Analysis of *Hoxd-13* and *Hoxd-11* misexpression in chick limb buds reveals that *Hox* genes affect both bone condensation and growth

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SUMMARY

Hox genes are important regulators of limb pattern in vertebrate development. Misexpression of *Hox* genes in chicks using retroviral vectors provides an opportunity to analyze gain-of-function phenotypes and to assess their modes of action. Here we report the misexpression phenotype for *Hoxd-13* and compare it to the misexpression phenotype of *Hoxd-11*. *Hoxd-13* misexpression in the hindlimb results in a shortening of the long bones, including the femur, the tibia, the fibula and the tarsometatarsals. Mutations in an alanine repeat region in the N-terminus of *Hoxd-13* have recently been implicated in human synpolydactyly (Muragaki, Y., Mundlos, S., Upton, J. and Olsen, B. R. (1996) *Science* 272, 548-551). N-terminal truncations of *Hoxd-13* which lack this repeat were constructed and were found to produce a similar, although slightly milder, misexpression phenotype than the full-length *Hoxd-13*.

The stage of bone development regulated by *Hox* genes has not previously been examined. The changes in bone lengths caused by *Hoxd-13* misexpression are late phenotypes that first become apparent during the growth phase of the bones. Analysis of tritiated thymidine uptake by the tibia and fibula demonstrates that *Hox* genes can pattern the limb skeleton by regulating the rates of cell division in the proliferative zone of growing cartilage. *Hoxd-11*, in contrast to *Hoxd-13*, acts both at the initial cartilage condensation phase in the foot and during the later growth phase in the lower leg.

Ectopic *Hoxd-13* appears to act in a dominant negative manner in regions where it is not normally expressed. We propose a model in which all *Hox* genes are growth promoters, regulating the expression of the same target genes, with some *Hox* genes being more effective promoters of growth than other *Hox* genes. According to this model, the overall rate of growth in a given region is the result of the combined action of all of the *Hox* genes expressed in that region competing for the same target genes.

Key words: *Hoxd-13*, *Hoxd-11*, viral misexpression, chick limb development

INTRODUCTION

Hox genes play a key role in regulating pattern during vertebrate morphogenesis. The *Hox* genes are believed to regulate developmental fate in sequential rostrocaudal domains in the forming neural tube, paraxial mesoderm, and gut. In these embryonic tissues, the expression patterns of the *Hox* genes obey rules of both spatial and temporal colinearity: within each *Hox* cluster the more 3′ genes are expressed earlier in development and in domains that are more rostral. Yet the situation in the limb bud appears to be more complex. The genes at the 5′ end of the *Hoxd* and *Hoxa* clusters are indeed activated in spatially and temporally overlapping domains (Dollé et al., 1989; Yokouchi et al., 1991). However, subsequently the *Hox* expression patterns are quite dynamic, with distinct phases and complex domains for each gene (Duboule, 1994; Nelson et al., 1996). Moreover, loss-of-function studies have not given results compatible with the simple model that the *Hoxd* genes pattern the anterior-posterior axis and the *Hoxa* genes pattern the proximal-distal axis, as proposed on the basis of the initial phase of nested expression domains.

Many of the *Hox* genes which are expressed in the limb have been deleted in mice using the technique of homologous recombination in embryonic stem cells. Disruption of each gene results in a distinct limb phenotype, generally affecting the pattern of skeletal elements within the region of the limb where that gene is normally expressed. While some mutants show defects in both forelimbs and hindlimbs, consistent with their expression in both limbs, other targeted alleles show limb-specific defects. For example, *Hoxd-13* mutants have defects in both forelimbs and hindlimbs, while *Hoxd-9* and *Hoxd-12* mutants have only forelimb defects and *Hoxa-10* mutants have only hindlimb defects (Dollé et al., 1993; Fromental-Ramain et al., 1996; Davis and Cappechi, 1996; Favier et al., 1996).

