Function of posterior *HoxD* genes in the morphogenesis of the anal sphincter

Takashi Kondo[1], Pascal Dollé[2], Jozsef Zákány[1] and Denis Duboule[1],*

[1]Department of Zoology and Animal Biology, University of Geneva, Sciences III, Quai Ernest Ansermet 30, 1211 Geneva 4, Switzerland
[2]Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, BP 163, 67404 Illkirch Cédex, CU de Strasbourg, France

*Author for correspondence (e-mail: Duboule@sc2a.unige.ch)

SUMMARY

Vertebrate 5′-located *HoxD* genes are expressed in the most caudal part of the digestive tract and their potential functions during gut development have been assessed by gene disruptions. We have inserted reporter *lacZ* sequences within the *Hoxd-12* gene and analysed the morphology of the gut in these mice as well as in *Hoxd-13* mutant animals. When homozygous, both mutations induce an important disorganization of the anorectal region. In particular, severe alterations of the smooth muscle layers of the rectum led to defective morphogenesis of the internal anal sphincter. Similarly, *Hoxd-12* and *Hoxd-13* functionally overlap during digit development. The function of these genes in the morphogenesis of the digestive system as well as their functional evolution are discussed.

Key words: mouse, *HoxD*, anal sphincter, gut development, digit development, digestive system, evolution, muscle

INTRODUCTION

The key role of *Hox* genes in the organization of the vertebrate body plan is well documented. Genetics experiments involving either gain-of-function or loss-of-function alleles have shown that they are required to properly build structures along the major body axes (reviewed in Krumlauf, 1994). Their developmental expression patterns have revealed that genes located at the 3′ extremities of the complexes are expressed anteriorly while genes located at 5′ positions are expressed in progressively more restricted posterior areas (Gaunt et al., 1988; Dollé et al., 1989; see Krumlauf, 1994). This type of colinearity, analogous to that observed in *Drosophila* (Lewis, 1978), is visible not only within presumptive skeletal structures such as vertebrae or appendicular bones, but also in internal organs or systems such as in the genitourinary organs (Dollé et al., 1991a,b) or the digestive tract (Yokouchi et al., 1995; Roberts et al., 1995). As a consequence of colinearity, genes located at the 5′ extremity of the *Hox* complexes, and related to the *Drosophila* gene *AbdB*, are expected to exert their functions at the distal ends of the various body axes. This was first verified by the inactivation of the *Hoxd-13* gene, which led to alterations in the sacrum, the digits and the penian bone (Dollé et al., 1993).

Amongst the various sites of expression of *Hox* genes, the alimentary canal is of particular interest as it represents one of the phylogenetically oldest structures wherein such genes have been speculated to have a function. Unlike skeletal elements, which are present only in a minority of metazoa and emerged rather recently in evolution, most animals have a 'digestive' tube. It may thus represent one of the oldest signs of an anterior-posterior asymmetry and, thereby, be associated with the original requirement for a rostrocaudal specification. The regionalization of the gut, a necessity for the achievement of a variety of important functions, may thus rely on the *Hox* gene complexes (Roberts et al., 1995; Yokouchi et al., 1995).

We previously reported the expression of the two most posterior *Hoxd* genes, *Hoxd-13* and *Hoxd-12*, in the terminal part of the hindgut (Dollé et al., 1991a,b). More recently, expression analyses revealed that 5′-located genes belonging to either the *HOXD* or *HOXA* chicken complexes are expressed in a colinear fashion and that the transition between expression domains correlates with anatomical divisions (Roberts et al., 1995; Yokouchi et al., 1995). Therefore, it seems plausible that colinear expression of *Hox* genes in the hindgut may be informative regarding changes in morphologies, much like what is known to happen in the vertebral column. Such a function for vertebrate *Hox* genes in the gut is particularly intriguing as some *Drosophila* homeotic genes too, are required for proper development of midgut mesoderm, mutant flies showing morphological alterations in muscle constrictions (e.g. Bienz, 1993). The potential importance of *AbdB*-related genes in terminal gut morphogenesis is also supported by the expression of *egl-5*, the *C. elegans* gene orthologous to *AbdB*, in rectal blast cells, a group of cells lining the anus (Chisholm, 1991), as well as by the presence of both *Hoxd-13* and *Hoxa-13* transcripts in the developing cloaca of zebrafish (van der Hoeven et al., 1996a; P. Sordino, T. K. and D. D., unpublished).

