOXA and HOXD clusters, *Hoxb-13* expression remained restricted to posterior regions in the three embryonic germ layers during subsequent differentiation. However, the 13 group paralog from the HOXC cluster is only detected in the spinal cord and posterior mesoderm, and not in the digestive or urogenital tracts (Peterson et al., 1994).

In situ hybridization on frozen sections at E12.5 showed *Hoxb-13* expression in what are considered the most posterior regions of the embryo (Fig. 8A,B). The expression domain seen earlier in the tailbud remained strong (Fig. 8E). *Hoxb-13* transcripts in the spinal cord were restricted to the caudal extent of the mantle layer, with higher levels found in the ventral grey horn (Fig. 8D). This result is surprising, because other genes in the HOXB cluster are dorsally restricted in the spinal cord (Graham et al., 1991), whereas the HOXA and HOXC cluster genes demonstrate a ventral and central/ventral restriction respectively (Gaunt et al., 1990). In the digestive tract, *Hoxb-13* transcripts were confined to the lining of the hindgut and did not reach the full caudal extent of the structure, the anal canal (Fig. 8C), reminiscent of the expression pattern described for *Hoxd-13* (Dolle et al., 1991a). *Hoxb-13* was posteriorly restricted in the urogenital system, as it was expressed

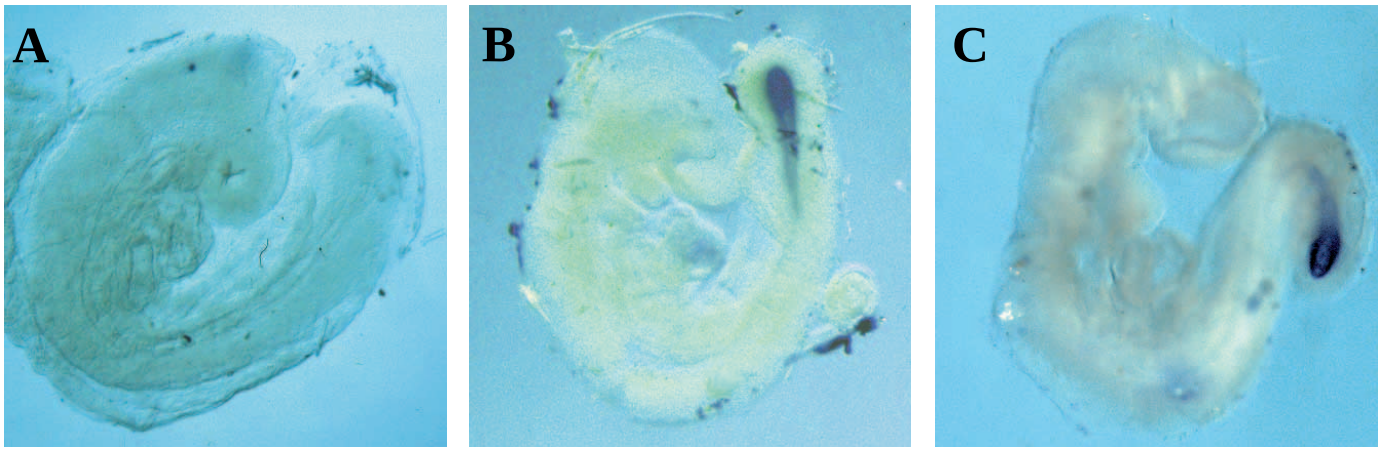

Fig. 7. Onset of *Hoxb-13* expression. Whole-mount in situ hybridization on mouse embryos from E8.75 to E9.25 showing the expression in the hindgut diverticulum. (A) An E8.75 embryo, which has almost completed the process of turning. (B) An early E9.0 embryo, which has just completed turning and neural tube closure in the cephalic region. (C) An approx. E9.25 embryo.

in the lining of the urogenital sinus and not in the genital ridge or metanephros.

