Development 124, 4523-4536 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV2210

Hoxd-12 differentially affects preaxial and postaxial chondrogenic branches in the limb and regulates *Sonic hedgehog* in a positive feedback loop

Vladimir Knezevic[1,*], Ron De Santo[1,*], Klaus Schughart[3,†], Ulrike Huffstadt[3], Chin Chiang[2], Kathleen A. Mahon[2,‡] and Susan Mackem[1,§]

[1]Laboratory of Pathology, NCI and [2]Laboratory of Mammalian Genes and Development, NICHD, National Institutes of Health, Bethesda, MD 20892 USA
[3]GSF-National Research Center for Environment and Health, Institute of Mammalian Genetics, Neuherberg, Ingolstadter Landstrasse 1, 85764 Neuherberg, Germany
*Both authors contributed equally to this work
†Present address: Transgene S.A., 11 rue de Molsheim, F-67082, Strasbourg, France
‡Present address: Department of Cell Biology, T721, Baylor College of Medicine, 1 Baylor Plaza, Houston, Tx 77030, USA
§Author for correspondence: (e-mail: smack@helix.nih.gov)

SUMMARY

Several 5′ members of the *Hoxd* cluster are expressed in nested posterior-distal domains of the limb bud suggesting a role in regulating anteroposterior pattern of skeletal elements. While loss-of-function mutants have demonstrated a regulatory role for these genes in the developing limb, extensive functional overlaps between various different *Hox* genes has hampered elucidation of the roles played by individual members. In particular, the function of *Hoxd-12* in the limb remains obscure. Using a gain-of-function approach, we find that *Hoxd-12* misexpression in transgenic mice produces apparent transformations of anterior digits to posterior morphology and digit duplications, while associated tibial hemimelia and other changes indicate that formation/growth of certain skeletal elements is selectively inhibited. If the digital arch represents an anterior bending of the main limb axis, then the results are all reconcilable with a model in which *Hoxd-12* promotes formation of postaxial chondrogenic condensations branching from this main axis (including the anteriormost digit) and selectively antagonizes formation of 'true' preaxial condensations that branch from this main axis (such as the tibia). *Hoxd-12* misexpression can also induce ectopic *Sonic hedgehog* (*Shh*) expression, resulting in mirror-image polydactyly in the limb. Misexpression of *Hoxd-12* in other lateral plate derivatives (sternum, pelvis) likewise phenocopies several luxoid/luxate class mouse mutants that all share ectopic *Shh* signalling. This suggests that feedback activation of *Shh* expression may be a major function of *Hoxd-12*. *Hoxd-12* can bind to and transactivate the *Shh* promoter in vitro. Furthermore, expression of either exogenous *Hoxd-11* or *Hoxd-12* in cultured limb bud cells, together with FGF, induces expression of the endogenous *Shh* gene. Together these results suggest that certain 5′ *Hoxd* genes directly amplify the posterior *Shh* polarizing signal in a reinforcing positive feedback loop during limb bud outgrowth.

Key words: *Hoxd-12*, limb development, *Sonic hedgehog*, luxate mutants, luxoid mutants, *extra-toes*, chondrogenesis

INTRODUCTION

Vertebrate limb development has served as an excellent model for unravelling mechanisms of pattern formation during embryogenesis. Recent insights have been gained as to the molecular nature of some of the secreted signals that regulate growth and pattern along the anterior-posterior (AP), dorsoventral (DV) and proximodistal (PD) limb axes. Limb outgrowth occurs in a proximal-to-distal order, with branching and segmentation of more proximal cartilaginous anlagen to produce more complex distal structures, and is regulated by FGF signals from the overlying specialized ridge of ectoderm running along the DV edge of the bud, the apical ectodermal ridge or AER (reviewed by Hinchliffe and Johnson, 1980; and Cohn and Tickle, 1996). A functionally defined zone of polarizing activity (ZPA) located in the posterior limb bud mesenchyme regulates AP polarity (reviewed by Hinchliffe and Johnson, 1980), which is mediated by *Sonic hedgehog* (*Shh*). Anterior misexpression of *Shh* produces polarity reversals and consequent mirror-image digit duplications (Riddle et al., 1993; Chang et al., 1994). DV polarity is regulated by the limb ectoderm (reviewed by Hinchliffe and Johnson, 1980). Cross regulation of secreted signals along different axes coordinates growth and patterning (reviewed by Cohn and Tickle, 1996). *Fgf4* expression is initially induced in posterior AER by *Shh* and subsequently FGF4 forms part of a positive feedback loop that stimulates *Shh* expression in posterior mesenchyme. Likewise, *Wnt7a* (a dorsal polarizing signal) together with FGF4, stimulates *Shh* expression.

While some of the potential downstream nuclear mediators of these signalling events have been identified, particularly multiple members of the homeodomain class of transcriptional

regulators, how these genes function and interact to regulate limb development is less well understood, although clearly differential growth regulation is an important feature (see for example, Dolle et al., 1993; Yokouchi et al., 1995; Goff and Tabin, 1997). Along the AP axis, 5′ members of the *Hoxd* cluster are expressed in overlapping, nested, posterior and distal zones of the limb bud colinearly with their chromosomal order, and ZPA grafts induce duplicated *Hoxd* expression domains that correlate with subsequent skeletal duplications (reviewed by Izpisua-Belmonte and Duboule, 1992). By analogy with the key roles of homeobox genes in specification of segmental identity in *Drosophila* (see review by Krumlauf, 1994), these results suggested a *Hox* code in which AP positional identity would be specified by the combinatorial expression of different *Hoxd* genes along the limb AP axis. Proximodistal identity might be similarly regulated by the clustered 5′*Hoxa* genes, which are expressed in nested domains along the limb PD axis (Yokouchi et al., 1991). In fact, for specification of axial mesoderm in vertebrates, there is support for the operation of a 'Hox code' (reviewed by Krumlauf, 1994; see also Duboule, 1995). However, such simple models break down in the limb. While ectopic *Hoxd-11* expression in chick embryo limb buds results in 'posterior' transformations (Morgan et al., 1992) and *Hoxa-13* misexpression yields apparent 'distal' transformations (Yokouchi et al., 1995) that are compatible with a code, similar experiments with *Hoxd-13* do not produce an analogous outcome (Goff and Tabin, 1997). Most notably, targeted disruption of several *Hoxd* and *Hoxa* genes has generally resulted in complex and sometimes subtle limb phenotypes affecting multiple skeletal elements that are not readily reconciled with *Hox* code models (eg. Dolle et al., 1993; Small and Potter, 1993; Davis and Capecchi, 1994, 1996; Favier et al., 1995; Fromental-Ramain et al., 1996; Kondo et al., 1996). These analyses have also revealed a high degree of functional redundancy and interaction between both linked *Hox* genes and paralogous (homologous) and non-paralogous genes in different *Hox* clusters (Davis and Capecchi, 1996; Davis et al., 1995; Favier et al., 1996; Fromental-Ramain et al., 1996; Kondo et al., 1996; Zakany and Duboule, 1996). In the case of *Hoxd-12* in particular, loss-of-function results in minimal limb defects, and hints at potential function only begin to be revealed in the context of compound mutants with other *Hox* genes (Davis and Cappechi, 1996; Kondo et al., 1996). In fact, the high level of functional overlap and interaction between various *Hox* genes in the limb has raised speculation that this serves to expand limb size and morphology repertoire in a population, increasing plasticity and adaptability both during development and evolution (Duboule, 1994).

To gain further insight into the role of *Hoxd-12* in limb development, we have used a well-characterized mouse *Hoxb-6* promoter (Schughart et al., 1991; Eid et al., 1993; Becker et al., 1996) to selectively drive *Hoxd-12* transgene expression in the developing limb bud and lateral plate mesoderm of mouse embryos. Depending on location relative to the main limb axis, *Hoxd-12* misexpression can either promote or inhibit formation and proliferation of chondrogenic condensations that give rise to limb skeletal elements. Furthermore, *Hoxd-12* misexpression can activate ectopic *Shh* expression and produce mirror-image digit duplications. We propose that *Hoxd-12* is normally part of a positive feedback loop within the posterior mesenchyme that reinforces polarizing signals during limb outgrowth. Such a role for *Hoxd-12* in regulating *Shh* is further supported by the finding that misexpression of *Hoxd-12* in several lateral plate derivatives phenocopies luxoid/luxate mouse mutants shown to have ectopic *Shh* signalling (see Chan et al., 1995; Masuya et al., 1995, 1997). In vitro binding and activation experiments suggest that *Shh* is a direct target of *Hoxd-12*. *Hoxd-12* or *Hoxd-11* (which shares some similarities with *Hoxd-12* in gain-of-function phenotype) will activate endogenous *Shh* expression in limb mesenchymal cells in the presence of FGF, suggesting that certain 5′*Hoxd* genes may participate together in a positive feedback loop, in conjunction with FGF signals from the AER, to amplify the *Shh* signal in the posterior limb bud.

MATERIALS AND METHODS

Preparation and analysis of DNA and RNA

Routine isolation, cloning, labeling, blotting, PCR and sequencing procedures were performed using standard techniques (Sambrook et al., 1989). The nucleotide sequence of the mouse *Shh* *Eco*RI-*Xho*I promoter fragment has been deposited in GenBank (accession no. AF019387).