Specialization of the gut epithelia is thought to occur through an induction from the visceral mesoderm (e.g. Haffen et al., 1987), while earlier inductive signals emanating from the endoderm may instruct mesodermal tissue to undergo gut differentiation (Kédinger et al., 1990). The molecules involved in these early interactions are unknown, although expression
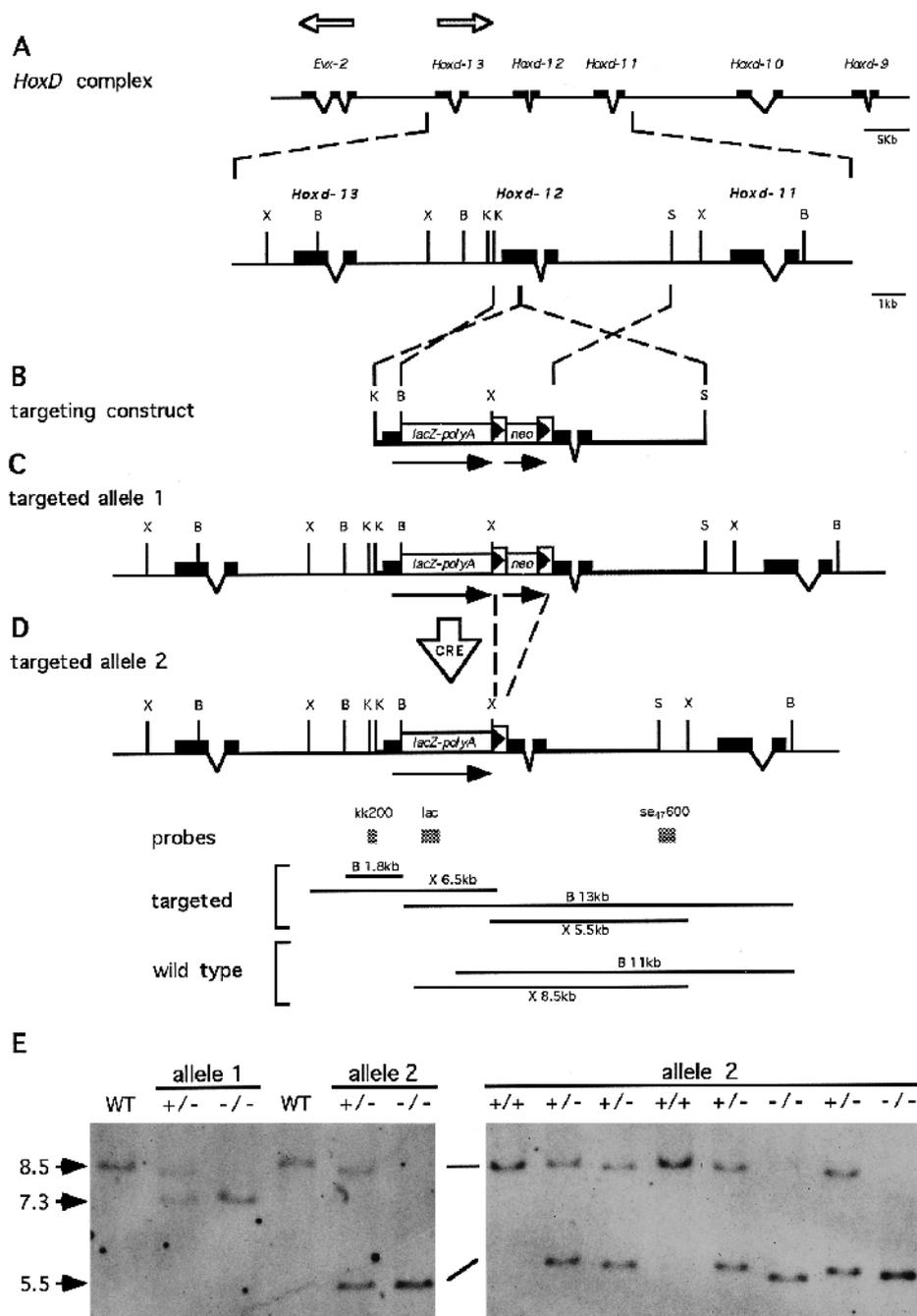
Fig. 1. Schematic description of the *Hoxd-12* locus and of constructs used for gene inactivation. (A) Diagram of the posterior part of the *HoxD* complex. (B-D) targeting construct as well as the different alleles produced before (C) or after treatment with *Cre* recombinase (D). Black rectangles indicate exons and directions of transcription are shown by arrows. Hatched boxes in D are probes used for verifying the clones and a summary of the expected restriction fragments obtained in Southern hybridization are described below. B, *Bam*HI; K, *Kpn*I, S, *Sal*I; X, *Xho*I. (E) Southern blot analysis of an F2 progeny. *Xho*I-digested DNA was hybridized to the se₄₇600 probe. Wild-type locus gave 8.5 kb band, while the targeted allele 1 in C and allele 2 in D gave 7.3 kb and 5.5 kb bands, respectively.

studies suggest that the signaling molecule *Sonic hedgehog* (*Shh*) may be part of the cascade, upstream of *Hox* genes (Roberts et al., 1995). So far, however, a genetic demonstration of the function of these genes during gut morphogenesis was lacking. In order to document this point, we have analysed the effect of the inactivation of two *Hoxd* genes on the morphology of the anorectal region. Both the *Hoxd-13* and *Hoxd-12* genes were shown to be expressed, during mouse development, in the terminal part of the hindgut mesoderm, in the presumptive musculosa, which will generate, after birth, the circular and longitudinal layers of muscles. Expression of both genes was strong and overlapping, up to the most distal part, i.e. the level of the internal sphincter that demarcates the anorectal transition (Dollé et al., 1991). In addition, *Hoxd-13*,

but not *Hoxd-12*, was heavily expressed in rectal epithelium, which is derived from definitive endoderm, while undetectable in squamous anal epithelium, which derives from the ectoderm.