Functional redundancy between paralogs and non-paralogs has been established through the analysis of double mutants and *trans*-heterozygotes (Davis et al., 1995; Favier et al., 1996; Fromental-Ramain et al., 1996; Davis and Capecchi, 1996). In every mutant combination examined so far, synergism between different genes has been detected. One extreme example is the effect of the 11th paralogs on the determination of the radius/ulna segment of the forelimb. Individually, *Hoxd-11* and *Hoxa-11* mutations cause minor defects in the shape of the distal ends of the radius and ulna (Davis and Capecchi, 1994; Favier et al., 1995; Small and Potter, 1993). However, when mice mutant in these genes are mated to produce progeny that

are doubly mutant, an almost complete loss of the radius and ulna in the forelimbs results (Davis et al., 1995). Non-paralogs also collaborate in the proper formation of this segment, as shown by the analysis of the misshapen bones in the *Hoxa-11/Hoxd-12* double mutants and the *Hoxd-11/Hoxa-10* double mutants (Davis and Capecchi, 1996; Favier et al., 1996).

The compound *Hoxd-11/Hoxa-11* mutant indicates that these genes function in specifying the radius/ulna segment. However, since the entire segment is missing, it is difficult to use this phenotype to ascertain the cellular properties differentially regulated by the *Hox* genes. This is particularly problematic because the multiple phases of *Hox* gene expression in the limb suggest that they may act over a range of limb stages. If the limb segment fails to form because of early *Hox* action, the later roles of the genes within that segment will be masked.

Complementary information can be provided by gain-of-function experiments. In the chick, retroviral vectors have been used to misexpress *Hoxd-11* and *Hoxa-13* in the developing limb (Morgan et al., 1992; Yokouchi et al., 1995). Retroviral misexpression experiments cause both a temporal and a spatial misexpression of the transgene throughout the entire injected limb bud for all of its development. Misexpression of *Hoxd-11* in the hindlimb results in an extra phalanx in digit I (Morgan et al., 1992). This suggests that the *Hox* genes may act early at the time of cartilage condensation, consistent with the complete loss of bones in severely affected *Hoxa-11/Hoxd-11* double mutants. In contrast, misexpression of *Hoxa-13* in the hindlimb causes a reduction in the length of the tibia and fibula and a failure of the cartilage in these long bones to undergo a normal transition to hypertrophic cartilage (Yokouchi et al., 1995), suggesting that *Hox* genes also influence the skeletal elements after they have condensed.

Given the complexity of their expression patterns, in order to fully understand the mechanism of Hox protein action, it is essential to determine the time at which the Hox proteins are active and the nature of the cellular responses to them. Here we report the result of misexpressing *Hoxd-13* throughout the developing limb bud, and compare the consequent phenotype to that resulting from *Hoxd-11* misexpression. We show that *Hox* genes can affect at least two different stages of skeletal development. We also show that one mechanism by which the *Hox* genes lead to pattern alterations is by specifically altering the growth rate of cells in cartilage elements. Our data suggest a model in which Hox proteins regulate the same growth-controlling target genes, but different Hox proteins have distinct effects by differentially activating those target genes.

MATERIALS AND METHODS

Retrovirus construction

There appear to be at least two *Hoxd-13* transcripts expressed in the limb bud, the two transcripts differing at their N-termini (B. Morgan, personal communication). The putative initiator methionine of the shorter transcript is in-frame at amino acid position 98 of the longer transcript. The two encoded proteins are otherwise identical, including 136 amino acids N-terminal of the homeobox, the homeobox itself, and 8 amino acids C-terminal to the homeobox (B. Morgan, personal communication; Rogina and Upholdt, 1993).

Hoxd-13 cDNAs were isolated as previously described (Nelson et al., 1996) from which three *Hoxd-13* viruses were constructed. RCAS-*Hoxd-13* (X) (Fig. 1A) was constructed using a cDNA corresponding to the longer of the two transcripts whose putative coding region is identical to the published sequence, with the exception of 9 glycines encoded by ggc repeats starting at amino acid position 10, instead of the reported 7 ggc repeats (Rogina and Upholdt, 1993). This cDNA was cloned into the shuttle vector SLAX-13 (Riddle et al., 1993), and then subcloned into the *Cla*I site of RCAS(BP)A (Hughes et al., 1987).
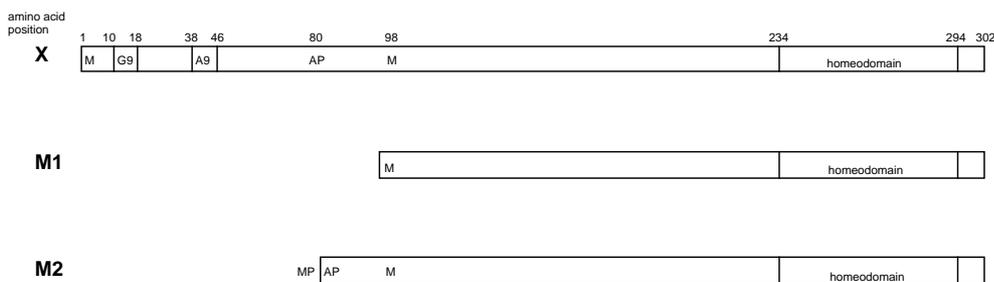
A.