The timing of activation and localization of *Hoxb-13* expression coincided with other Hox-13 genes with several notable exceptions. *Hoxb-13* was not detected in the prevertebrae, genital tubercle or the limbs. The developing limb bud is a model system to study the patterning of secondary axes in the embryo. *AbdB*-related genes from the HOXA, HOXC and HOXD clusters each play different roles in specifying skeletal elements of the limb (Dolle et al., 1989; Izpisua-Belmonte et al., 1991b; Nohno et al., 1991; Peterson et al., 1994; Yokouchi et al., 1991). While *Hoxa-13* and *Hoxd-13* paralogs are both expressed in the limb (Dolle et al., 1991a; Yokouchi et al., 1991), neither *Hoxc-13* (Peterson et al., 1994) nor *Hoxb-13* is. Quantitative RT-PCR was used to confirm the absence of *Hoxb-13* expression in the limbs. RNA samples from head, trunk and limb regions of E9-14 embryos were analyzed for expression of *Hoxd-12* and *Hoxb-13*. While *Hoxd-12* was detected in both the trunk and limb samples, *Hoxb-13* was found only in the trunk (Fig. 9). Therefore, *Hoxb-13* expression reflects colinearity in the main A/P axis of the embryo, but it is not expressed in the secondary axes such as the limb and genital tubercle. This may reflect the absence or disruption in the HOXB complex of cluster-specific *cis* regulatory elements for these axes that may be found in the region between *Hoxd-9* and *Hoxd-13* (D. Duboule, personal communication).

The search for the other missing paralogs

Two different approaches, involving degenerate PCR and low-stringency hybridization, were taken to identify any of the missing members of paralogous groups 10, 11 and 12 in the region between *Hoxb-9* and *Hoxb-13*. Degenerate PCR primers, corresponding to highly conserved sequences within paralogous homeodomains, were designed to amplify any member of a specific paralog group. Each of the primer pairs amplified a product of the correct size from genomic DNA. Additionally, the Hox-12 primer pair worked on a cosmid clone which contained *Hoxd-12*. However, only the Hox-13 group primers amplified the correct product when the *Hoxb-13* cosmid, YACs and BACs were used as templates (data not shown). A major caveat to the degenerate PCR experiments was that they relied on strong sequence conservation, particularly at the 3′ end of the primer. It is possible that another homeobox in the region may have diverged from its paralogs, as is the case for *Hoxb-13*, and thus lost the ability to bind one of the primers. Therefore, the second approach we employed to search for other Hox genes involved low-stringency hybridization to the region encoding the highly conserved third helix in the homeodomain.

The ‘universal Hox oligo’ (kindly provided by R. Krumlauf) hybridized to three bands with different intensities in the BAC spanning *Hoxb-9* and *Hoxb-13* (data not shown). The blot was rehybridized under high stringency conditions with *Hoxb-9* and *Hoxb-13* probes to identify the bands corresponding to