Both *Hoxd-13* (Dollé et al., 1993) and *Hoxd-12* (this work) have been inactivated and we report here that, in homozygous mutants of either genes, the morphology of the internal sphincter was deeply disorganized. In particular, the longitudinal muscle layer was severely reduced or partially absent. We show that this defect becomes apparent soon after birth, after the two muscle layers differentiate. In the case of *Hoxd-13*, a high proportion of aged homozygous males developed an anal prolapsus, likely due to an incapacity of the internal sphincter. These results demonstrate the requirement of *Hox* genes for proper gut morphogenesis. The phylogenetic impli-
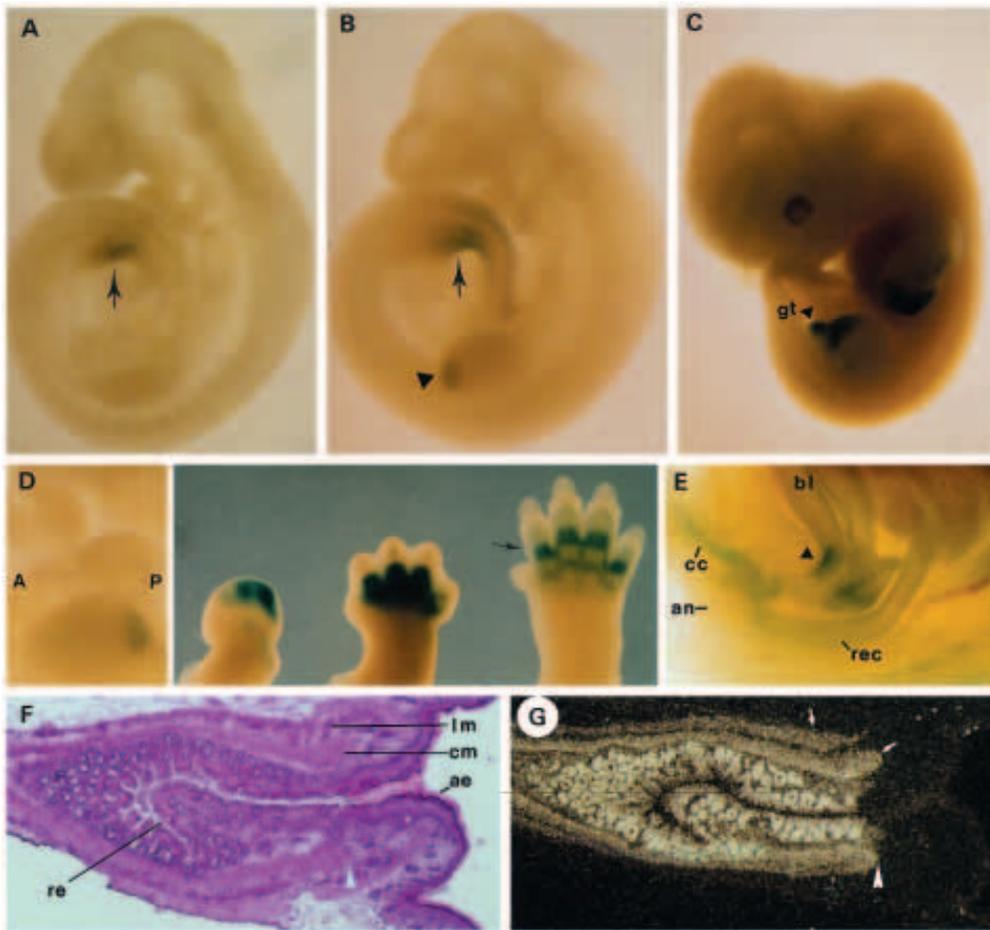
Fig. 2. Expression of *Hoxd-12* and *Hoxd-13*. (A-E) Whole-mount β-galactosidase staining shows *Hoxd-12* expression pattern in fetuses. (A) Earliest detection of *Hoxd-12* expression was around the proctodeum (arrow), in 9.5 days p.c. embryos. (B) At 10.5 days p.c., embryos showed expression in forelimb bud (arrowhead), together with a more widespread expression posteriorly. Arrow as in A. (C) 11.5 days p.c. embryo with expression in both limbs and genital tubercle (gt). (D) *Hoxd-12* expression in forelimbs, at 10.5, 11.5, 13.5 and 15.5 days p.c., from left to right. All panels are with anterior (A) left and posterior (P) right. The arrow points to the expression in phalange 1 of 15.5-day-old forelimb. (E) Expression of *Hoxd-12* in the posterior region of a 15.5 p.c. foetal trunk. an, anus; bl, bladder; cc, corpus cavernosum; rec, rectum. (F) Histological section of the anorectal region of a newborn mouse. ae, anal epithelium; cm, circular muscle; lm, longitudinal muscle; re, rectal epithelium. (G) In situ hybridization of *Hoxd-13* RNAs in the anorectal region seen under F. *Hoxd-13* is expressed in the epithelium of the rectum as well as in the two muscle layers (arrows), but not in the anus (starting at the white arrowhead).

cations of this observation are discussed in the context of a potential origin of colinearity in evolution.

MATERIALS AND METHODS

Targeted mutagenesis of the *Hoxd-12* gene

For the construction of the replacement targeting vector, a *Kpn*I-*Sal*I 5.5 kb fragment of mouse genomic DNA containing the *Hoxd-12* gene was subcloned from a cosmid clone (cosB, Izpisúa-Belmonte et al., 1991). The bacterial *lacZ* gene, coding for the β-galactosidase (β-gal) enzyme, followed by a SV40 poly(A) sequence (*lacZ* cassette), was inserted in frame in the *Hoxd-12* protein coding sequence at the *Sty*I site in the first exon and a *loxP*-PGK*neo-loxP* cassette was introduced next to the *lacZ* cassette, in the same transcriptional orientation. 10 μg of the construct, digested with both *Kpn*I and *Sal*I, was electroporated into 1×10^7 ES cells (D3, Doetschman et al., 1985). One internal and two external probes were used to verify the targeting; the internal probe was a 0.3 kb *Cla*I-*Eco*RV fragment within *lacZ* (Fig. 1; lac probe), while the external probes were either a 0.2 kb *Kpn*I fragment (Fig. 1; kk200 probe) or a 0.6 kb *Sal*I-*Eco*47III fragment (Fig. 1; se$_{47}$600 probe). Two out of 108 G418 resistant colonies were correctly targeted, amplified and injected into C57BL/6 blastocysts. As both clones gave equivalent results, as judged by β-gal staining of progenies from chimeric males, only one of them was selected to excise the *loxP*-PGK-*neo-loxP* cassette. This was done by electroporating 10 μg of the pMC-CRE plasmid producing the *Cre* recombinase from bacteriophage P1 (Gu et al., 1993). Clones were selected for the deletion of the PGK-*neo* cassette by using the se$_{47}$600 DNA

fragment as a probe (Fig. 1) and one such clone was injected into blastocysts and transferred into the germ line.

β-*gal* staining, histological analyses and in situ hybridizations

Whole-mount enzyme histochemical detection of β-galactosidase activity in embryos was carried out as described previously (Zákány et al., 1988). Embryos were isolated at appropriate stages, fixed in 4% paraformaldehyde and stained with X-gal cocktail. Stained animals were embedded in paraffin and sectioned. As β-gal staining was weak on 10 μm sections, the picture in Fig. 2E was taken directly from the paraffin block, at a mid-sagittal level. For histological analyses, animals and organs were fixed with Bouin's fluid and embedded in paraffin following standard procedures. 7 μm thick sections were stained with eosin and hematoxylin or van Guison staining solution (for goblet cells). The *Hoxd-13* probe as well as the protocol used for in situ hybridizations were as described previously (Dollé et al., 1991).

Skeletal preparations

For skeletal preparations, animals at 1, 6, 13 and 70 days after birth were killed. Kidneys or tails were taken for isolation of DNA and animals were genotyped by Southern hybridization. Whole-mount skeletal preparations of newborn, juvenile and adult mice were carried out according to established procedures (Inouye, 1976; Dollé et al., 1993).