Construction of *Hoxd-12* transgene expression vector

Expression constructs containing the chick *Hoxd-12* coding sequence and its native 5′ untranslated region (5′UTR) translate very poorly in vivo (S. Mackem, unpublished observations). Therefore the 5′ UTR sequences were replaced with those from the RSV *src* 5′UTR, which supports efficient translation (Hughes et al., 1987). The coding sequence of *Hoxd-12* 5′ of the *Bgl*II site was replaced with a 23 bp *Nco*I-*Bgl*II oligonucleotide that converted the ATG to an *Nco*I site and changed the second amino acid from cysteine to glycine. This altered coding sequence, extending from *Nco*I to *Bam*HI in the 3′UTR, was cloned into the *Nco*I and *Bam*HI sites of the Cla12Nco vector containing the *src* 5′UTR (Hughes et al., 1987). SV40 virus late splice signals and poly(A) addition signals were added to this construct by PCR amplification. For the intron, a mini-intron containing late 16S donor and acceptor sites (including nucleotides 486-555 fused to 1411-1497 of the SV40 genome, in pOBCAT4 provided by C. C. Baker) was amplified flanked by *Bgl*II and *Sal*I sites and introduced into the *Bam*HI and *Sal*I sites of *Hoxd-12*/Cla12Nco. The SV40 late poly(A) addition signal (from nucleotides 2545-2765 of the SV40 genome) was amplified flanked by *Sal*I and *Hin*dIII sites and introduced into the same sites of the *Hoxd-12*/Cla12Nco construct. The final construct was inserted as a *Cla*I fragment downstream of a 3.6 kbp *Hoxb-6* promoter (Schughart et al., 1991), and the transgene excised from plasmid sequences with *Bss*HII for zygote injections.

Generation and analysis of transgenic mice

The transgene was injected into either FVB/N or CD1 zygotes, as described by Hogan et al. (1994). Embryos were transferred to foster mothers and recovered after birth for establishing lines or immediate analysis, or at various stages of intrauterine development for analysis. Transgenicity of embryos was determined by DNA extraction from viscera (or heads for E10-13) and Southern analysis using either a 600 bp chick *Hoxd-12* probe (as below) or the SV40 250 bp poly(A) signal fragment described above. Whole-mount in situ hybridizations and preparation of riboprobes was essentially as described by Conlon and Rossant (1992). For transgene detection, a 600 bp *Sac*I probe from chick *Hoxd-12* that does not cross hybridize with the murine *Hoxd-12* was used (Mackem and Mahon, 1991). A mouse *Sonic hedgehog* probe was provided by A. McMahon and mouse *Hoxd-12* probe by D. Duboule. Skeletons were visualized by staining with either 0.1% Alcian green in acid-alcohol followed by alcohol destaining and

clearing in methyl salicylate for some E13-E15 embryos; or with Alcian blue and alizarin red followed by alkaline hydrolysis and glycerol clearing for most embryos E14.5 and older (as described by Kessel and Gruss, 1991). Long-bone lengths were measured by micrometer and compared to nontransgenic siblings.

Exonuclease protection assay

The 1 kbp *Shh Eco*RI-*Xho*I promoter fragment was ^{32}P-5′-end-labeled and incubated, in the presence of a 200-fold excess of nonspecific carrier ΦX DNA, with *Hoxd-12*/glutathione-s-transferase (GST) fusion protein (GEX-15, R. Hutson, S. Aguanno and S. Mackem, unpublished data) bound to glutathione sepharose. Bound beads were incubated and washed with PBS/0.1% Tween 20/5 mM DTT, digested with T7 Gene 6 exonuclease (50 units, USB) for 5 minutes using manufacturer's conditions, and DNA released by phenol extraction followed by ethanol precipitation and analysis on 6% polyacrylamide-urea gels.

Transfection and retroviral infection of cultured limb bud cells

Early (72 hours incubated) chick limb buds were dissected into PBS and divided into anterior and posterior halves or used whole, and were trypsinized (0.25% trypsin/PBS) and washed in DMEM with 10% FCS. For transfections, the 700 bp *Pst*I-*Xho*I mouse *Shh* promoter region was cloned from Bluescript as a *Pst*I-*Kpn*I fragment into a promoterless chloramphenicol acetyltransferase (CAT) reporter. Full-length *Hoxd-12* with the sarc 5′UTR from Cla12Nco (see above) was cloned into the expression vector pSG5 (Stratagene). A *Hoxd-12* construct containing the HSV-1 VP16 activation domain was generated by PCR amplification of sequences encoding VP16 amino acids 398-479 (provided by T. Kristie) and cloning in frame into the amino terminal end of full-length *Hoxd-12* in Cla12Nco, just 3′ of the initiator ATG, followed by transfer into pSG5. Cells were transfected by electroporation (BioRad, 0.3 kV, 350 μF, 5×10^6 cells in DMEM with 10%FCS) with from 5 to 10 μg of reporter and expression vector, plated onto 35 mm dishes and cultured for 36 to 48 hours at 38.5°C in DMEM with 10% FCS. Extracts were prepared and CAT assays performed using standard protocols (Sambrook et al., 1989). *Hoxd-12*-expressing retrovirus was generated by inserting the *Hoxd-12*/Cla12Nco construct into RCAS BP and transfecting chick embryo fibroblasts (Hughes et al., 1987), a *Hoxd-11* RCAS BP construct was provided by C. Tabin and RCAS BP was used as a negative control. For infections, cells were plated at a density of 1.5×10^6 in 12-well plates and exposed to viral supernatants (about 10-fold concentrated final) for 2-3 hours, after which fresh media containing 300 ng/ml FGF4 (gift from Genetics Institute, Inc.) and 200 ng/ml heparin sulfate were added and incubation continued for 48 hours. Cells were harvested in RNAzol and northern analysis performed on 1.2% agarose-formaldehyde gels using a chick *Shh* probe (provided by R. Riddle) and a chick β-actin probe.

RESULTS

Targeting *Hoxd-12* misexpression to limb bud and lateral plate in transgenic mice

To misexpress *Hoxd-12* selectively, a 3.6 kbp *Hoxb-6* promoter was used to drive expression of the transgenic construct (diagrammed in Fig. 1A). This promoter, previously characterized extensively in transgenic mice (Schughart et al., 1991; Eid et al., 1993; Becker et al., 1996), directs expression specifically to the posterior lateral plate mesoderm including the limb buds.

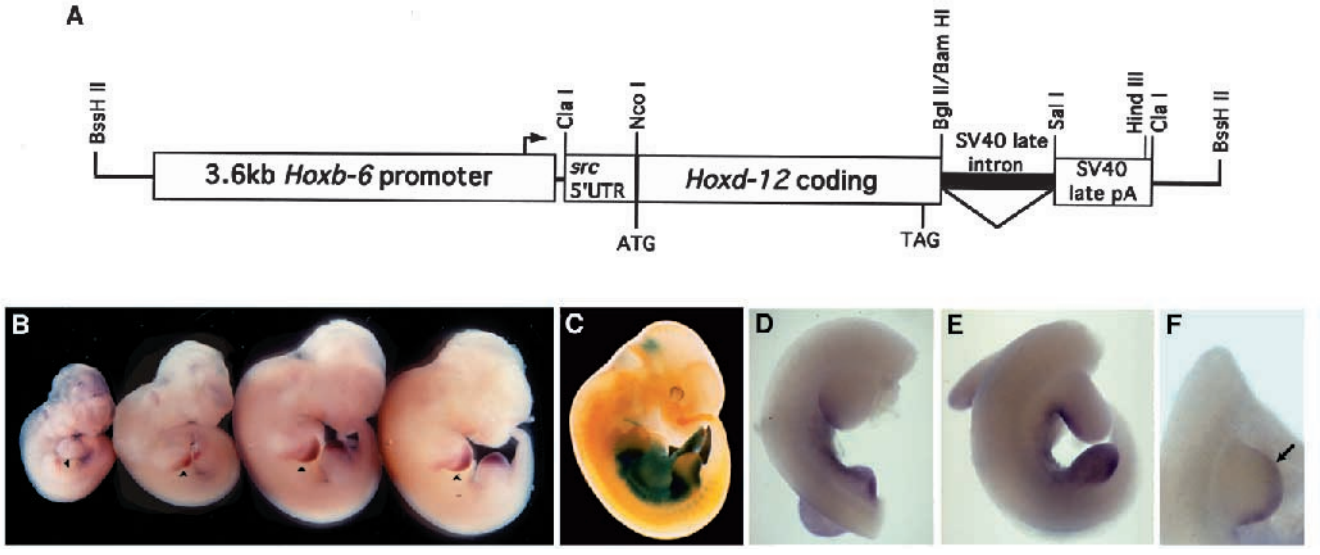

Fig. 1. Expression of endogenous and transgenic *Hoxd-12*. (A) Diagram of *Hoxd-12* expression construct used to generate transgenic mice. Note that the *Hoxd-12* 5′ untranslated region (5′UTR) was replaced by 40 bp of RSV *src* 5′UTR and initiator ATG (materials and methods) to obtain efficient translation of *Hoxd-12* message in vivo. (B) Expression of the endogenous *Hoxd-12* message at E10, E11, E11.5 and E12 (left to right), as visualized by whole-mount in situ hybridization. Normal expression is initially restricted to the posterior mesenchyme in each limb bud and later becomes more distally restricted (arrow heads indicate forelimb signals). (C) Expression of *lacZ* protein under the control of the 3.6 kbp *Hoxb-6* promoter in transgenic mice at E12, visualized with Xgal. Note high level expression in the lateral plate region between limb buds, in posterior forelimb bud and in dual anterior and posterior domains of hindlimb bud. At earlier stages, *LacZ* expression was observed throughout the entire hindlimb bud (Schughart et al., 1991). Expression in the midbrain region, as seen in this animal, was variable. (D-F) Expression of *Hoxd-12* transgene message in E10.5-11 transgenic embryos from intermating of line 2917. Expression is visualized by whole-mount in situ hybridization in the lateral plate region, the posterior forelimb bud and throughout the hindlimb bud (D,E). Note that forelimb expression can extend into the anterior region of the distal limb bud (F, arrow). Heads of embryos did not show significant hybridization and were removed.

In the limb buds, the *Hoxb-6* promoter directs expression selectively to the posterior mesenchyme of the forelimb bud and throughout the mesenchyme of the hindlimb bud. The hindlimb expression later (E12-E12.5) splits into dual anterior and posterior domains (see Fig. 1C). A chick *Hoxd-12* transgene was used since the chick and mouse proteins are very similar both in structure and expression, but the mouse coding sequence contains an ambiguity with respect to splicing within its coding region (Mackem and Mahon, 1991; Izpisua-Belmonte et al., 1991), and because use of the chick gene allowed transgene expression to be easily distinguished from endogenous mouse *Hoxd-12* . Since *Hoxd-12* expression is normally restricted to the posterior and later to the posterior-distal limb bud mesenchyme (eg. Fig. 1B), ectopic expression of the *Hoxd-12* transgene was expected in the anterior hindlimb and in the lateral plate mesoderm.