Fig. 1. *Hoxd-13* viruses. (A) *Hoxd-13* inserts for virus constructions. Amino acid positions are relative to published sequence (Rogina and Upholdt, 1993). Viruses are designated X, for the full-length insert, and M1 and M2 for truncated constructs. The X insert is derived from the longer of two putative *Hoxd-13* transcripts, and the M1 and M2 truncations are derived from the shorter transcript. M1 starts at the putative initiator methionine for the shorter transcript, and M2 starts at the beginning of this transcript and initiates with two heterologous amino acids, methionine and proline. (B) Stage 10 infection results in complete limb bud infection by stage 22. A whole-mount in situ hybridization using an RCAS-specific RNA probe shows complete infection of the right hindlimb at stage 22 (arrow). The contralateral hindlimb is uninfected and serves as a stage-matched control. Scale bar, 1 mm.

The other two *Hoxd-13* viruses were constructed using a cDNA corresponding to the shorter of the two transcripts. Both of these viruses are N-terminal truncations relative to the longer cDNA, lacking two homopolymeric stretches of amino acids: the 9 glycines described above as well as a stretch of 9 alanines starting at amino acid position 38 of the published sequence. RCAS-*Hoxd-13* (M1) (Fig. 1A), begins at this transcript's putative initiator methionine corresponding to position 98 of the longer cDNA. RCAS-*Hoxd-13* (M2) (Fig. 1A) begins 18 amino acids upstream of the putative initiator methionine at amino acid position 80 of the longer cDNA. To form an intermediate deletion construct, leaving more of the coding sequence downstream of the alanine repeat, the M2 truncated construct was engineered using convenient restriction sites in the shorter cDNA. The amino acids MP, encoded by the linker used in the cDNA library construction, were used to define the 5′ end of the M2 truncated molecule for insertion into the *Nco*I site of the SLAX-13 shuttle vector. Both the M1 and the M2 truncated constructs were cloned into the *Cla*I site of RCAS(BP)A. All molecular techniques were standard (Ausubel et al., 1989), and the enzymes were purchased from Boehringer Mannheim Biochemicals.

Primary chick embryo fibroblasts were transfected with the RCAS(A)/*Hoxd-13* plasmid DNAs to generate virus stocks which were harvested and concentrated as described previously (Morgan et al., 1992; Fekete and Cepko, 1993a). The concentrated virus stocks were titered on chick embryo fibroblasts (Fekete and Cepko, 1993b), and the titer was found to be approximately 1×10^8 cfu/ml for each virus.

The *Hoxd-11* virus used in this study has been described previously (Morgan et al., 1992).

Misexpression protocol

Embryos were obtained by incubating fertilized White Leghorn eggs (SPAFAS, Norwich, CT) at 37°C and were staged according to Hamburger and Hamilton (1951). Stage 10 hindlimb injections were done as described by Morgan et al. (1992). Briefly, the eggs were incubated for 1.5 days, at which time about 2 ml of albumen were removed through a hypodermic needle inserted into the shell, and a window was cut into the top of the egg to expose the embryo. Virus was injected into the embryos in the area fated to become the right hindlimb, infecting the area with a series of 5-10 closely spaced injections, and the eggs were sealed with cellophane tape and returned to the incubator for varying lengths of time. Forelimb injections were done in the same way, targeting the sites of injection to positions more anterior than those of the hindlimb injections. Whole-mount in situ hybridizations (Riddle et al., 1993) were performed at stage 22-24 with a riboprobe specific to the viral message to assay for extent of infection. In 33 of 50 embryos examined, this infection protocol resulted in complete infection of the right hindlimb or forelimb, leaving the contralateral limb as a stage-matched uninjected control (Fig. 1B). Viral RNA expression caused by RCAS infection has been shown to correlate with viral protein misexpression (Marigo et al., 1996), as detected by staining with a monoclonal antibody (3C2) which recognizes the gag protein of the retrovirus, suggesting that protein encoded by the transgene is also misexpressed at this stage.