RESULTS

Production of *Hoxd-12*-deficient mice

In order to follow the expression of *Hoxd-12*, we inserted the

lacZ reporter sequences in frame with the *Hoxd-12* gene. To preserve all *Hoxd* regulatory elements, no deletion of genomic sequences was generated by the targeting construct (Fig. 1A-C). The PGK*neo* selectable gene was subsequently removed in order to avoid possible interference with the surrounding *Hox* transcription units. The targeted allele was identified as a 6.5 kb or 7.5 kb *Xho*I fragment with probe kk200 or probe se$_{47}$600, respectively, instead of the 8.5 kb band expected for both probes in the wild-type configuration (Fig. 1D). A *lacZ* probe was used to verify that a single insertion event had occurred. Excision of the PGK*neo* cassette (Fig. 1D) was produced by subsequent electroporation of a plasmid expressing the *Cre* recombinase of bacteriophage P1. The PGK*neo* deleted locus was identified as a 5.5 kb *Xho*I fragment with probe se$_{47}$600 (Fig. 1D,E). Two *neo*-positive ES cell clones as well as one *neo*-negative clone (derived from one of the former) were injected into C57BL/6 blastocysts. In all cases, germ-line chimeras were obtained and heterozygous animals from F$_1$ progenies were crossed to generate homozygous *Hoxd-12*-deficient mice. Such animals were viable and fertile.

Hoxd-12/lacZ expression

β-galactosidase activity was first assayed in undifferentiated ES cell clones containing the *Hoxd-12/lacZ* fusion gene. No β-gal staining could be recovered, either in the absence or in the presence of all-*trans* retinoic acid (4 days of treatment), in contrast to results obtained in the human NT2/D1 teratocarcinoma cell line where this gene was reported to be transcribed in absence of RA (Simeone et al., 1991). Similar results were obtained with a *Hoxd-13/lacZ* fusion gene (T. K. and D. D, unpublished) and may be explained by expression levels undetectable by this technology.

We used the β-gal activity of the targeted *Hoxd-12* allele to visualize the expression of *Hoxd-12*. At both 9 and 15 days post coitum (p.c.), heterozygous and homozygous animals derived from all three clones were stained. All mouse lines showed identical staining patterns. This indicated that the function of the *Hoxd-12* promoter was not modified by the presence of the PGK promoter. Secondly, as no qualitative staining difference was observed between heterozygous and homozygous animals, we concluded that autoregulation of *Hoxd-12* does not have an essential role in the expression of the gene. This did not exclude, however, the possibility of cross-regulation by other paralogous or non-paralogous *Hox* genes, such as *Hoxc-12* or group 11 and/or 13 genes.

The earliest detection of β-gal activity was at mid day 9 p.c. Embryos of this age showed a restricted expression in the area of the proctodeum, the region from which the anus subsequently forms (Fig. 2A, arrow). The staining was visible along each side of the proctodeal membrane, extending towards the future base of the hindlimb buds. At 10.5 days p.c., the staining became visible in the posterior region of the forelimb buds (Fig. 2D) as well as in more dorsal parts of the tail (Fig. 2B,D). In 11.5 days p.c. embryos, expression in limbs evolved with the appearance of a second domain covering the presumptive digit area (Fig. 2C,D). This distal domain was similar to that observed for *Hoxd-13* (Dollé et al., 1993) and *Hoxd-11* (Sordino et al., 1995) and likely originated at the posterodistal margin of the developing bud (Duboule, 1994). Interestingly, however, this *Hoxd-12* digit domain was slightly smaller than that of *Hoxd-13* and did not involve the future thumb, as pre-

viously described for birds (Fig. 2; Yokouchi et al., 1991). At this stage, weak expression was detected in the zeugopodium region (Fig. 2D). In older animals, the staining became more restricted. While the distal tips of the developing digits and the zeugopodium were negative in day 15 p.c. fetuses, strong expression was observed at the levels of both the first phalangeal (P1) and the distal part of the metacarpal chondrogenic condensations (Fig. 2D). Staining did not extend into the most anterior handplate, in contrast to both *Hoxd-11* and *Hoxd-13* (Dollé et al., 1993).

Hoxd-12 was also expressed in the genital eminence (Fig. 2C). After a rather homogenous expression at day 12 p.c., 14- and 15-day-old fetuses showed strong staining in the future corpus cavernosum of the penis (Fig. 2E). The outer layer of the bladder, especially at the junction with the penian urethra, was positive as well as the presumptive sites of origin of accessory glands such as the prostate, at the level of the urogenital sinus. The mesodermal layer of the rectum, from the anorectal junction up to the first few turns of the hindgut expressed *Hoxd-12* (Fig. 2E). In contrast, *Hoxd-13* was transcribed both in the rectal epithelium and mesodermal derivatives (Fig. 2F,G), until adulthood (not shown). While both *Hoxd-12* and *Hoxd-13* were expressed in the internal and external muscle layers, their anterior boundaries of expression in hindgut were not precisely established. Neither *Hoxd-12* nor *Hoxd-13* were expressed in the anal epithelium (Fig. 2G; data not shown).

Function of 5′-located *Hoxd* genes in the rectum

Mice homozygous for the *Hoxd-12* mutation appeared normal in all respects. No particular phenotype could distinguish them from wild-type littermates and a detailed macroscopic observation and anatomical dissections did not reveal any obvious alteration of structures or internal organs which expressed this gene during development. Upon microscopic analysis, however, a consistent histological defect was uncovered in the rectum of both male and female homozygous animals.

The terminal part of the large intestine, the rectum, contains two well-defined subserosal smooth muscles layers, the inner, circular muscle layer (cm) and the outer, longitudinal muscle layer (lm; Fig. 3A). While the outer muscle layer is rather thin anteriorly, in the colon, it becomes almost as thick as the inner muscle layer in the terminal part of the rectum (Fig. 3A,B). In its terminal part, at the level of the anorectal transition, the inner layer extends slightly more distal than the outer layer and thickens to form a circular ring at the basis of which the outer layer comes to attach. This thick muscular structure, the internal anal sphincter, delimits the end of the rectum. This autonomous sphincter is surrounded by an external sphincter, composed of striated muscle masses, which do not express either *Hoxd-12* or *Hoxd-13*. Smooth muscle layers of the rectum are particularly important as they receive enteric innervation responsible for the constipation reflex. As shown in Fig. 2G, both smooth muscle layers, including the internal sphincter, expressed the *Hoxd-12* and *Hoxd-13* genes, from mid-gestation stages up to adulthood.