In initial experiments, two stillborn primary transgenics displayed a phenotype (described below), while several live born primary transgenics appeared entirely normal. Liveborn founders produced transgenic embryos with no detectable expression of the transgene, with a single exception (data not shown, discussed below). Therefore, assuming that high level transgene expression may be incompatible with postnatal survival, several additional primary transgenic embryos ranging from E16.5 up to newborn were analyzed. Among the liveborn founders, a single line (#2917) was identified with no phenotype but in which variable, weak *Hoxd-12* expression was detectable in the lateral plate mesoderm and limb buds of transgenic embryos. Mating of this line to generate homozygous embryos substantially increased transgene expression and produced about 20% of offspring with phenotypic changes that were all very similar to those seen in abnormal primary transgenic embryos (described below). Thus it is unlikely that the line 2917 phenotype is related to transgene integration site. Sixteen consecutive litters from E13.5 up to newborn were analyzed (summarized in Table 1). About 80% of the expected number of homozygotes displayed one or more features of a characteristic phenotype (Tables 1-3). As expected, a subset of embryos from line 2917 matings showed consistent, easily detectable transgene expression in the lateral plate mesoderm and limb buds (Fig. 1D-F). Notably, variability in expression level was observed even between paired limb buds of the same embryo (not shown), and extension of transgenic *Hoxd-12* expression to include the anterior edge of the distal forelimb bud was also seen (arrow in Fig. 1F). Occasional expression in the anterior distal forelimb has been previously observed in transgenic analyses of the *Hoxb-6* promoter (K. Schughart, unpublished observations). Consistent with observed transgene expression, phenotypic changes were often not bilaterally symmetric and sometimes involved the distal forelimb (anterior digits), as well as the hindlimb and certain other lateral plate derivatives. The same phenotypic variability (between limbs within one embryo and between different embryos) was observed among primary transgenic embryos. No abnormalities were seen in non-transgenic embryos.

Hoxd-12 misexpression causes posterior transformations and duplications of distal anterior skeletal elements and selective reductions of certain proximal elements in the limb

In both primary transgenics and in offspring of line 2917 matings, limb phenotypes generally involved only ‘anterior’ structures. Five out of six primary transgenics with a phenotype had abnormalities in either hindlimb and/or forelimb, and comparable hindlimb and/or forelimb abnormalities were seen in 26 out of 29 abnormal offspring from line 2917 matings (Table 2). In the hindlimb, the anterior autopod (hand/foot) was most frequently affected. Digital changes consisted of conversion of the anteriormost digit I (big toe) to a triphalangeal digit with longer metatarsal (similar to digit II or occasionally III in morphology) and/or anterior digit duplications (Fig. 2B,C,E,F; Table 2). Changes in the tarsal elements of the autopod were generally limited to the anterior tarsals in the distal row and again consisted of ‘posterior’ transformations of cuneiforme I to II or III, and/or duplications of cuneiforme I (Fig. 2I,J; Table 2). Fusions of the naviculare with cuneiforme I or II were also observed. The proximal row of tarsals (tibiale, talus, calcaneus) were either unaffected or mildly reduced in size. In the long bones of the hindlimb, abnormalities were seen in the tibia (anterior long bone), which was shortened to a variable degree (hemimelia, Fig. 2B,C; Table 2). Concomitant bowing of the fibula was interpreted as a secondary change related to failure of the tibia to elongate. The femur was relatively unaffected.

Table 1. Summary of offspring from transgenic line 2917 intercrosses

	Overall (total observations = 146)		Of expected homozygotes (total no. = 36.5)	
	No. abnormal	% abnormal	No. abnormal	% abnormal
Limb phenotype	26	18	26	71
Limb and/or sternal phenotype	29	20	29	80

Occasional, usually unilateral abnormalities in the anteriormost digits of the forelimb were also observed; again consisting of digit I (thumb) conversions to a triphalangeal digit with longer metacarpal and/or anterior digit duplications (Fig. 2G; Table 2). In a single case, an extra digit-like element arose from the posterior pisiform (Table 2, ks10). Forelimb changes were restricted to the digits and were never observed in more proximal elements (carpals, long bones), consistent with the distally restricted *Hoxd-12* transgene expression in the anterior forelimb (see Fig. 1F).

Abnormalities were generally not observed in posterior limb structures; hence overexpression of *Hoxd-12* in domains where endogenous expression normally occurs did not produce any apparent phenotype. The pelvis is traditionally considered part of the hindlimb and is derived entirely from lateral plate mesoderm (Chevalier, 1977). Four out of six abnormal primary transgenics and an additional 30% of abnormal embryos examined from line 2917 matings displayed abnormalities of the pelvis. The most commonly affected component was the pubic bone (in 7/13 embryos), which was reduced (incomplete or absent superior ramus, Fig. 3F arrows), or shortened so that the pubic symphysis remained widely open (Fig. 3E,F; Table 2). A proportionately small pelvis, probably due to decreased growth of both pubis and ischium, was also observed (in 5/13 embryos; see Fig. 3E; Table 2). In a single case, nearly complete agenesis of the pelvis was observed, with only a small ilium remaining (Fig. 3G). Interestingly, the pubic bone

Table 2. Summary of limb phenotypes of *Hoxb-6pro*/*Hoxd-12* transgenic mice

		Hindlimb			Forelimb	
Animal	Age	Digits	Tarsals	Tibia	Digits	Pelvic girdle
Primary transgenics:						
ks38	E16.5	NL	NL	NL	R: 1,2,2†,3,4,5	NL
kmA1	SB	R: 2*,2,3,4,5 L: 2*,2,3,4,5	R: 3,2,3,4, fus. L: 3,2,3,4, fus.	R: 25% NL L: absent	NL	pelvic agenesis (iliac remnant)
km34	SB	L: 1,2,2,3,4,5	NE	NL	NL	R: pubis incomplete
ks9	NB	NL	NE	NL	L: 1,2,2,3,4,5	short pelvis, open symphysis
ks10	NB	R: 1,1†,2,3,4,5	NL	R: 75% NL L: 75% NL	R: 1,2,3,4,5,5′ L: 2,2,3,4,5	short pelvis, open symphysis, acetabular region open
line 2917:						
618	E13	L: 1,1,2,3,4,5	NE	NL	NL	NE
793	E13.5	NL	NE	NL	L: 1,1,2,3,4,5	NE
794	E13.5	NL	NE	NL	R: 1,1,2,3,4,5	NL
959	E13.5	R: 3*,2,3,4,5 L: 3*,2,3,4,5	NE	NL	NL	NL
977	E13.5	L: 3*,2,3,4,5	NE	NL	NL	NL
980	E13.5	NL	NE	NL	L: 1,2,2,3,4,5	NL
982	E13.5	R: 3*,2,3,4,5	NE	NL	NL	NL
986	E13.5	NL	NE	NL	R: 2*,1,2,3,4,5	NL
733	E14	R: 1,1,2,3,4,5	NE	NL	NL	NL
734	E14	R: 1,1,2,3,4,5	NE	NL	NL	NL
721	E15	R: 2*,2,3,4,5 L: 2*,2,3,4,5	NE	L: 50% NL	NL	NL
722‡	E15	R: 2*,2,3,4,5 L: 2*,2,3,4,5	R: 3,2,3,4 L: 3,2,3,4, fus.	R: 50% NL L: 75% NL	NL	open symphysis
725	E15	R: 1,1,2,3,4,5	R: 1,1,2,3,4 L: 1,1,2,3,4, fus.	NL	NL	NL
954‡	E16	R: 2*,2,3,4,5	R: 3,2,3,4 L: fus.	NL	NL	NL
760	E17	R: 3*,1,2,3,4,5 L: 2*,2*,1,2,3,4,5	R: 2,2,3,4, fus. L: 2,2,2,3,4, fus.	L: 25% NL	NL	NE
777	SB (est.E17)	R: 2*,2,3,4,5 L: 1,2†,1,2,3,4,5	R: 1,2,2,3,4, fus. L: 1,1,2,3,4, fus.	R: 75% NL L: 50% NL	NL	NL
432	E19	R: 2*,2,3,4,5 L: 1,3*,2,3,4,5	R: 1,3,2,3,4, fus. L: 1,1,2,3,4, fus.	L: 50% NL	L: 1,1,2,3,4,5	short pelvis, L:pubis incomplete; open symphysis
1114	E20	R: 2*,2,3,4,5 L: 3*,2,3,4,5	R: 3,2,3,4, fus. L: 2,2,3,4, fus.	R: 75% NL L: 25% NL	R: 1,2†,2†,3,4,5	short pelvis, R,L: pubis incomplete; open symphysis
1117	E20	NL	NL	NL	R: 1†,1†,2,3,4,5	NL
1124	E20	NL	R and L: fus.	NL	R: 1,2,2,3,4,5	NL
1126	E20	NL	R: 1,1,2,3,4, fus. L: fus.	R: 75% NL	NL	short pelvis
1129	E20	NL	R and L: fus.	NL	NL	short pelvis
1109	NB	NL	L: fus.	NL	NL	NL
1111	NB	R: 1,1,2,3,4,5 L: 1†,2,3,4,5	R: 1,1,2,3,4, fus. L: fus.	NL	NL	short pelvis
1112	SB	R: 1,1,2,3,4,5	R: 1,1,2,3,4, fus. L: fus.	R: 75% NL L: 50% NL	NE	L: pubis incomplete
1131	NB	R: 1,1,2,3,4,5	R and L: fus.	NL	NL	short pelvis

*phalanges compatible with digit identity of 2,3, or 4 and possible identity assigned based on total length.

†bifurcated digit.

‡scoliosis also present.

For tarsals: 1,2,3= first, second, and third cuneiform and 4=cuboideum morphology, fus.=fusion of navicular tarsal to cuneiforms 2 and/or 3. Note that in some early embryos (E13-14), small size and poor delineation of condensations precluded evaluation of tarsals; in some older transgenics, poor staining of tarsal elements also precluded their evaluation.