Stage 22 injections were done similarly, after approximately 3 days of development, targeting multiple locations on the distal-most portion of the right hindlimb bud.

Skeleton preparations and analysis

Embryos were harvested at various stages after injection for phenotypic analysis. For staining the intact skeletons, the embryos were fixed in 4% paraformaldehyde overnight at 4°C and washed in phosphate-buffered saline. The skeletons were visualized by staining in Alcian blue (20% in 70% ethanol/30% glacial acetic acid) for 6 hours, followed by clearing in 0.5% KOH and a graded series of 0.5% KOH/glycerol solutions to 100% glycerol. Injected and contralateral limbs were dissected from stained and cleared embryos and pho-

tographed through a dissecting microscope. For quantitation of the phenotype, the limbs were dissected into proximal-distal segments, and the lengths of the bones were measured with the aid of a dissecting microscope using an ocular reticule. To analyze fibula ankle articulation, embryos which had been infected at stage 10 were harvested on day 8, fixed in Bouin's fixative for several days to decalcify the tissue, then embedded in paraffin and cut into 10 μm sections in the frontal plane through the ankle region. Sections were dewaxed and stained with Weigert-Safrinin stain to identify cartilage.

[³H]thymidine studies

The analysis of cell proliferation in the limbs was a modification of a published method (Janners and Searls, 1970). The right presumptive hindlimb was injected with the *Hoxd-13* (M2) virus at stage 10 as described above. On day 5.5, 10 μCi of [³H]thymidine was injected into the yolk of each infected embryo. The embryos were incubated for a further 8 hours, killed and fixed in 4% paraformaldehyde/2% glutaraldehyde for 1 hour. Both hindlimbs were dissected from the embryos, dehydrated and embedded in the same paraffin block. Frontal 5 μm serial sections were collected, dewaxed and exposed to NTB2 photographic emulsion for 4 weeks at 4°C under dessicant prior to development. The slides were counterstained with hematoxylin to visualize the tissue.

The number of labeled cells in the distal ends of the tibias and fibulae was quantified for injected and control limb pairs. For each tibia and fibula, every 5th section was photographed at 200× magnification and a collage of overlapping photographs was generated to include all of the distal end of each bone in the section. The collages were used to count cells within the distal halves of the skeletal elements whose nuclei were heavily labeled with silver grains. For each bone, the number of labeled cells in each counted section were summed to represent an overall proliferation index for that bone.

RESULTS

Hoxd-13 misexpression leads to a decrease in the length of limb bones where it is not normally expressed

In order to investigate the role of *Hoxd-13* in limb development, a recombinant retroviral stock which encodes the full-length *Hoxd-13* coding sequence was prepared. Embryos were infected with the virus in the right prospective hindlimb at stage 10 and allowed to develop until day 10, at which time they were harvested, stained with Alcian blue and cleared in KOH/glycerol to reveal the cartilaginous skeleton. Fig. 2A shows an example of the resultant phenotype. With the exception of the digits, which are unaffected, all the bones of the limb are reduced in length, including the femur (data not shown), the tibia, the fibula, and the tarsometatarsals. Most dramatic is the change in the relative lengths of the tibia and fibula. Normally in the chick, the tibia (the 'drumstick') is significantly longer than the fibula; however in *Hoxd-13* infected limbs the tibia and fibula are roughly the same length. The fibula now extends to the ankle and makes a novel articulation, as shown by a histological frontal section through that region (Fig. 2E).