Histological analysis of homozygous *Hoxd-12* mutant mice showed a marked disorganization of rectal muscle layers in the region of the sphincter (Fig. 3C-E). Most frequently, the outer muscle layer was abnormally thin, or had a degenerated aspect (Fig. 3C). Often, it was interrupted at many places, so that

isolated blocks of muscles cells were found scattered in the most distal region of the rectum (Fig. 3E). In addition, the muscle layers of *Hoxd-12*-deficient mice did not properly stop at the anorectal transition. Instead of forming the internal sphincter, muscle cells extended to a more distal position, such that the circular layer often overlapped with the position of the anal epithelium (e.g. Fig. 3C, left), in contrast to the very regular organisation of this terminal structure in wild-type mice. In each observed specimen, the sphincter was ill-formed and this alteration allowed us to identify mutants animals on histological sections independently from genotyping.

Hoxd-13-deficient animals showed a similar, though stronger, rectum defect (Fig. 4). As for *Hoxd-12*-deficient animals, the longitudinal muscle layer was abnormally thin (Fig. 4A) and, sometimes, locally absent (Fig. 4E, right side). The usual thickening of this muscle layer in the terminal part of the rectum (Fig. 4C) was never clearly detected in these mutant mice (Fig. 4D-F). This severe defect was observed from the very beginning of gut muscle differentiation, as newborn animals already exhibited some histological alterations (data not shown). 14 days after birth, *Hoxd-13* mutant animals showed the same defect as in adults (Fig. 4E), indicating that the cause was likely a morphogenetic problem rather than a late degenerescence. At this age, while the two smooth muscle layers were already well apparent in wild-type littermates, mutant animals had disrupted muscle blocks (Fig. 4E) which did not reach the distal end of the circular layer, thus preventing formation of a sphincter. In some cases, the longitudinal layer was spectacularly absent (Fig. 4E). In other *Hoxd-13*-deficient animals, the terminal part of the smooth muscle layer turned towards the external sides leading to a kind of 'internally prolapsed' muscle (Fig. 4A,D).

Upon dissection, the anus of mutant animals appeared abnormally opened and relaxed, with a funnel-like shape, probably due to insufficient muscular tonus in the sphincter. This was also visible on histological sections, where the muscle masses of the circular layer adopted an open, evaginated position (Fig. 4D,F,G). In such animals, the internal anal sphincter was virtually absent and this situation eventually led to an anal prolapse (Fig. 4B). More than 30 percent of the males after the age of 18 months developed this severe pathology (Fig. 4B,G). Interestingly, this was rarely observed in females, even though histology revealed identical alterations of the sphincter.

Anal prolapses can result from a variety of causes and may occur spontaneously in most animal colonies, for example due to the presence of intestinal parasites. While we sporadically observed such cases, the observed frequency of prolapse amongst the *Hoxd-13* mutant mice, as well as the quasi absence of such manifestations in mice from other genotypes, makes an epigenetic etiology very unlikely. In addition, histological sections of the prolapsed material revealed that the external (longitudinal) smooth muscle layer was absent (Fig. 4G,H). In contrast, histological analyses of an anal prolapse that occurred in an unrelated mouse showed the presence of normal muscle layers (not shown). In the case of *Hoxd-13*, heterozygous animals already showed a tendency for disorganization of the sphincter and, in a single case, a prolapse was seen. As the *Hoxd-12* mutation was produced more recently, we cannot exclude that anal prolapses will appear in older animals. Using different types of colorations (for enterocytes and goblet cells),

we did not detect any alterations in the rectal epithelium of *Hoxd-13* mutant mice, even though this gene was strongly expressed there (Fig. 4F). In summary, the *Hoxd-13* phenotype was comparable, though stronger, to that of *Hoxd-12*.

Function of *Hoxd-12* in digit morphogenesis

Hoxd-12 mutant animals were analysed for their limb skeletal patterns. Surprisingly, no obvious defect was scored. However, upon direct measurement of the length of the skeletal elements, homozygous animals exhibited a minor reduction of the second phalange (P2) of digit V (Fig. 5A). As such a reduction of P2 was reminiscent of the *Hoxd-13* phenotype, and since the two genes are co-expressed in digits, we produced *Hoxd-12*/*Hoxd-13* *trans*-heterozygous animals. *Trans*-heterozygous intercross generated progeny that had segregated the various alleles so that phenotypes could be compared between littermates. Animals homozygous for the *Hoxd-13* mutation showed a strong digit phenotype, as previously reported (Dollé et al., 1993). *Hoxd-13* heterozygous mice had no significant defects except that about half of them had a sign of postminimus. In *Hoxd-12*/*Hoxd-13* *trans*-heterozygote animals, reduction of digits V and II was readily observed in forelimbs (Fig. 5), together with an occurrence of postminimus in 80% of the animals (not shown). The reduction in digit size was most prominent in the second phalanges, as expected for a *Hoxd-13*-derived phenotype, confirming a recent study from Davis and Capecchi (1996). This result indicated that *Hoxd-12* can have a genuine function during limb development. However, this function is largely masked in the presence of a full complement of *Hoxd-13* product.

DISCUSSION

We have examined the function of the *Hoxd-12* gene by targeted mutagenesis. Introduction of the *lacZ* gene in the *Hoxd-12* reading frame allowed us to reexamine its expression pattern during development. While only a minor phenotype was detected in the skeleton, a fully penetrant malformation appeared in the anal sphincter. A related defect was uncovered in mice mutant for the 5′ neighbouring gene and most posterior one of the *HoxD* complex, *Hoxd-13*.

Anus horribilis

In several instances, *Hox* genes have been reported to be expressed during gut development (e.g. Dollé et al., 1991). More recently, comparative analyses have suggested that they may contribute to the establishment of morphological transitions in the intestine through the colinear distribution of their products (Yokouchi et al., 1995; Roberts et al., 1995), similarly to segmented mesodermal derivatives such as vertebrae. In such a view, the most posterior *Hox* genes of the *HoxD* complex were expected to be involved in the functional organization of the most distal hindgut structure, i.e. the anal sphincter. We show here that both *Hoxd-12* and *Hoxd-13* are required for proper morphogenesis of this terminal structure.