R, right; L, left; NL, normal; %NL, percent of normal; NB, newborn; SB, stillborn; NE, not evaluated.

Mice with digit transformations and/or duplications having partial mirror-image symmetry are designated by number in boldface type.

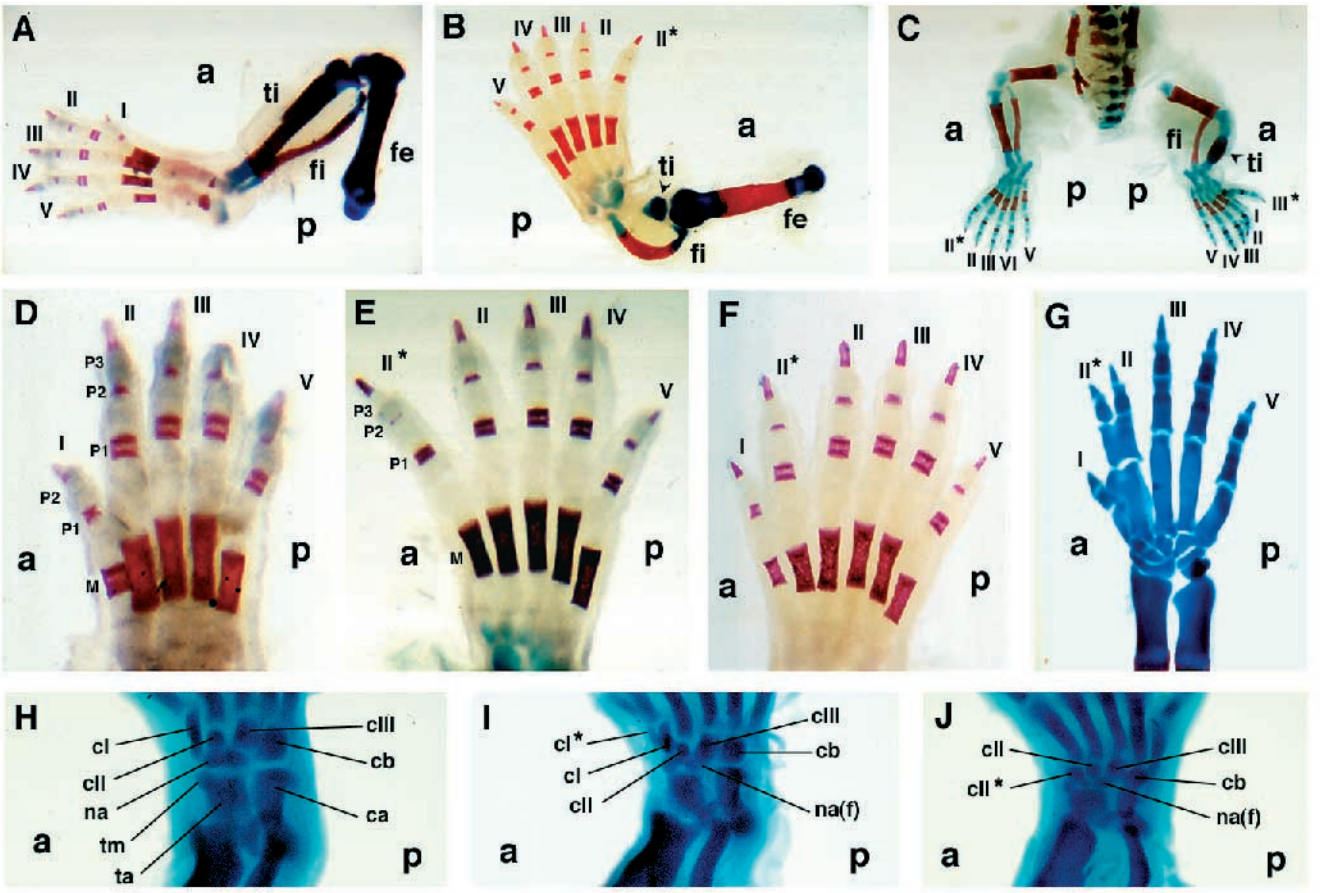

Fig. 2. Alterations in limb skeletal pattern in transgenic mice misexpressing *Hoxd-12*: conversions of anteriormost digits to posterior morphologies, with or without associated duplications and tibial hemimelia. (A,D) Normal newborn hindlimb and hindfoot; (B,E) stillborn primary transgenic kmA1 hindlimb and hindfoot; (C) E19 line 2917 embryo #432 hindlimbs; (F) stillborn primary transgenic km34 hindfoot; (G) E16.5 primary transgenic ks38 forelimb; (H) normal E15 hindfoot closeup of tarsal elements; (I) E15 line 2917 embryo #725 tarsals; (J) E15 line 2917 embryo #722 tarsals. a, anterior; p, posterior; ti, tibia; fi, fibula; fe, femur; I-V, digit designations with asterisk for transformations; P1-3, phalangeal elements; M, metatarsal; cI-cIII, cuneiforme tarsals I-III; cb, cuboideum tarsal; na, naviculare tarsal; tm, tibiale mediale tarsal; ta, talus tarsal; ca, calcaneus tarsal element. Asterisks denote digits and tarsals with duplications and/or morphologic transformations. na(f) indicates fusions and morphologic alterations of naviculare. Arrowheads indicate tibial shortening (hemimelia).

is phylogenetically the most anterior component of the pelvis; the ilium subsequently rotates anteriorly, and the pubis and ischium more posteriorly (Hinchliffe and Johnson, 1980). Consequently, if this is reflected in mammalian ontogeny, then the most frequent pelvic abnormalities seen in the *Hoxd-12* transgenics also seemed to selectively involve more anterior components.

Hoxd-12 misexpression in the lateral plate also causes sternal dysgenesis and associated rib abnormalities

Three of six primary transgenics with phenotypes and 62% of abnormal embryos examined from line 2917 matings displayed abnormalities in the sternum. The sternal phenotype ranged from mildly split posterior sternebrae to severe splitting and near total sternal agenesis (Fig. 3B-D; Table 3). The latter, more severe phenotypes would produce a 'flail chest' that would compromise respiration and account for the high frequency of neonatal mortality in transgenic animals with phenotypes. These sternal changes appeared to follow a posterior-to-anterior order in severity and so could represent a kind of 'posterior transformation' due to repression by *Hoxd-12* expression, since the paired sternal bands (which arise in the lateral plate and later fuse in the midline) do not normally form at all in the posterior-most lateral plate (Chen, 1952a,b; Chevallier, 1977).

Abnormalities consistent with posterior transformations were also seen in the ribs, usually in conjunction with sternal changes (with two exceptions). These included both fusions of ribs prior to joining the sternum (normally seen only in more posterior 'free' ribs), and a reduction in the number of sternal articulating ribs from seven to six (Fig. 3B,D; Table 3). In a total of two transgenic animals, an eighth sternal rib was observed (usually considered an anterior transformation), and this was thought to represent a sporadic event since it occurred so infrequently and was also observed in non-transgenic littermates in the CD1 mouse strain used.

Although the ribs are derived from somitic mesoderm, the rib tips develop in close association with the lateral plate mesoderm and potentially could receive signals from this region (Huang et al., 1996), explaining the rib abnormalities. Transgene expression was never detected in somitic mesoderm

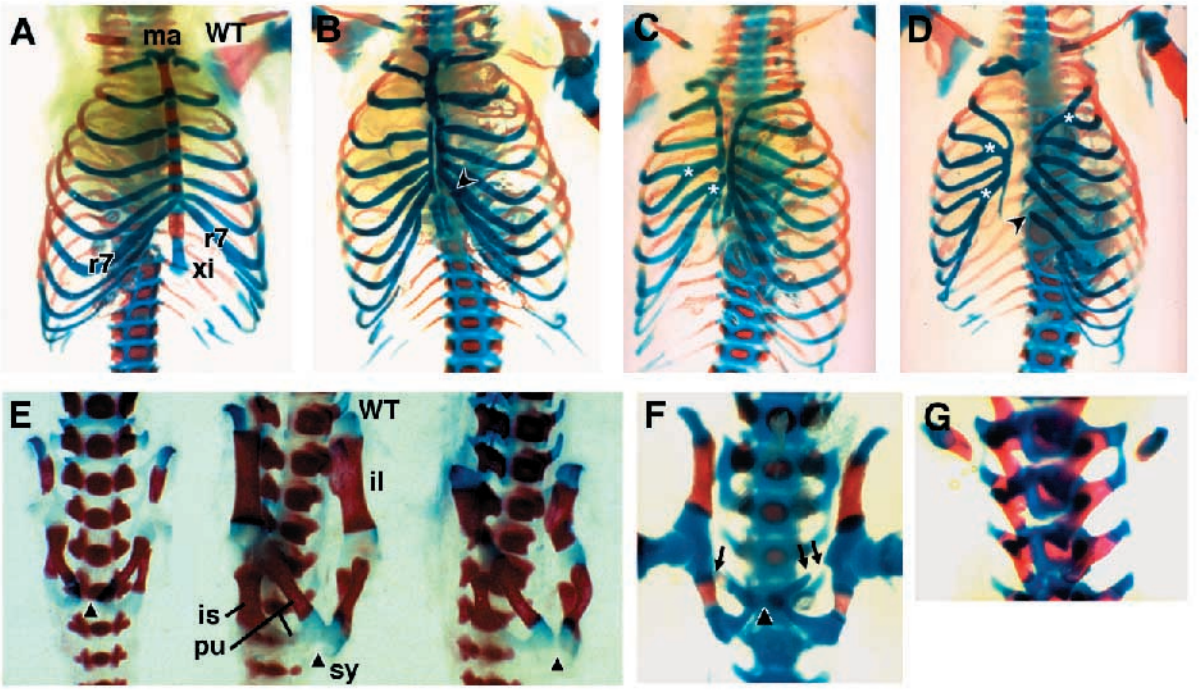

Fig. 3. Sternal, rib and pelvic abnormalities in transgenic mice misexpressing *Hoxd-12*. (A) Compared to normal littermates, (B-D) chest abnormalities in transgenic mice included mild to severe split sternebrae, free 7th rib (arrowheads) and abnormal fusions of sternal ribs (asterisks). (E-G) Pelvic abnormalities included overall shortening of the pelvis (compare transgenics to WT at same magnification in center of E), incomplete pubic bone (F, arrows show loss of superior pubic ramus), open symphysis pubis (arrowheads) and (G) one instance of near total pelvic agenesis. (A) Normal (WT) newborn; (B) line 2917 #1124 E20 embryo; (C) line 2917 #1111 newborn; (D,F) line 2917 #1114 E20 embryo; (E) primary transgenic ks10 (left), normal (WT, center) and primary transgenic ks9 (right) newborns; (G) primary transgenic kmA1 stillborn. WT, wild type; ma, manubrium; xi, xiphoid; r7, 7th rib; il, ilium, is, ischium, pu, pubis; sy, symphysis pubis.