The effects of *Hoxd-13* misexpression were quantified by comparing measurements of the lengths of equivalent bones from injected and contralateral sides of the same embryo. Ratios of these measurements for 33 injected animals are displayed graphically (Fig. 3A). A ratio of less than 1 indicates that the affected bone is shorter than the contralateral bone, a

Fig. 2. *Hoxd-13* hindlimb misexpression phenotype. (A-D) Frontal views of lower leg and foot skeletons harvested on day 10, stained with Alcian blue and cleared with KOH-glycerol. In each case, the injected right hindlimb is pictured to the left of its contralateral uninjected control limb. The embryos were injected at stage 10 with either (A) the full-length X *Hoxd-13* virus, (B) the M1 truncated *Hoxd-13* virus, or (C) the M2 truncated *Hoxd-13* virus. (D) Tibias and fibulae dissected from an M2-injected embryo are shown to highlight differences in lengths between injected and uninjected leg bones. Scale bars, 1 mm. (E) Tissue sections through the ankle region show that the fibula makes a novel articulation at the ankle in *Hoxd-13*-infected hindlimbs. Stage 10 embryos were injected in the presumptive right hindlimb with the M2 truncated *Hoxd-13* virus and were allowed to develop until day 8, at which time embryos were harvested and fixed in Bouin's fixative, the hindlimbs were embedded in paraffin, and frontal sections were taken through the ankle region. Sections were stained with Weigert-Safrinin to visualize the cartilaginous skeleton. The arrow marked with * indicates the novel articulation of the fibula with the tarsus in the infected limb. Scale bar, 0.25 mm.
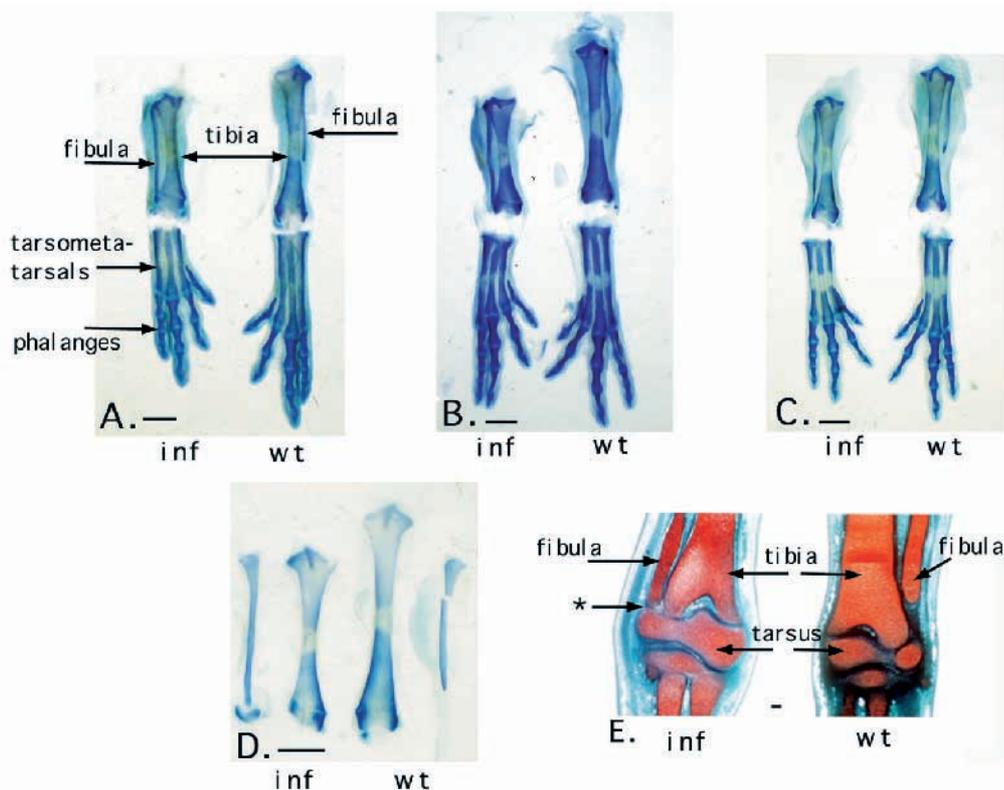
ratio of 1 indicates the bones are the same length, and a ratio of greater than 1 indicates that the affected bone is longer than the contralateral bone. Ratios of the measurements for equivalent contralateral bones in either uninjected controls, or in controls injected with a virus carrying the histochemical marker alkaline phosphatase, are 1. The measurements indicate that, on average, *Hoxd-13* misexpression causes the tibia length to be shortened to 73% of its normal length, the fibula to be shortened to 95% of its normal length, the femur to be shortened to 84% of its normal length, the metatarsals to be shortened to 79% of their normal length, and the phalanges of the digits to be unchanged in length.