The rectal musculosa is characterized by a marked thickening of the external layer, while the internal layer remains more or less of the same width. At the distal end, however, a conspicuous thickening of the internal layer forms the internal anal sphincter, at the base of which it comes into contact with the
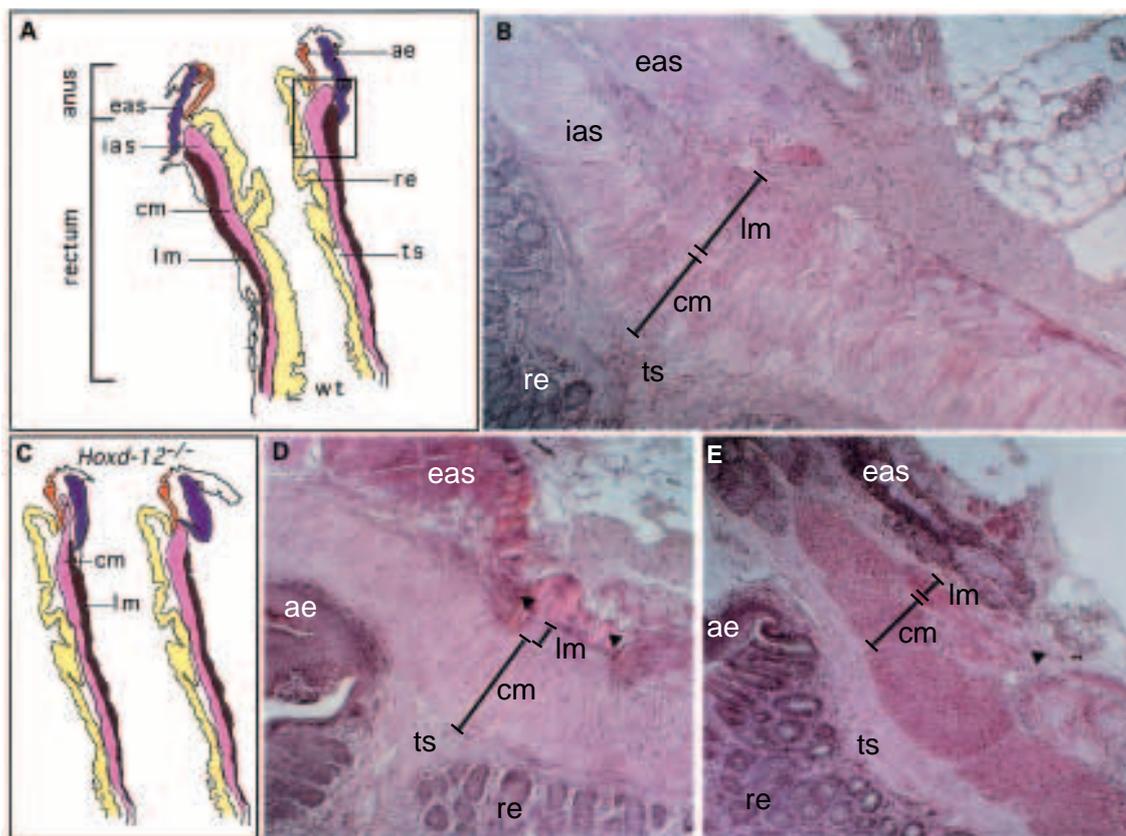
Fig. 3. Anorectal defects in *Hoxd-12* mutant mice. (A) Scheme of a wild-type rectum. Both muscle layers are thickened at the end, as shown in B, on a histological section corresponding to the rectangle in A. (B) Histological section of a 10 week-old wild-type animal showing a proper internal anal sphincter. (C) Schemes of two half-rectum observed amongst *Hoxd-12*-deficient animals. In the right, a thin circular muscle extends beyond the anorectal transition, as shown in D and E, which are two examples of *Hoxd-12* mutant animals seen as under B. In the left, the internal muscle appears more or less normal, but the longitudinal muscle is thin and disorganized, as observed in all *Hoxd-12*-deficient animals. (D,E) Sections of the anorectal transition in *Hoxd-12* mutant animals. In both cases, the longitudinal muscles are severely disrupted (arrowheads), occasionally invaded by striated muscles from the external anal sphincter (D). In E, an important reduction in the thickness of the circular muscle layer is visible. ae, anal epithelium; cm, circular muscle; eas, external anal sphincter; ias, internal anal sphincter; lm, longitudinal muscle; re, rectal epithelium; ts, tunica submucosa. Slides were stained with hematoxylin and eosin.

external layer. In both *Hoxd-12* and *Hoxd-13* mutant mice, the thickening of the external layer failed to occur. As a consequence, the muscle layers became disorganized and no efficient sphincter formed. This absence of thickening led to a structural aspect of the rectal muscle layers which resembled more anterior parts of the large intestine, the sigmoid for example, where no thick external layer is present. In this view, it seems that both *Hoxd-12* and *Hoxd-13* are required to build up the terminal smooth muscle structure, on the top of a 'hindgut-like' generic muscle distribution. The function of both genes may be to increase the thickness of smooth muscles, leading to the formation of a sphincter. Consequently, the absence of this 'terminal' signal results in the distal extension of the thin muscle layers. No alteration was observed in more anterior parts of the intestine.

In aged *Hoxd-13* mutant males, the disorganization of the internal anal sphincter led to an evagination of both the anus and the terminal part of the rectum. While the cause(s) of such anal prolapses are difficult to establish, it likely resulted from an overall weakness in the muscular tonus in the anorectal region. This weakness could result from both the disorganization of the muscle mass and the consequent problem in the

innervation by the enteric system controlling the constipation reflex. In the prolapsed material, the rectal epithelium appeared hyperplastic and somewhat disorganized. However, this was probably a secondary effect as both the number and histological aspect of epithelial cells in the rectum, in similarly aged but not yet prolapsed mutants, was apparently normal. While females showed the same alterations of the internal sphincter, they did not develop anal prolapses, indicating that, for some as yet unknown reasons, males are more susceptible than females to this alteration.