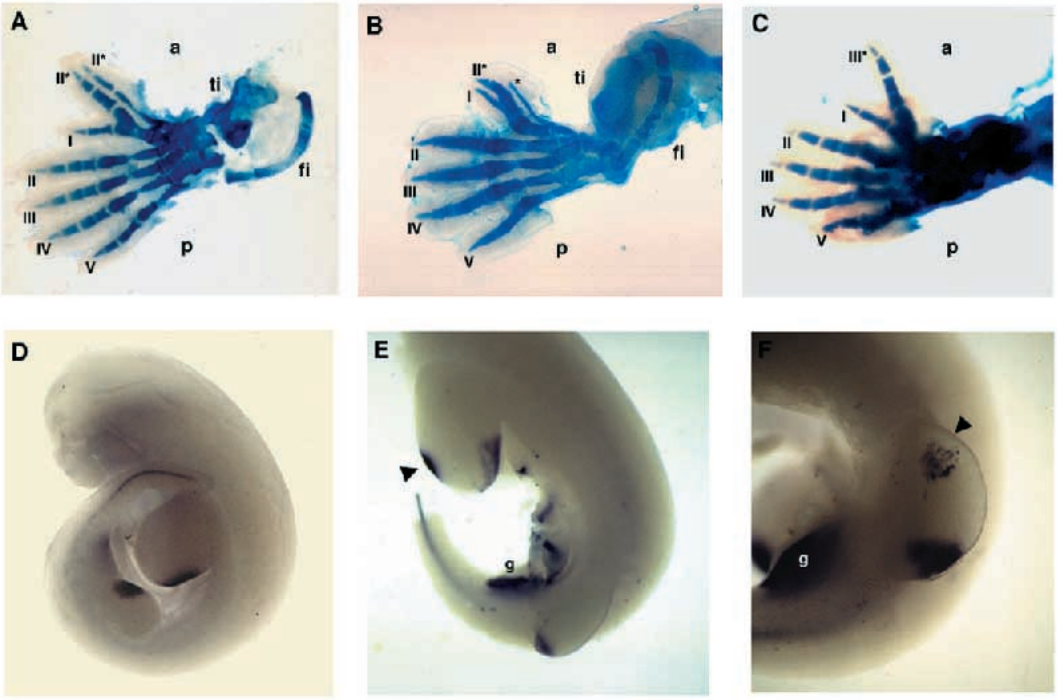

Fig. 4. A subset of *Hoxd-12* transgenic mice display characteristics consistent with ectopic activation of polarizing signals in the anterior limb bud. (A-C) Stained skeletons of transgenic mice showing digit duplications with partial mirror-image symmetry, often associated with hemimelia (short tibia (ti) and bowed fibula (fi)). Asterisks indicate transformed, duplicated digits. (D-F) Whole-mount in situ hybridization detecting *Sonic hedgehog* (*Shh*) message in normal (D) and transgenic mated line 2917 embryos (E,F) at E10.511.5. Note normal expression of *Shh* in the posterior limb buds, the notochord extending into the tail and the gut (g). The transgenic embryos display an ectopic focus of *Shh* expression in the anterior forelimb (E) or hindlimb (F) bud (arrowheads). (A,C) Line 2917 #760 E17 embryo (C shows ventral view of right foot). (B) Line 2917 #777 stillborn (est. E16). a, anterior; p, posterior.

Table 3. Sternal phenotypes of *Hoxb-6pro*/*Hoxd-12* transgenic mice

Animal	Age	Sternum	Rib fusions		No. of vertebrosternal ribs	
			Right	Left	Right	Left
Primary transgenics:						
ks67	E18.5	NL	r4-5; r6-7	---	7	7
kmA1	SB	open, split-severe	r1-2; r3-5	r1-2; r4-5	6	6
km34	SB	NL	---	r1-2	7	7
ks9	NB	split below r2	---	r6-7	6	7
ks10	NB	open, agenic	r1-2; r4-6	r1-2	7	7
line 2917:						
721	E15	open, split	r4-6	---	7	7
722	E15	complete agenesis	---	---	---	---
725	E15	split below r3	---	---	7	7
734	E15	open, split	---	---	7	7
954	E16	open, split	---	---	7	7
777	SB(~E17)	open, split-severe	r6-7	r2-3	7	6
760	E18	split with no ossif. below r2	---	---	7	8
1114	E20	open, split-severe	r3-4; r6-7	r1-2	7	6
1117	E20	no ossif. below r2-mild	---	---	7	7
1124	E20	split with no ossif. below r2	---	---	7	6
1126	E20	split with no ossif.-severe	r3-4; r7-8	---	6	7
1129	E20	split with no ossif.	---	---	7	7
688	NB	open, split-severe	r4-5	r2-3	7	6
1109	NB	split below r2	---	---	7	7
1111	NB	open, split, no ossif.-severe	r4-5; r6-7	---	7	7
1131	NB	no ossif. below r2	r7-8	---	7	7
1134	NB	split with no ossif. below r3	r4-5	r1-2	8	7
1138	NB	split with no ossif. below r4	---	---	7	7

R, right; L, left; NB, newborn; SB, stillborn; NL, normal; r, rib; ossif., ossification centers.

and axial skeletal changes were not seen in primary transgenics or in mated line 2917 offspring. Vertebral numbers and morphologies were entirely normal with a single exception of 5 rather than 6 lumbar vertebrae in one animal, which was considered a sporadic event since it occurs as a natural variant in some mouse strains (see for eg. Kessel and Gruss, 1991). Therefore, the rib abnormalities were interpreted as being secondary to alterations in the lateral plate mesoderm.

A subset of *Hoxd-12* transgenic embryos have mirror-image duplications of digits correlating with ectopic activation of *Sonic hedgehog* in the anterior limb bud

Among *Hoxd-12* transgenic mice with limb abnormalities, some animals (8 out of 26) displayed a severe phenotype that included transformations of digit I to a very long triphalangeal digit (digit III-like morphology) and frequent associated duplications of anterior digits, resulting in an appearance of partial mirror symmetry (Fig. 4A-C; Table 2). Many of these transgenics also displayed associated tibial hemimelia (shortening). Such phenotypes (mirror-symmetric duplications, transformations of anterior digits, associated hemimelia) were very reminiscent of several naturally occurring luxoid/luxate mouse mutants (Carter, 1951; Forsthoefel, 1962; Johnson, 1967) and also suggested the presence of ectopic anterior polarizing activity. In fact, ectopic *Shh* expression and ZPA activity have recently been demonstrated in several different luxoid mutants (Chan et al., 1995; Masuya et al., 1995, 1997).

We examined *Shh* expression in early embryos from line 2917 matings. In a subset of embryos, an ectopic focus of *Shh* expression was detectable by whole-mount in situ hybridization in either the anterior hindlimb or forelimb bud (Fig. 4E,F). This was never observed in parallel hybridizations with control embryos. In multiple hybridizations (20 litters total), about 12% of the expected homozygous embryos displayed an ectopic focus of *Shh* in anterior limb bud, compared to a 22% occurrence of mirror-symmetric digital patterns in the predicted number of line 2917 homozygotes. Thus, ectopic *Shh* expression was seen with about half the frequency of mirror-symmetric limb phenotypes. This imperfect correlation may be related to some variability in embryonic stages (which ranged from E9.5-E13) and/or lower sensitivity of the whole-mount in situ detection compared to the level of *Shh* expression necessary for bioactivity (abnormal polarization). The occurrence of mirror-symmetric limb changes in only a subset of phenotypically affected transgenics (8 out of 29 analyzed) could reflect variable transgene expression, with only the higher levels of *Hoxd-12* misexpression resulting in a high enough level of *Shh* activation to alter limb polarization. Notably, ectopic foci of *Shh* expression were usually seen subjacent or near to the AER, suggesting involvement of AER signals together with *Hoxd-12* misexpression.

The mouse *Sonic hedgehog* promoter contains *Hoxd-12-binding* sites and is transactivated by *Hoxd-12* in vitro

The apparent correlation between mirror-symmetric limb phenotypes and ectopic *Shh* expression in a subset of transgenic embryos suggested that *Hoxd-12* can activate *Shh*. We examined the *Shh* promoter to assess whether such activation of *Shh* might be a direct effect of *Hoxd-12* misexpression.