Truncated *Hoxd-13* viruses which lack the N-terminal alanine repeat produce a slightly milder misexpression phenotype

The N-terminus of *Hoxd-13* contains a stretch of alanine residues which has recently been identified as an important functional domain of the protein in humans (Muragaki et al., 1996). In this study, hereditary synpolydactyly was found to be caused by an expansion of this alanine repeat in three different pedigrees. We deleted this repeat in the two different truncated *Hoxd-13* viruses to determine whether the absence of this domain would have an effect on the misexpression phenotype. One virus (M1) corresponds to a putative alternative transcript missing the first 98 amino acids of the full-length transcript, the other encodes an artificial transcript, (M2), containing an additional 18 amino acids (Fig. 1A).

Embryos were injected with the two truncated viruses at stage 10. They were harvested at day 10 and their skeletons stained as for the full-length *Hoxd-13* virus. Both truncated viruses gave phenotypes similar to the full-length virus (Fig. 2B,C). The effects of misexpression of the truncated viruses were quantified by measuring the lengths of the bones and expressing them as ratios of injected bone lengths vs. contralateral bone lengths. Misexpression of the truncated viruses causes reduction in the lengths of the tibia, the femur, and the metatarsals and has no effect on the length of the phalanges (Fig. 3B,C). The M1 truncated form has a more severe effect than the M2 truncated form; for example, the M1 virus causes the tibia to be shortened an average of 74% of its normal length, while the M2 virus shortens the tibia to an average of 84% its normal length.

As with the full-length viral infection, the tibias and fibulae in affected limbs are approximately the same length. However, in absolute terms, the effect of the truncated viruses on the fibula was different from that of the full-length virus in that, while the full-length virus shortened the fibula to an average of 95% its normal length, the truncated viruses caused an average of 5% increase in length (Figs 2D, 3D). In the case of the M2 virus, all the injected fibulae were lengthened, while in a few examples injected with the M1 virus the fibulae were of normal length or slightly longer. Thus the M2 truncated form of *Hoxd-13* differentially affects the two bones of the lower leg, lengthening the fibula while shortening the tibia.

Fig. 3. Box plots showing bone length ratios of *Hoxd-13* injected and contralateral hindlimbs on day 10 of development. (A) Right hindlimbs were injected at stage 10 with the full-length X *Hoxd-13* virus, and embryos were allowed to develop to day 10, at which time the skeletons were stained with Alcian blue and the lengths of the bones of the hindlimb were measured. *n*=33 embryos. For each bone, the length (in millimeters) of the right hindlimb bone was divided by the length of the contralateral bone to determine the ratio of affected vs. unaffected bones. For the metatarsal measurements, the third metatarsal was measured. For the digit measurements, all the phalanges of digit III were measured together without individual dissection. (B) Ratio data for embryos injected with the M1 truncated *Hoxd-13* virus. Data was collected as for A. *n*=23 embryos. (C) Ratio data for embryos injected with the M2

truncated *Hoxd-13* virus. Data was collected as for A. *n*=26 embryos. Kaleidagraph™ was used to generate the box plots, which are a statistical plot in which, for each column, 50% of the data is enclosed within the box and the line within the box indicates the median. The bars extend to include data points which lie outside the boxed data within an interval which is defined as 1.5 times the range of values which define the box. Any data point which lies outside this interval is represented as an individual point (see Chambers et al., 1983 for discussion of box plots). (D) The M1 and M2 truncated viruses cause lengthening of the fibula. The fibula boxes for each virus are replotted next to each other on the same plot to point out that while, on average, the full-length X virus causes a shortening of the fibula, the M1 and M2 truncated viruses cause a lengthening of the fibula. A Student's *t*-test was used to determine the probabilities that the injected bone lengths did not differ significantly from the contralateral bone lengths; for the tibia, femur, and metatarsal measurements, $P<0.001$; for the fibula measurements, $P<0.02$; for the digit measurements, $P=0.36$.

Truncated *Hoxd-13* misexpression in the wing results in shortening of both the radius and ulna

When they first form, the wild-type tibia and fibula are of equal length. The difference in their relative lengths depends on interactions between the growth plates of these two bones (Hampé, 1960; Archer et al., 1983; Müller, 1989). The differential effect of the M2 truncated *Hoxd-13* misexpression on the lengths of these two bones could therefore involve disruption of that interaction. In contrast, the homologous skeletal elements of the chick wing, the radius and ulna, never interact in this manner during development and as a consequence the two bones are normally the same length.