Hoxd-12 function in digits

β-gal staining of embryos and fetuses gave a precise account of the *Hoxd-12* expression pattern during limb development. The dynamics of the transcript domain was very similar to that observed for both *Hoxd-13* (Dollé et al., 1993) and *Hoxd-11* (Sordino et al., 1995). An initial posterior (post-axial) domain extended distally and anteriorly in subsequent developmental stages, to finally cover most of the distal autopodium. The similarity between the three *Hox* digit domains (*d-11* to *d-13*), likely due to the use of one and the same control mechanism (van der Hoeven et al., 1996b), suggested that the three genes
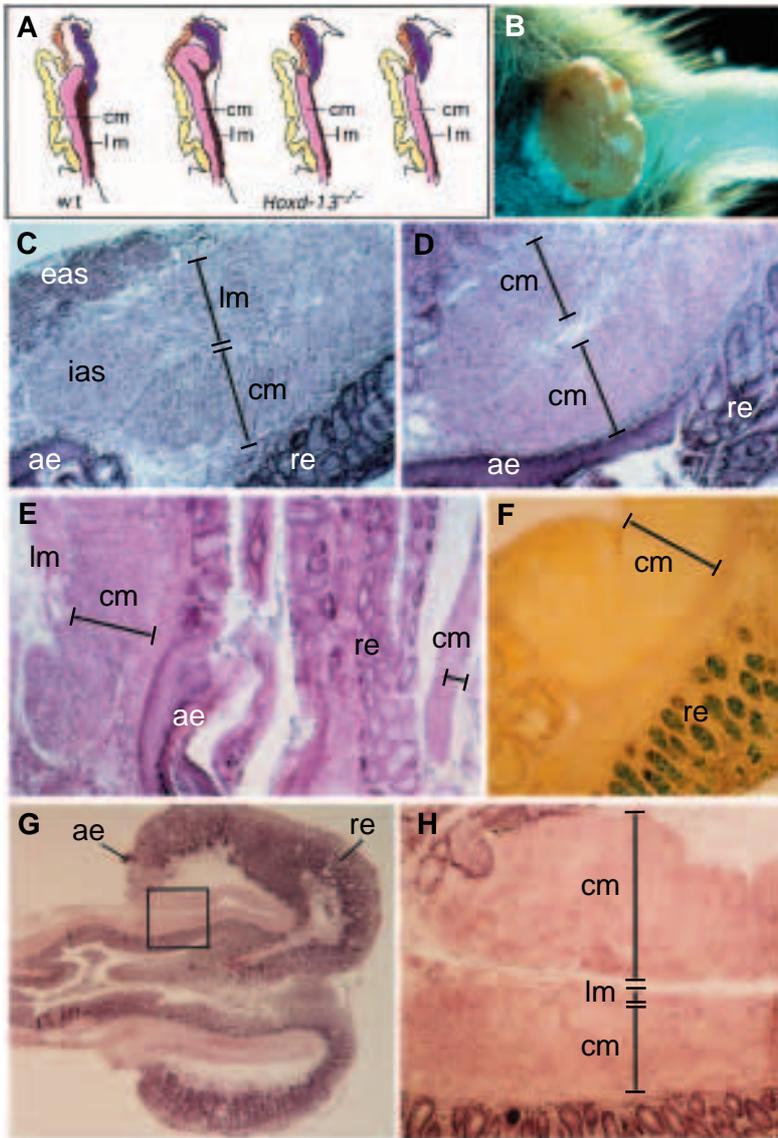
Fig. 4. Anorectal defects in *Hox-13* mutants mice. (A) Schemes of four half-rectums, one wild type on the left, and three mutants on the right, to illustrate some of the observed alterations. The second to the left shows a circular muscle layer that did not stop at the proper level and turned outside at the end (also in D). In the second to the right, the inner muscle stopped rather anteriorly, without thickening, while the one on the right almost totally lacked the outer muscle layer. (B) Cauliflower-like outlook of a prolapsed *Hoxd-13* mutant anus. (C-H) Histological sections of control (C) and mutant animals in the anorectal region. (C) 3 week-old control animal. (D) *Hoxd-13*-deficient littermate which shows an extension of the circular muscle turning outside. Longitudinal muscles are virtually absent. (E) 2-week-old *Hoxd-13*-deficient animal with a disorganized sphincter, in the left, and an absence of longitudinal muscle, in the right. (F) Van Guison staining of a *Hoxd-13* mutant rectum shows the start of an evagination leading to an anal prolapse. The number and appearance of goblet cells are normal. (G) Section through a prolapsed anus. The magnification seen under H, corresponding to the rectangle in G, shows that almost no trace of longitudinal muscle is observed in the prolapsed portion (upper part). Stainings were with hematoxylin and eosin, except for F, with van Guison solution. Magnifications are the same throughout, except for G which is 10 times lower. Symbols are as for Fig. 3.
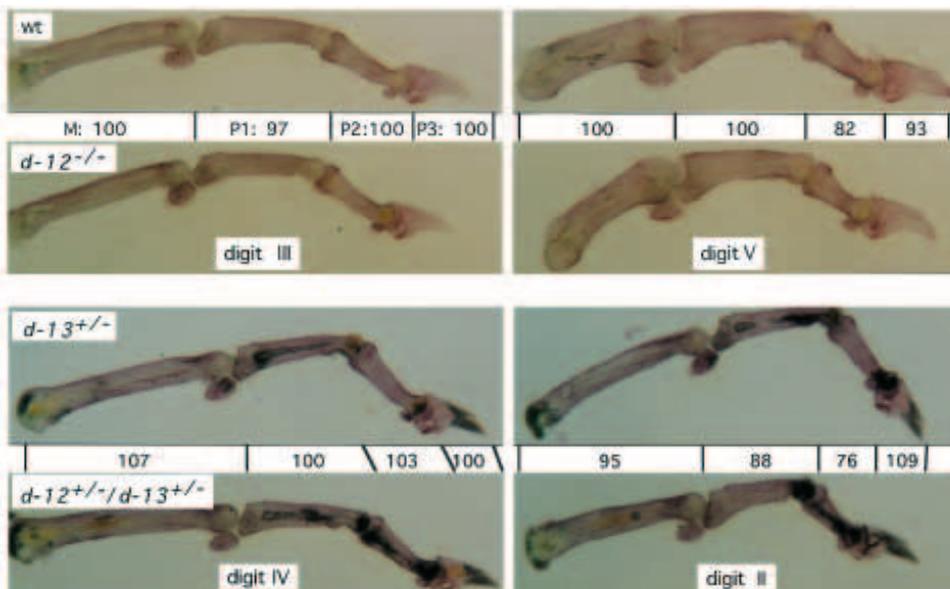

Fig. 5. Digit phenotype associated with the *Hoxd-12* mutation. Wild-type and *Hoxd-12*-deficient littermates are compared in the upper two lines. Digit III was taken as a control. Digit V showed a slight reduction in the length of the second phalange (82%). The lower panels show comparison between *Hoxd-13*^{+/−} and *Hoxd-12*^{+/−}/d-13^{+/−} *trans*-heterozygous littermates. Digit IV is shown as a control. Digit II is clearly shorter, the reduction being maximal in P2 (76%). These numbers are comparable to those reported by Davis and Capecchi (1996).