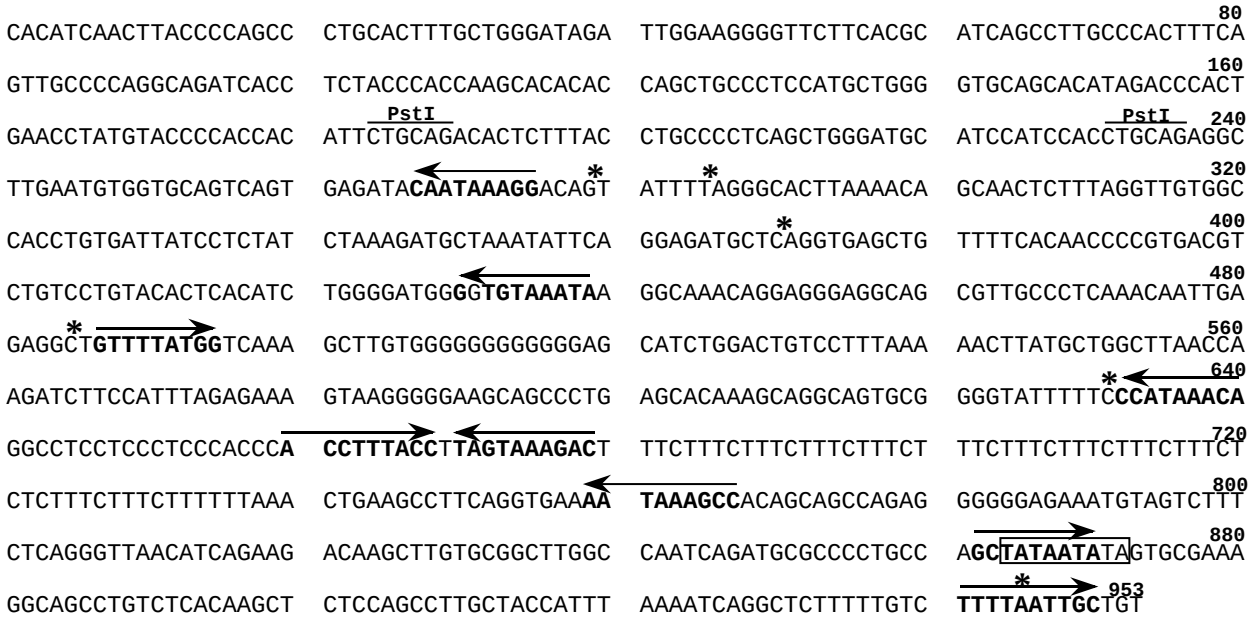

Fig. 5. Sequence of 1 kbp of 5′ upstream region of the mouse *Shh* promoter showing *Hoxd-12* consensus binding sites and positions of *Hoxd-12* protein binding mapped in vitro. The sequence, extending for about 900 bp upstream from near the start of the *Shh* transcribed region, is shown with a consensus TATA promoter sequence boxed and PstI sites overlined (the *Eco*RI and *Xho*I sites at the 5′ and 3′ ends, respectively, are not included). *Hoxd-12* TAAT and TTTAY type consensus binding sites (R. Hutson, S. Aguanno and S. Mackem, unpublished data) are shown with conserved nucleotides in boldface and relative orientations indicated by arrows. The asterisks indicate the positions of T7 exonuclease stops generated by *Hoxd-12* protein binding in vitro (see Fig. 6). The nucleotide sequence of the mouse *Shh Eco*RI-*Xho*I promoter fragment has GenBank accession no. AF019387.

Hoxd-12 transactivates through binding to either a typical TAAT motif or to a variant TTTAY motif (R. Hutson, S. Aguanno and S. Mackem, unpublished data); a variant TTAT core is also preferred by several other *Abd B* subtype homeobox genes (Benson et al., 1995; Ekker et al., 1994). 1 kbp of DNA upstream from the transcribed region of the mouse *Shh* gene was sequenced and found to contain several *Hoxd-12* consensus binding sites, all within the first 700 bp, as well as a TATA motif in the expected location (Fig. 5). This DNA was ^{32}P-end-labeled, bound to recombinant *Hoxd-12*/GST fusion protein and digested with T7 exonuclease (T7 exo) to map *Hoxd-12*-binding sites. Four out of eight consensus matches identified by sequence comparison were adjacent to T7 exo stop sites generated by *Hoxd-12* binding (Fig. 6A-C), while two were not, and two could not be evaluated due to overlap with the position of T7 exo limit-digestion product of naked DNA. Of the total of six exonuclease stops detected, only one was not closely positioned near a good *Hoxd-12* consensus site match in the promoter region analyzed.

The 700 bp *Shh* promoter region containing *Hoxd-12*-binding sites was ligated to CAT coding sequences to assay expression driven by the *Shh* promoter in cell culture. This reporter was transfected into primary cultured early limb bud cells from 72 hour chick embryos. Cotransfection with a *Hoxd-12* expression vector reproducibly stimulated expression of the *Shh*-CAT reporter an average of 2.5-fold over baseline, suggesting that *Hoxd-12* can transactivate expression of the *Shh* promoter (Figs 6D, 7). High baseline expression was not simply due to endogenous *Hoxd-12* in the limb cells, as this was observed even when cells from only anterior limb bud halves were used (not shown), and may reflect the presence of elements permitting inappropriate basal *Shh* promoter activity from the fragment used, because other presumptive negative regulatory elements are missing. To confirm that the activation by *Hoxd-12* was mediated by direct binding to the *Shh* promoter, the full-length *Hoxd-12* protein fused to the potent VP16 activation domain was tested and found to strongly stimulate expression of the *Shh*-CAT reporter (Fig. 7).

Exogenous *Hoxd-11* or *Hoxd-12* activates expression of the endogenous *Sonic hedgehog* gene in cultured limb mesenchymal cells

To obtain independent confirmation of the ability of *Hoxd-12* to regulate the natural *Shh* promoter, we assessed whether infection with a *Hoxd-12*-expressing retrovirus could activate the resident *Shh* gene in cultured chick limb bud mesenchymal cells. Since *Hoxd-11* misexpression in chick results in phenotypes somewhat similar to the *Hoxd-12* transgene (leg triphalangeal digit I, shortened tibia, wing digit duplication; see Morgan et al., 1992), it was of interest to determine whether this *Hoxd* member might also be able to regulate *Shh* expression. Cultured limb cells were infected with either *Hoxd-11*- or *Hoxd-12*-expressing retrovirus and, after 48 hours, were analyzed for *Shh* transcript levels. As shown if Fig. 8, the introduction of either *Hoxd-11* or *Hoxd-12* in these cells stimulates expression of the endogenous *Shh* gene compared to the retroviral expression vector alone. Interestingly, this induction was dependent upon inclusion of FGF in the culture, again suggesting that AER signals may cooperate with *Hoxd* genes to induce *Shh* expression.

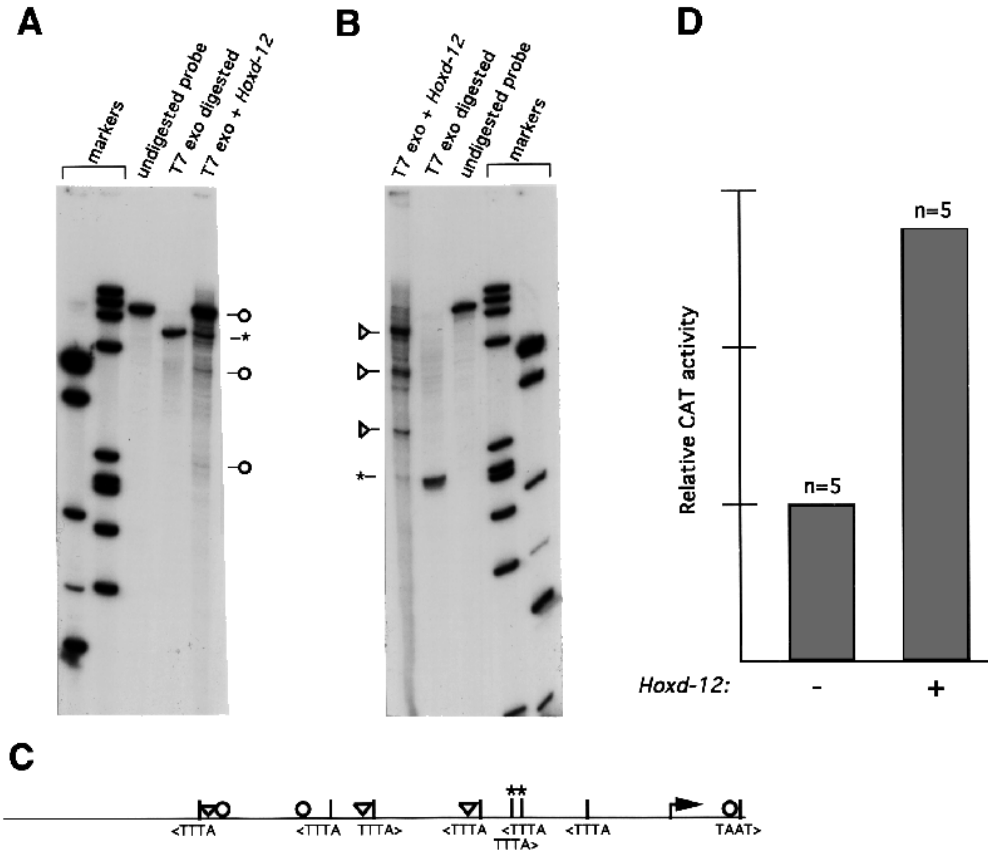

Fig. 6. T7 exonuclease mapping of *Hoxd-12* protein binding sites on the *Shh* promoter in vitro and transactivation of the *Shh* promoter by *Hoxd-12*. The *Shh* promoter was end-labeled at the 5′ *Eco*RI site (A) or the 3′ *Xho*I site (B), incubated with *Hoxd-12*/GST fusion protein, digested with T7 exonuclease and analyzed on a 6% acrylamide-urea gel. Free probes were also digested to determine the size of limit digestion 'half-molecules' (asterisks) and labeled bluescript *Msp*I (outer lanes) and ΦX *Hae*III (inner lanes) fragments were included as size markers. The positions of exonuclease stops (circles for *Eco*RI, triangles for *Xho*I labeled) are indicated next to the digestion products (A,B) and are also shown below on a diagrammatic representation of the *Shh* promoter with *Hoxd-12* consensus sites indicated (C). A 700 bp *Shh* promoter fragment including the detected binding sites was cloned into a CAT reporter and cotransfected into cultured limb bud cells together with a *Hoxd-12* expression vector or control vector pSG5 (D). In five independent experiments, *Hoxd-12* stimulated CAT expression an average of 2.5-fold over baseline.