To see whether the radius and ulna would be differentially affected by misexpression of the M2 form of *Hoxd-13*, we injected the full-length X virus and the M2 virus in the presumptive forelimb at stage 10 and allowed embryos to develop to day 10. The humerus and metacarpals are shortened and the phalanges of the digits are unaffected, as in the hindlimb injections, but, unlike the lower leg, both bones of the lower arm, the radius and ulna, are shortened to an average of 80% the contralateral bone length by both viruses (Fig. 4). This suggests that as with the full-length *Hoxd-13*, the primary effect of misexpressing the truncated M2 version of *Hoxd-13* may be a decrease in bone growth for both bones of the lower leg as well,

and the ultimate increase in fibula length could be a secondary consequence of disrupting the interactions with the tibia.

Hoxd-13 appears to act only at later stages of limb development

To understand the phenotypes resulting from *Hoxd-13* misexpression, a key question concerns the stage at which the *Hox* gene acts. This is particularly important because of the dynamic nature of the *Hox* expression patterns in the limb, which localizes their expression in different domains at different times. In order to address the time of bone development at which the full-length *Hoxd-13* and the truncated *Hoxd-13* act, injected and contralateral limbs were examined at successive days after infection to see when the phenotype first becomes apparent (Fig. 5A-C and data not shown). The onset of the phenotype was the same for all three *Hoxd-13* viruses. No differences were observed between injected and contralateral limbs at condensation stages (day 5). It was not until day 8 that the phenotype first manifests itself, which interestingly is approximately the same time that the tibia and fibula normally become different in length. To examine the kinetics of the phenotype quantitatively, the lengths of the bones were measured. The lengths of the injected tibia and fibula were plotted relative to the contralateral tibia or fibula length, which
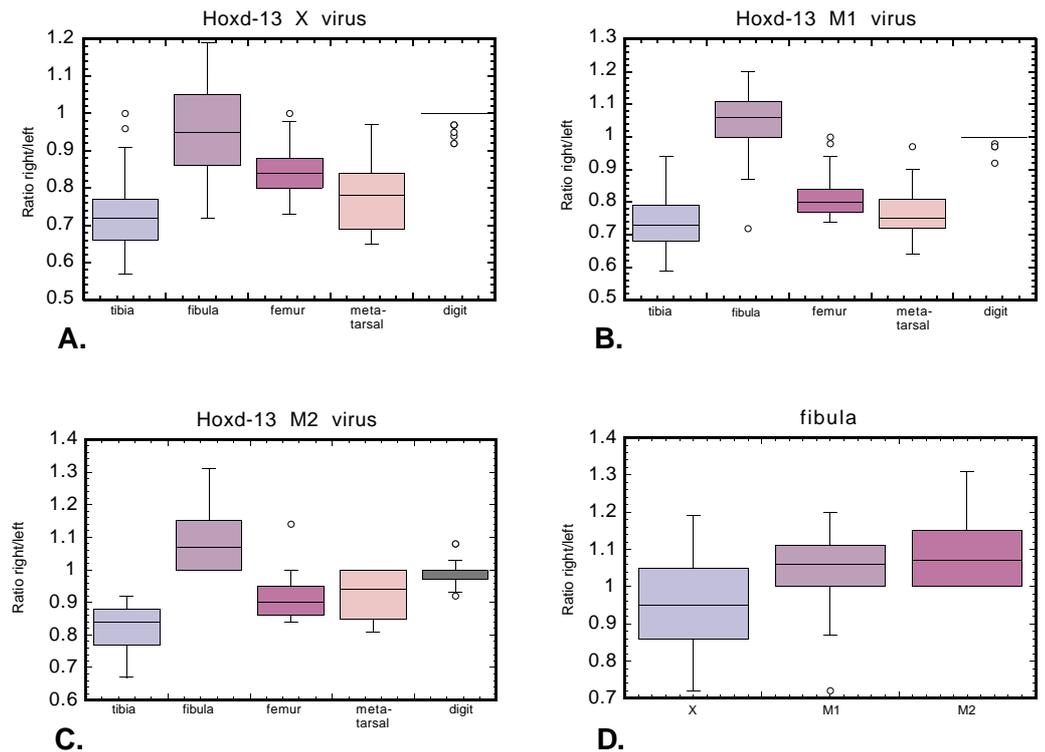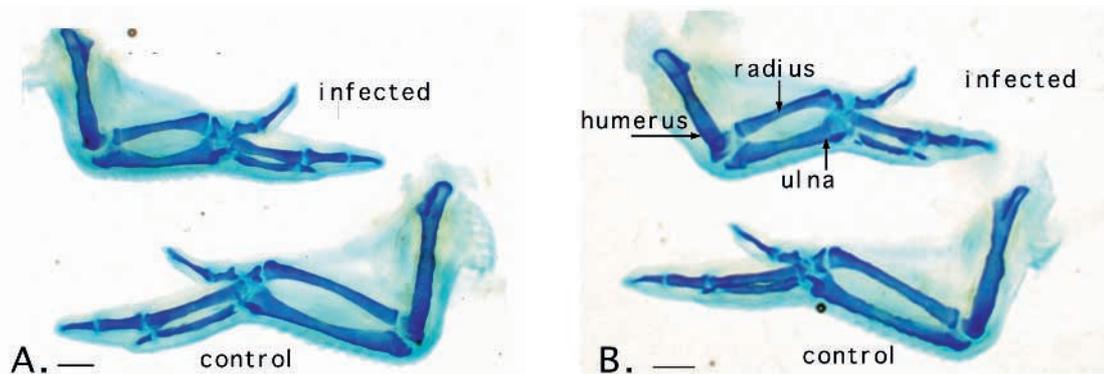
Fig. 4. *Hoxd-13* forelimb misexpression phenotype. Dorsal view of wing skeletons, harvested at day 10 and stained with Alcian blue and cleared with KOH-glycerol. In each case, the right injected wing is pictured above its contralateral uninjected control limb. Stage 10 embryos were injected in the presumptive wing region with either (A) the full-length X *Hoxd-13* virus, or (B) the M2 truncated *Hoxd-13* virus. Scale bars, 0.9 mm.