may collaborate in digit morphogenesis. However, in contrast to *Hoxd-13*, but similarly to *Hoxd-11*, *Hoxd-12* mutant animals showed only minor alterations in the skeleton of digits. This may have different causes. Firstly, *Hoxd-12* may not exert a function in digits and may be restricted to more proximal regions (Davis et al., 1995; Davis and Capecchi, 1996). Alternatively, *Hoxd-12* may function in digits but in a way somehow redundant with *Hoxd-13* so that the latter gene could 'rescue' or compensate for the absence of *Hoxd-12* function. Finally, *Hoxd-12* may have a function in digits only in absence of *Hoxd-13* product, consistent with a strict interpretation of posterior prevalence (Duboule and Morata, 1994). In such a view, *Hoxd-12* would be dispensable in digits of normal animals, but may exhibit functional capacities whenever the level of the *Hoxd-13* (or *Hoxa-13*, or both) gene product is reduced.

Trans-heterozygous *Hoxd-12*/*Hoxd-13* animals did show some alterations in the digits, thus ruling out the first possibility. However, this result did not allow us to discriminate between the second and third alternatives. Therefore, in contrast to the original proposal of Davis et al. (1995), *Hoxd-12* does have an apparent function in digits which became visible when one dose of *Hoxd-13* was removed. This confirmed that *AbdB*-related genes functionally cooperate during the morphogenesis of digits (Sordino et al., 1995; Davis and Capecchi, 1996). These results also suggested that *Hox* gene loss-of-function experiments could lead to the functional 'activation' of other *Hox* genes in some areas where their functions would otherwise be suppressed. We believe that this point is important to consider, as apparent loss-of-function phenotypes may be partly contributed by artificial gain-of-function of other *Hox* genes. For example, it is possible that part of the described *Hoxd-13* phenotype (Dollé et al., 1993) may result from a functional (not transcriptional) gain-of-function of the otherwise suppressed *Hoxd-12* function. The prevalence (or stronger functional impact) of *Hoxd-13* over *Hoxd-12* in limbs is also evident in the gut, where the *Hoxd-13$^{-/-}$* phenotype was clearly more severe than that of *Hoxd-12*. Inactivations of several such genes at once will be informative in this respect.

Evolutionary aspects

Based on comparison of expression patterns, it was proposed that vertebrate *Hox* genes exert their functions within a morphological window that extends from the anterior to the posterior parts of the digestive tract, i.e. from the level of the foregut pocket, in the branchial region, to the proctodeal area (van der Hoeven et al., 1996a). One argument in support of this hypothesis is the systematic expression, in various metazoans, of the most posterior *Hox* genes (*AbdB*-related) in the region of the future anus or in the terminal part of the hindgut (with the exception of shrimps, where transcripts of the *AbdB* cognate were not reported in the analia; Averof and Akam, 1995). This suggested that the terminal *Hox* genes are involved in the morphogenesis of the distal part of the digestive system. The results presented here support this proposal, as they show that both *Hoxd-12* and *Hoxd-13* are required for the proper making of the anal sphincter. The question remains as to how ancestral this 'gut' function is? It is conceivable that one of the most ancient signs of a longitudinal axial structure in a metazoan body plan was linked to the design of a genuine ali-

mentary tract, i.e. a region of transit demarcated, at both extremities, by specialized structures. It is therefore possible that colinearity in *Hox* gene expression co-evolved with the emergence of such a structure. In this view, the digestive tract is considered as the original site of deployment of *Hox* genes, this genetic system being recruited subsequently in neural and mesodermal derivatives.

Although such evolutionary arguments are, by definition, difficult to substantiate, two pieces of indirect evidence deserve to be discussed in this context: firstly, the search for terminal *Hox* genes in 'primitive' animals and secondly, the expression patterns of posterior *Hox* genes, as viewed from an phylogenetic perspective. In recent years, *Hox* genes have been extensively looked for in essentially all kind of animals, from the diblastic hydra (e.g. Schummer et al., 1992) to planarians (Balavoine and Telford, 1995), nematodes (Kenyon and Wang, 1991), crustaceans (Averof and Akam, 1995) and a variety of other animals. In all cases, genes have been isolated belonging to the major (ancestral) groups of paralogy. As far as *AbdB*-related genes are concerned, however, the situation is less clear since this class of gene has not yet been found, either in hydra, or in planarians. One cannot exclude that this is due to important divergences in DNA sequences (such genes may be soon isolated), but it is intriguing that neither cnidarians, nor planarians have a bipolarity in their 'alimentary tracts' which, in both cases, works through a unique syphoon or pharynx. It thus appears conceivable that an *AbdB* ancestral gene appeared together with the evolution of a 'posterior' opening in the digestive system.

The second, more complex, argument relies on the mechanistic bases of temporal colinearity. *Hox* genes are activated following a temporal sequence that reflects their positions along the complex (Duboule, 1994). Experimental *Hox* gene transpositions at an ectopic position within the *HoxD* complex have revealed that early active genes are repressed until late stages, when placed at a more posterior position (van der Hoeven et al., 1996b). This latter set of experiments supports a view in which posterior (late) genes are 'closed for business' early on, for instance through a mechanism relying on a high order regulatory control such as a progressive transition in the structure of chromatin. As a consequence, vertebrate *Hox* genes may have been refractory to an evolutionary recruitment for additional functions earlier on during development, as this would have implied a premature opening of the complex, a situation probably detrimental to the animal. Therefore, the earliest sign of expression of a given *Hox* gene (in animals where temporal colinearity applies) may indicate its most ancestral functionality. In the case of *Hoxd-12*, transcripts are first visible in the proctodeal region. We do not wish to give a strict Haeckelian interpretation to this reasoning, i.e. to imply that the order of appearance of transcript domains during development would reflect the phylogeny of the functions, since, for instance, some functions may have been subsequently lost. Nevertheless, the initial site of expression during ontogeny may well represent the ancestral function, for it is unlikely that the function for which the system of colinear activation would have originally evolved (whatever it is) be lost in subsequent evolutionary steps.

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