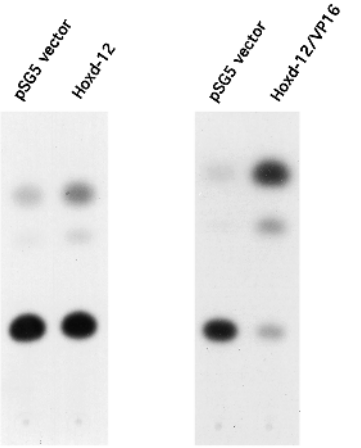

Fig. 7. The *Sonic hedgehog* promoter is activated in transient transfection assays by either the native *Hoxd-12* protein or by a *Hoxd-12*/VP16 fusion protein containing a stronger activation domain. *Shh*-CAT reporter, expression vectors and cells were as in Fig. 6.

DISCUSSION

Misexpression of *Hoxd-12* in the limb: differential effects on chondrogenic blastema arising as preaxial and postaxial branches

Normal *Hoxd-12* expression begins in the posterior half of the limb bud mesenchyme and later becomes more distally restricted and also extends more anteriorly within the distal presumptive digit region, but never encompasses digit I (Yokouchi et al., 1991; Kondo et al., 1996, Nelson et al., 1996). *Hoxd-12* misexpression phenotypes resemble 'posterior' transformations in some respects. In the distal autopod (hand/foot), where the transgene is expressed in both the forelimb and hindlimb, it is the anterior part of the autopod that is affected; anterior digits (eg. digit I) are converted to more posterior digits and/or become duplicated. In the hindlimb, where the *Hoxd-12* transgene is also expressed proximally, the anterior-most element in the distal row of tarsals displays 'posterior' transformations as well. However, the anterior long bone (tibia) is shortened. Likewise, the phylogenetically 'anteriormost' pubic bone in the pelvis is reduced (discussed further below). At the same time, other proximal elements (femur, ilium) are unaffected.

There are several salient features of the phenotype. (1) The effects of *Hoxd-12* on the skeletal pattern are evident as soon as condensations become visible and affect the formation of condensations as well as their subsequent growth, as evidenced by digit duplications and triphalangeal digit I transformations. (2) The transgene exerts its effects exclusively in domains where endogenous *Hoxd-12* is not normally expressed: the anteriormost part of the autopod (digit I region), the anterior zeugopod (tibia) and the anterior pelvis. This suggests that *Hoxd-12* levels are already saturating in regions where it is normally expressed, as observed for other *Hox* genes in the limb (Morgan et al., 1992; Yokouchi et al., 1995; Goff and Tabin, 1997). (3) In domains where *Hoxd-12* is not normally expressed, the transgene promotes, represses, or has no effect on the formation and growth of chondrogenic condensations, depending on the particular locale. In contrast to effects on the autopod, reductions occur in the tibia and pubic bone while other proximal elements that are also outside of the normal *Hoxd-12* expression domain are

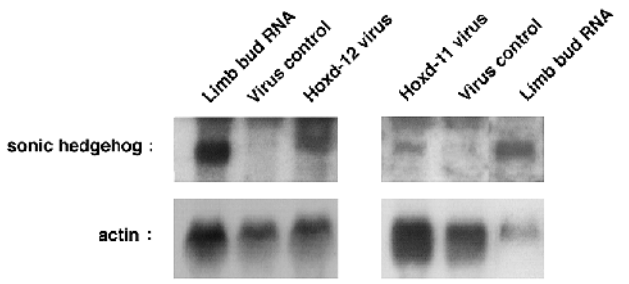

Fig. 8. Activation of expression of the endogenous *Shh* gene in cultured limb mesenchymal cells following infection with *Hoxd-12* or *Hoxd-11*-expressing retroviruses. RNA from cells infected with either *Hoxd-11*-expressing, *Hoxd-12*-expressing, or control RCAS BP virus was analyzed on Northern blots probed with either chick *Shh* or chick actin. Intact limb bud RNA was included as a positive control. In the absence of exogenous *Hoxd* gene expression (RCAS BP virus control), endogenous *Shh* RNA typically declined to undetectable levels under the culture conditions used, despite the addition of FGF-4 (300 ng/ml) to the cultures.

unaltered (femur and ilium). Such results are not easily reconciled with 'homeosis' models.

The formation of skeletal elements from proximal to distal (hip to toe) proceeds by progressive branching and segmentation of chondrogenic condensations to produce the more distal elements (eg. the femur branches distally to produce tibia and fibula). Comparisons of the branching pattern in various tetrapods has generated a model in which the autopod forms by an anterior bending of the main (metapterygial-like) limb axis: the distal row of carpal/tarsals and the digits all arise as successive postaxial branching events from the continuation of the main limb axis along a curving 'digital arch' (Shubin and Alberch, 1986). In this view, the digits (including digit I) and the distal row of tarsals are in fact all 'postaxial' structures whereas the tibia or the radius are true preaxial branches (see Fig. 9). The effects of *Hoxd-12* misexpression in the limb are compatible with a model in which *Hoxd-12* promotes formation of postaxial condensations branching from the main limb axis (previously proposed for *Hoxd* genes; see Duboule, 1994), while selectively inhibiting formation or growth of preaxial condensations.

The pelvis arises from the lateral plate mesoderm (Chevallier, 1977) and has traditionally been considered a part of the hindlimb. Its development is less well understood, partly because it is specified prior to formation of a visible limb bud swelling and the early condensations are ill-defined (see for eg. Rogulska, 1965). Work with a chick mutant indicates that the pelvis can form in the absence of apical ridge and polarizing signals (Prahlad et al., 1979; Ros et al., 1996; Noramly et al., 1996; Grieshammer et al., 1996). In mouse and human, the pelvis and femur appear to arise by segmentation from a single condensation (Forsthoefel, 1963; Rooker, 1979), making the pelvic anlage part of the branching/segmenting limb axis. The pubic bone of the pelvis is phylogenetically its anteriormost component (rotations in mammals distort this relationship; Hinchliffe and Johnson, 1980), suggesting the pubis may represent a 'preaxial' branch from a pelvic condensation. Since *Hoxd-12* misexpression often results in pubic reduction in the affected pelvis, this phenotype may also be compatible with a model in which *Hoxd-12* exerts inhibitory effects on preaxial condensations (Fig. 9).

Misexpression of *Hoxd-13* or *Hoxa-13* in the chick causes reductions in proximal long bones, where these genes are not normally expressed (Yokouchi et al., 1995; Goff and Tabin, 1997). *Hoxd-11* misexpression in the chick produces a somewhat similar phenotype to that of transgenic *Hoxd-12*, including conversions of digit I to a triphalangeal digit and marked tibial reductions (Morgan et al., 1992; Goff and Tabin, 1997). Goff and Tabin (1977) have proposed that *Hoxd-11* promotes growth of distal elements (digits) and represses growth of more proximal elements (long bones), since mild reductions in the fibula and femur were also observed with *Hoxd-11* misexpression. However, transgenic *Hoxd-12* selectively retards tibial development and concomitant bowing of the fibula suggests that any mild fibular reduction results from the tibial remnant acting as a mechanical tether to impede normal growth. The relatively normal femur in *Hoxd-12* transgenics also indicates that proximal skeletal elements are not affected uniformly. Mild shortening of both forelimb zeu-

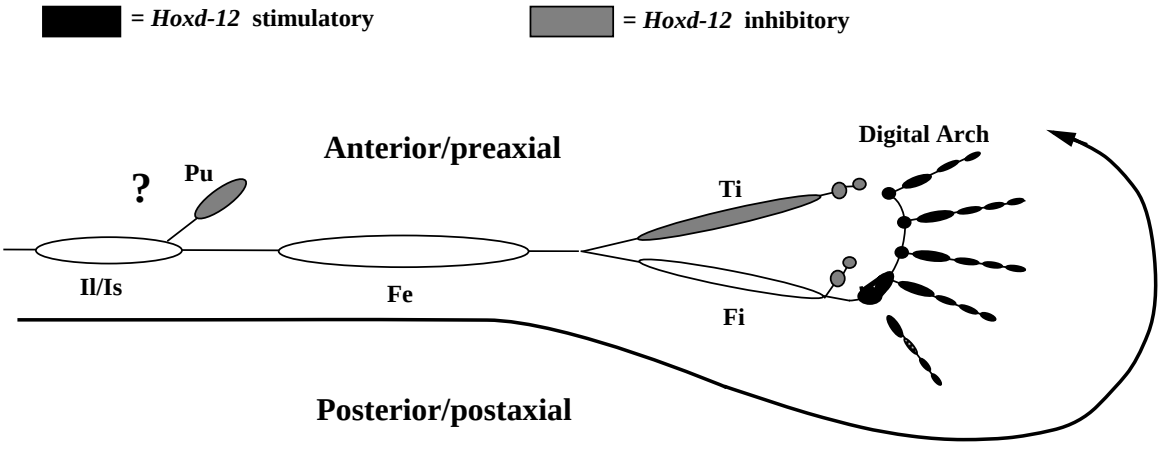

Fig. 9. Schematic of branching and segmentation pattern of chondrogenic blastema in hindlimb indicating 'preaxial' elements and 'postaxial' elements proposed to be differentially regulated by *Hoxd-12*. The arrow indicates the main limb axis which bends anteriorly to produce the digital arch. The branching pattern is according to that derived by Shubin and Alberch (1986), with the inclusion of the pelvis and representation of the pubis as an anterior branch of the ilium being hypothetical. Il, ilium; Is, ischium; Pu, pubis; Fe, femur; Ti, tibia; Fi, fibula.

gopodal long bones (radius and ulna) in *Hoxd-12*/*Hoxa-11* null mice (Davis and Capecchi, 1996) suggests that *Hoxd-12* may play some positive role in development of both zeugopodal long bones (tibia and fibula in hindlimb). In these loss-of-function mutants, the preaxial branch may be secondarily affected due to a primary reduction in the postaxial branch/main limb axis from which it bifurcates.

How opposing effects of *Hoxd-12* on different condensations may be mediated is unknown. When inappropriately expressed, *Hoxd-12* may interfere with the function of other *Hox* genes proximally, as proposed for *Hoxd-13* (van der Hoeven et al., 1996; Goff and Tabin, 1997). However with *Hoxd-12*, such dominant-negative effects would be restricted to preaxial condensations (pubis, tibia, as compared to ilium, femur). Furthermore, it is possible that any, or perhaps all, of the stimulatory and inhibitory effects of *Hoxd-12* on chondrogenic condensations may result indirectly from feedback induction of *Shh*, since very similar selective changes in the digits, tibia and pubis occur in several luxoid mouse mutants that misregulate *Shh* (see below).