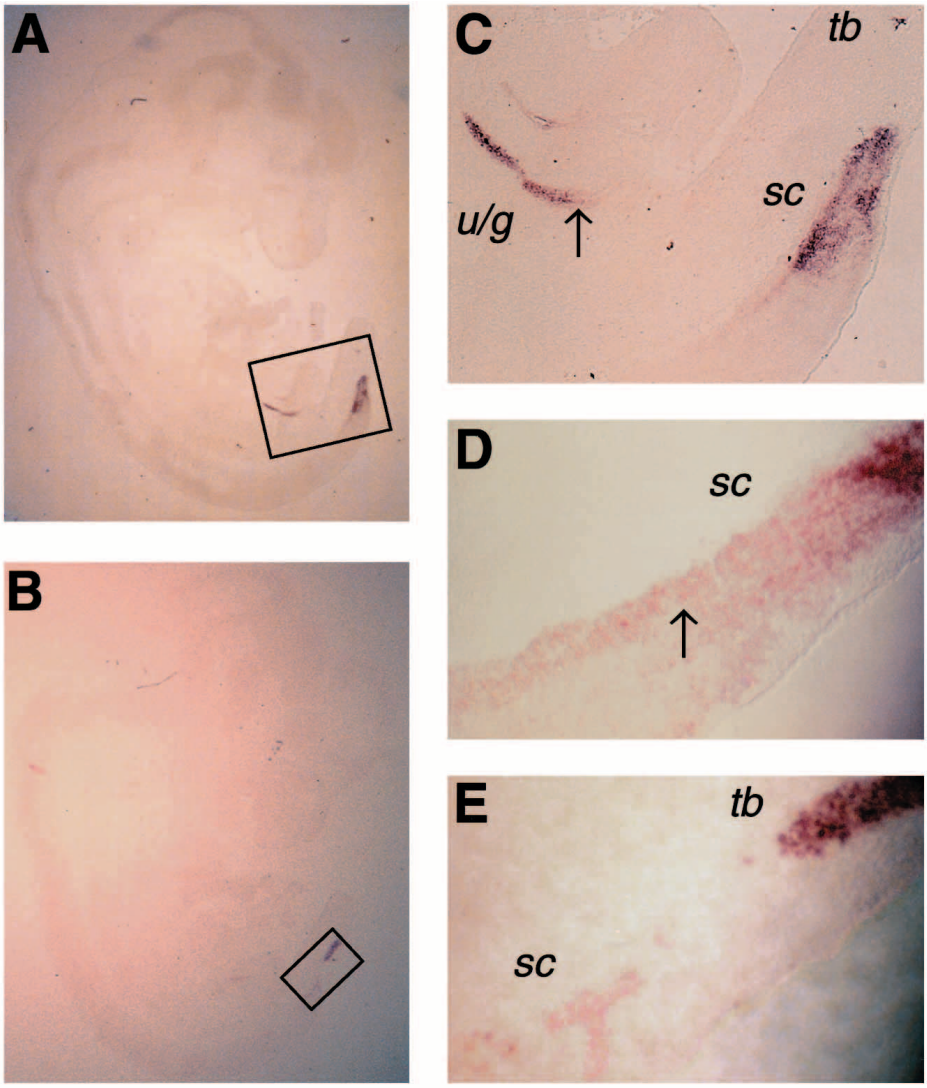

Fig. 8. Expression of *Hoxb-13* in posterior regions of the E12.5 embryo. **(**A-D**)** Sagittal sections of an E12.5 embryo hybridized with a digoxigenin-labeled probe. (A,B) Section as seen under low power to demonstrate that *Hoxb-13* is only detected in posterior regions. (C) Higher magnification of the boxed region in A showing expression in the hindgut (g), urogenital sinus (u) and a small region in the tailbud (tb). Note that the staining does not extend to the posterior extent of the hindgut (see arrow). (D) Higher magnification of a medial serial section showing the relatively uniform expression in the caudal extent of the spinal cord (sc), and the higher levels of expression found in the ventral gray horn in the rostral expression domain in the spinal cord. An arrow shows the point at which the expression becomes ventrally restricted. (E) Higher magnification of the boxed area of the lateral serial section shown in B demonstrating strong expression in the tailbud (tb) and the caudal region of the spinal cord (sc).

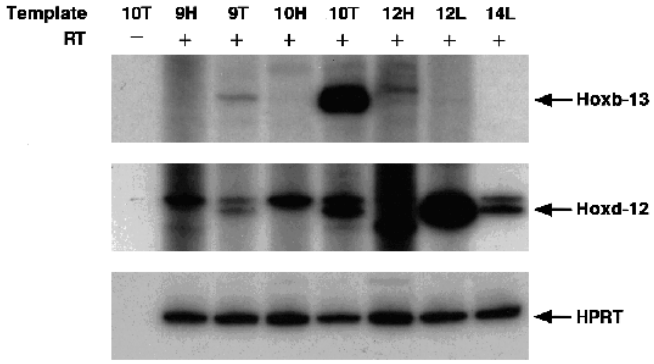

Fig. 9. *Hoxb-13* is expressed in the trunk and not in the head or limbs. RNA was extracted for reverse transcription-polymerase chain reaction (RT-PCR) from head, trunk and limb regions of E9-14 mouse embryos. *Hoxb-13* is detected at low levels in E9 trunk and at high levels by E10, but it is absent from head and limb samples. *Hoxd-12* is detected throughout E9-14 in both trunk and limb tissues.

those genes. The strongest signal corresponded to *Hoxb-9* and the intermediate signal to *Hoxb-13*. This result is consistent with the fact that *Hoxb-13* is more divergent in the third helix than *Hoxb-9*. The existence of the weakly hybridizing signal suggested that another, more divergent, Hox gene may reside between *Hoxb-9* and *Hoxb-13*. When the approx. 4.5 kb *Bam*HI fragment corresponding to the weak signal was used to probe the BAC blot, it detected the same *Eco*RI and *Hin*dIII bands that bound the oligo probe. This band mapped to the approx. 25 kb *Sac*II/*Cla*I fragment immediately downstream of *Hoxb-13* in the BAC (Fig. 6). The corresponding approx. 2.8 kb *Hin*dIII fragment was subcloned into bluescript and sequenced. Homology searches performed with standard and 'block' algorithms did not detect any homeodomain-like sequences, and instead revealed the presence of two different dinucleotide and B1 repeat elements. Since this band hybridized to the universal Hox oligo better than any other fragment in the BAC digest, aside from those corresponding to *Hoxb-9* and *Hoxb-13*, it is unlikely that any additional functional Hox genes exist in the region between *Hoxb-9* and *Hoxb-13* on mouse chromosome 11.