Sternal and rib phenotypes due to *Hoxd-12* misexpression: evidence for a 'Hox code' and/or differential growth regulation?

In *Hoxd-12* transgenic mice, reduction in the number of sternal ribs from seven to six and fusions of anterior sternal ribs could be considered posterior transformations, usually thought to result from an altered *Hox* code in the somitic mesoderm from which the ribs arise (see, for example, references in Krumlauf, 1994). Since the *Hoxd-12* transgene is expressed solely in the lateral plate mesoderm, in this case, the rib changes must be secondary, perhaps related to altered signals from the lateral plate or to associated sternal dysgenesis. The sternum develops from paired bands in the dorsal lateral plate mesoderm that condense and move ventrally to meet in the midline where they fuse and segment in association with the ribs (Chen, 1952a,b; Chevallier, 1977). While the sternal phenotype seen in *Hoxd-12* transgenics is often severest posteriorly (caudally), the formation and/or movement of the entire sternal band appears to be affected, ranging from split sternebrae to complete sternal agenesis. Primary effects of *Hoxd-12* on formation and growth of sternal chondrogenic condensations may produce a phenotype resembling homeosis, as proposed for the generation of apparent homeotic transformations in the axial skeleton due to altered *Hox* gene expression (see Duboule, 1995). The sternal and rib changes in *Hoxd-12* transgenics are also very similar to those seen in the *Xt* mutant, and may be mechanistically related (discussed below).

Hoxd-12 participates in a positive feedback loop with *Sonic hedgehog* to reinforce polarizing signals during limb outgrowth

Mirror-symmetric limb phenotypes seen in some *Hoxd-12* transgenic mice correlate with induction of ectopic *Shh* in the anterior limb bud. In vitro binding and activation of the *Shh* promoter, and induction of endogenous cellular *Shh* RNA by exogenous *Hoxd-12* all suggest that *Hoxd-12* may directly regulate *Shh*. *Hoxd* genes are thought to be downstream of polarizing signals (see Izpisua-Belmonte and Duboule, 1992) and misexpression of *Shh* induces de novo expression of *Hoxd* genes in the limb bud (Riddle et al., 1993). Thus, activation of *Shh* expression by *Hoxd-12* may represent the return half of a positive feedback loop; such loops are often used to amplify signals, particularly in the limb (reviewed by Cohn and Tickle, 1996). In chick, work with a limbless mutant and with limbs made of reaggregated anterior mesenchyme indicate that *Hoxd* genes can be expressed in the absence of a *Shh* signal and are posteriorly polarized in the early limb bud in the absence of *Shh* (Grieshammer et al., 1996; Hardy et al., 1995; Noramly et al., 1996; Ros et al., 1994, 1996). This raises the alternative possibility that some other earlier asymmetric signal initially induces posterior 5′*Hoxd* gene expression (e.g. *Hoxb-8*; Charite et al., 1994) and that certain 5′ *Hoxd* genes first activate *Shh* expression in the limb bud. *Shh* might then in turn induce the transition to a late 'de novo' distal domain of *Hoxd* expression in the limb bud (discussed by Duboule, 1994; Nelson et al., 1996). In any case, the ultimate outcome is similar; *Shh* and *Hoxd-12* activate each other, resulting in a positive feedback loop.

Hoxd-13 may not participate in this feedback loop, since misexpression phenotypes suggest no altered polarization (Goff and Tabin, 1997). *Hoxd-11* misexpression in chick has produced a phenotype somewhat similar to transgenic *Hoxd-12* (Morgan et al., 1992), but molecular evidence of ectopic polarizing signals was not observed, perhaps due to lower or non-uniform expression levels. Like *Hoxd-12*, *Hoxd-11* upregulates *Shh* expression in retrovirally infected limb cells in culture, suggesting that it too may participate in positive feedback regulation of *Shh*. In vivo feedback regulation by *Hoxd-12* (and *Hoxd-11*) may also require coincident FGF signals from the AER. Normal *Shh* expression occurs in a posterodistal domain subjacent to the AER that is more restricted than the expression domains of *Hoxd-11* and *Hoxd-12* (Riddle et al., 1993; Nelson et al., 1996). Similarly, the ectopic *Shh* domain seen in the anterior limb bud of *Hoxd-12* transgenic embryos was often closely associated with the AER. A co-requirement of FGF for the induction of cellular *Shh* by retrovirally expressed *Hoxd-11* or *Hoxd-12* would also support this view.

Feedback regulation also complicates interpretation of *Hoxd-12* transgene effects. Presumably, ectopic *Shh* induces expression of other *Hoxd* genes as well as other targets. Some, or all of the phenotypic changes seen could reflect indirect misregulation of these other 'downstream' genes. Identification of direct targets of *Hoxd-12*, as well as of *Shh* action, may help resolve this issue.

Hoxd-12 misexpression in various lateral plate derivatives phenocopies mouse luxoid mutations that misregulate *Sonic hedgehog*

The *Hoxd-12* transgenic phenotype in lateral plate derivatives strikingly resembles certain luxoid/luxate mouse mutants (Carter, 1951; Forsthoefel, 1962; Hinchliffe and Johnson, 1980; Johnson, 1967; Masuya et al., 1995; Mo et al., 1997). These mutants all have triphalangeal digit I and varying degrees of anterior digit duplications with hemimelia (selective shortening), usually of the anterior zeugopodal long bone (tibia or radius). Strong's luxoid (*lst*), Carter's luxate (*lx*) and *extra toes* (*Xt*), also have selective reductions in the pubic bone of the pelvis; particularly loss of the superior pubic ramus and open symphysis (Carter, 1951; Forsthoefel, 1962; Johnson, 1967). Additionally, *Xt* has very similar sternal abnormalities, ranging

from mildly split sternebrae to agenesis with open rib cage and reductions to six sternal ribs (Johnson, 1967; Mo et al., 1997).

Similarities between luxoid mutants and *Hoxd-12* transgenics also exist at a molecular level. Several, including *lst*, *lx* and *Xt*, express ectopic *Shh* in the anterior limb bud (Chan et al., 1995; Masuya et al., 1995, 1997), suggesting that the constellation of hemimelia and 'preaxial' (anterior) digital polydactyly may generally indicate aberrant *Shh* signaling. *Xt* results from a loss-of-function mutation in the *Gli3* zinc finger gene (Schimmang et al., 1992; Hui and Joyner, 1993), thought to function as a feedback repressor of *Shh* (Buscher et al., 1997; Marigo et al., 1996; Masuya et al., 1997; Mo et al., 1997). *Xt* mutants are haplo-insufficient, and heterozygotes and homozygotes resemble milder and more severe *Hoxd-12* transgenic phenotypes, respectively (Johnson, 1967; Mo et al., 1997). The strikingly similar sternal, rib and pelvic changes seen in *Xt* and in *Hoxd-12* transgenics suggest that these phenotypes in both may also result from altered *Shh* expression. If so, then detection of ectopic *Shh* in only a subset of *Hoxd-12* transgenics must reflect a lower detection sensitivity than bioeffect threshold; such a difference has also been seen between ectopic *Shh* detection and phenotype in *Xt* (Masuya et al., 1995).

Specificity of the *Hoxd-12* gain-of-function phenotype

Given similar DNA binding by different *Abd B* type *Hox* genes in vitro (Benson et al., 1995; Ekker et al., 1994), might *Hoxd-12* be mimicking the effect of some other posteriorly expressed *Hox* gene in vivo? Probably not, for several reasons. (1) In vivo DNA recognition must be considerably more specific than in vitro. In gain-of-function analyses of several *Abd B* type *Hox* genes in chick, including *Hoxd-10*, *Hoxd-11*, *Hoxd-13* and *Hoxa-13* (Morgan et al., 1992; Goff and Tabin, 1997; Yokouchi et al., 1995), only *Hoxd-13* and *Hoxa-13* have similar phenotypes. Of this group, only *Hoxd-11* phenotypically resembles *Hoxd-12* transgenics, which may reflect bona fide functional overlap in vivo. (2) There is ample evidence supporting the view that, particularly in the limb, *Hox* genes are present at saturating levels in their normal expression domains, since overexpression at these sites produces no phenotype (Morgan et al., 1992; Goff and Tabin, 1997; Yokouchi et al., 1995; this study). However, if different 5′ *Hox* genes can all substitute for one another when overexpressed (by binding each others targets), then misexpression of any *Abd B* type *Hox* gene should always produce a similar phenotype, regardless of the normal expression domain of that particular gene and saturation should not occur. (3) While *Hoxb-8* misexpression in transgenic mice induces *Shh* and produces some similar phenotypes to *Hoxd-12* (Charite et al., 1994), this gene belongs to the *Abd A* type, whose DNA-binding properties, even in vitro, are different from *Abd B* type *Hox* genes (see Ekker et al., 1994 and references therein). The similarity more likely reflects the in vivo complexity of *Shh* regulation.

Consequently, the *Hoxd-12* misexpression phenotype most likely reflects in vivo roles for this gene in patterning chondrogenic elements and in amplifying polarizing signals during limb bud outgrowth via a positive feedback loop with *Shh*. A number of different genes have been implicated in regulating *Shh* expression in the limb (including some 5′ *Hoxd* genes, *Hoxb-8*, *Gli3* and other luxoid mutant genes, as well as FGF and Wnt members), and may all be required in the complex regulatory circuitry modulating *Shh* expression in order to achieve precise and dynamic temporospatial regulation of this important signaling molecule.

We thank D. Levens and C. Tabin for comments and discussions; M. Federspiel and S. Hughes for advice on propagating retroviruses; C. Baker, D. Duboule, T. Kristie, A. McMahon, R. Riddle, and C. Tabin for probes and constructs; Genetics Institute, Inc. for recombinant FGF-4 protein; and W. Randolph and G. Best for expertise in photography and computer imaging. V. K. is a visiting fellow on leave from the University of Zagreb School of Medicine.

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(Accepted 26 August 1997)