DISCUSSION

Cloning *Hoxb-13*, a new member of the HOXB cluster

We have described a new member of the mammalian HOXB cluster. Sequence analysis in the homeodomain revealed that the novel Hox gene is a member of the Hox-13 paralogous group. We hypothesized that the novel gene represented the missing *Hoxb-13*, because the HOXB complex is the only one missing a Hox-13 paralog. This identification was confirmed with the genetic and physical mapping of the new gene to within approx. 70 kb of the *Hoxb-9*. Isolation of a BAC that spanned the regions between *Hoxb-9* and-*13* allowed the determination of the physical distance between the two genes on mouse chromosome 11. *Hoxb-13* is located approx. 70 kb upstream of the rest of the cluster in the same transcriptional orientation. This large distance is in marked contrast to the short spacing between other *AbdB*-related genes. The discovery of the *Hoxb-13* gene in close proximity to the HOXB cluster suggests that the other *AbdB*-related paralogs (*Hoxb-10*, *Hoxb-11* and *Hoxb-12*) might also be present. Extensive efforts to identify these genes in the region between *Hoxb-9* and *Hoxb-13* using degenerate PCR and low stringency hybridization have not succeeded. These efforts, coupled with the fact that this region contains highly repetitive DNA sequences characteristic of the flanking regions of HOX clusters, suggest that they may have been lost or highly diverged during rearrangement of this locus.

Implications for evolution of the HOX complex

The current compositions of the 5′ regions of the HOXA, C and D clusters are consistent with expansion of the ancestral *AbdB*-like gene to form five posterior paralogs, followed by large-scale duplications of the cluster, and the subsequent loss of the Hox-12 paralog from HOXA lineage (Kappen and Ruddle, 1993; Kappen et al., 1989). In the case of the HOXB cluster, in which *Hoxb-9* was the only known *AbdB* paralog, it was not clear whether it had lost the four posterior-most genes or had never acquired them. In the latter case, the HOXB cluster would represent the ancestral cluster (Izpisua-Belmonte et al., 1991a). The discovery of *Hoxb-13* in a distant region of the HOXB cluster suggests that gene loss is the probable explanation for the current composition of the cluster.

AbdB-related Hox genes are successively more divergent from paralog group 9 to 13. Although the *Hoxb-13* homeodomain has diverged slightly from the other Hox-13 paralogs, it is highly conserved between species. Thus, the human and mouse genes share 100% identity in the homeodomain, and 90% in the rest of the gene. Furthermore, in a zoo blot under conditions of high stringency, the non-homeobox portion of the human hybridized to DNA from several species including chicken and *Xenopus*. The lack of *Hoxb-13* hybridization to zebrafish genomic DNA does not rule out the existence of an ortholog. Zebrafish Hox genes are progressively more divergent than their mammalian counterparts at increasingly more 'posterior' regions in the cluster (van der Hoeven et al., 1996). In fact, the non-homeobox portion of the zebrafish *Hoxd-13* gene is virtually unrelated to its mammalian counterpart. This may explain why the probe used in the zoo blot, which encodes the non-homeobox portion of the human cDNA, could not detect a zebrafish ortholog.

Cloning *Hoxb-13* and determining its physical relationship to the HOXB cluster in other species may shed light on the point in evolution at which the gene was separated from the cluster and on the significance of the physical attachment to the cluster. The determination of the physical distance between *Hoxb-9* and *Hoxb-13* in a diverse range of vertebrate species will reveal whether the spacing between these genes is conserved, as is the case for other Hox genes. This line of research could also identify species in which *Hoxb-13* has been lost or separated from the cluster. The strong conservation of *Hoxb-13*, despite the disruption of the locus, suggests that there has been strong selective pressure to maintain it.

Similarity of *Hoxb-13* expression to other Hox-13 paralogs

Hoxb-13 expression in the main body axis of the embryo maintains both temporal and spatial colinearity. *Hoxb-13* was

first detected in the tailbud of E9.0 mice, consistent with the onset and posterior pattern of expression exhibited by other Hox-13 paralogs. In situ hybridization at E12.5 demonstrated *Hoxb-13* expression in the hindgut, urogenital tract, posterior extent of the spinal cord, and tailbud. This pattern is similar to other Hox-13 genes, except for the absence of expression in the limbs and genital tubercle.

Analysis of the expression patterns of the posterior Hox genes reveals that the developing limbs, digestive tract and urogenital tract are specified by different combinations of paralogs. The developing digestive and urogenital systems receive input from the HOXA, B and D clusters. The situation is different in the case of limb development, where all *AbdB*-related genes in the HOXA and D clusters contribute to patterning in both the fore- and hindlimbs (Duboule, 1992; Morgan and Tabin, 1993), whereas *Hoxc-9-11* are found only in the hindlimb (Peterson et al., 1994). The absence of *Hoxb-13* expression from the developing limbs may reflect the loss of an enhancer element in the region between *Hoxb-9* and *Hoxb-13*. Since this region is altered in the HOXB cluster (as evidenced by the expansion and presence of repetitive sequence elements), it is possible that the element was lost during this process. However, the acquisition of the limb regulatory element(s) may have occurred after the duplication of the clusters, and thus the HOXB cluster did not acquire it.

Hoxb-13 expression maintains colinearity

In light of the *Evx-1* data, which suggest that the physical limit of the regulatory mechanism(s) of the HOXA cluster is <45 kb, it was critical to determine whether *Hoxb-13* maintained colinear expression across a distance of 70 kb. The colinear expression of *Hoxb-13* in the main A/P body axis presents a paradox. On the one hand, two lines of evidence suggest that *Hoxb-13* lies outside the range of any global control mechanisms operating within the cluster. First, its position relative to the cluster is similar to *Evx-1*, which is regulated in a non-Hox manner. Second, the presence of repetitive sequence elements throughout the region spanning *Hoxb-9* and *Hoxb-13* (Fig. 6) would likely disrupt regulatory chromatin structure which may be required for global control. This suggests that *Hoxb-13* expression in the trunk is regulated independently of the cluster. On the other hand, the physical attachment to the cluster and its strong conservation among diverse vertebrate species suggest that *Hoxb-13* may receive regulatory information from the HOXB cluster.

Cis regulatory elements, 'autoregulation' by paralogs (Popperl et al., 1995), and cross regulation from neighboring genes contribute to the regulation of Hox genes (Krumlauf, 1994). An additional level of regulation may be achieved through the activity of a global 'locus control'-like mechanism operating in the cluster (Duboule, 1994). If *Hoxb-13* is regulated in a complex-independent manner, it resembles the situation of a transgene that is inserted in the genome at random. A *Hoxd-13* transgene, for example, is expressed in the same pattern as the endogenous gene, except that it is not expressed in the limbs or genital tubercle (Denis Duboule, personal communication). One explanation for this observation, is that the *cis*-acting regulatory sequences are able to confer proper expression in the main body axis, but that expression in the secondary axes requires the context of the cluster. The *cis*-acting regulatory region may inherently have the ability to direct proper expression of the transgene, or it may rely on the 'autoregulatory' activity of paralogs, which we shall refer to as para-regulation.

Thus, the *Hoxb-13* gene may contain the elements required to direct proper expression from its distal location in the cluster, or it may require para-regulation. The ventral restriction of *Hoxb-13* expression in the spinal cord, in contrast to the dorsal restriction of the other HOXB cluster members, suggests that it may be regulated differently. This ventral expression domain may reflect changes in the response of *Hoxb-13* to the regulatory mechanism of the complex, or the presence of different upstream regulators (i.e., the other paralogs). The role of other paralogs could be directly tested by analyzing *Hoxb-13* expression in the digestive and urogenital tracts of *Hoxa-13* and/or *Hoxd-13* loss-of-function and gain-of-function mutants, where its expression pattern should be altered if para-regulatory mechanisms are involved. If *Hoxb-13* expression is not changed, it could not distinguish between the possibilities that the *cis*-acting elements are sufficient to drive expression, or that a global mechanism operating in the cluster can exert its influence over the 70 kb separation. The *Hoxb-13* situation is ideal to analyze the role of paralogs in this way, because its physical separation from HOXB and the lack of paralogs 10-12 reduces the opportunity for cross-regulatory interactions with other cluster members that could complicate analysis in other clusters.

We thank D. Duboule and R. Krumlauf for helpful advice and insightful discussions. R. Krumlauf also provided the universal Hox oligo. We also thank Dillon Patterson for sectioning embryos; L. Niswander for useful advice on whole-mount hybridizations and for reviewing the manuscript; M. Barter for her assistance with the Jackson Laboratory Backcross; and A. Hemmati-Brivanlou, S. Dinardo, P. Wilson, J. De la Torre, and K. Platt for scientific advice. We thank the members of the Heintz and Desplan laboratories for comments and discussions, and J. Zuo and J. Miwa in particular for their contributions to the project. L. Zeltser is an Arnold and Mabel Beckman fellow. This work was supported by the Howard Hughes Medical Institute.

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(Accepted 5 May 1996)