is a function of the age of the embryo but also accounts for genetic variability in the size of individual embryos (Fig. 6A,B). At early times after condensation, from days 5-7, the lengths of the tibia and fibula are the same as the contralateral bones. As the bones grow from days 7-12, the infected tibia is seen to be progressively shorter than the contralateral tibia. The affected fibula is shorter than the contralateral fibula from days 7-9, but in some embryos between days 9 and 12 the injected fibula can grow longer than the contralateral fibula (Fig. 6B). This analysis suggests that the change in cellular behavior that results in the *Hoxd-13* phenotype occurs late, during the growth phase of the cartilage elements of the lower limb.

The *Hoxd-11* misexpression phenotype is apparent at condensation stages in the foot

In contrast to the *Hoxd-13* misexpression phenotype, the phenotype of *Hoxd-11* misexpression was previously reported to include an extra segment in digit I (Morgan et al., 1992). This change must occur prior to the cartilage growth phase, at the time of initial cartilage condensation. To verify this conclusion, we re-analyzed the *Hoxd-11* misexpression phenotype at successive days after infection (Fig. 5D-F). It is apparent that the *Hoxd-11*-induced alterations are present in digit I at the earliest time of condensation (Fig. 5D, arrow).

Fig. 5. (A-C) *Hoxd-13* acts late in skeletal development. Stage 10 embryos were injected into the right presumptive hindlimb with the full-length X *Hoxd-13* virus and harvested at successive days after infection. The skeletons were stained with Alcian blue and cleared in KOH-glycerol. Limbs are pictured in frontal view, with the injected limb shown to the left of the contralateral limb. (A) day 5 limbs; (B) day 6 limbs; (C) day 8 limbs. (D-F) The *Hoxd-11* misexpression phenotype is apparent early at the time of condensation. Frontal view of lower leg and foot skeletons, stained with Alcian blue and cleared with KOH-glycerol. In each case, the injected right hindlimb is pictured to the left of its contralateral uninjected control limb. The embryos were injected at

stage 10 with *Hoxd-11* virus and harvested at (D) day 6, (E) day 7, or (F) day 9. The arrow points to the abnormal digit I condensation in D. Scale bars: (A) 0.17mm; (B) 0.33 mm; (C) 0.4 mm; (D) 0.33 mm; (E) 0.42 mm; (F) 0.57 mm.
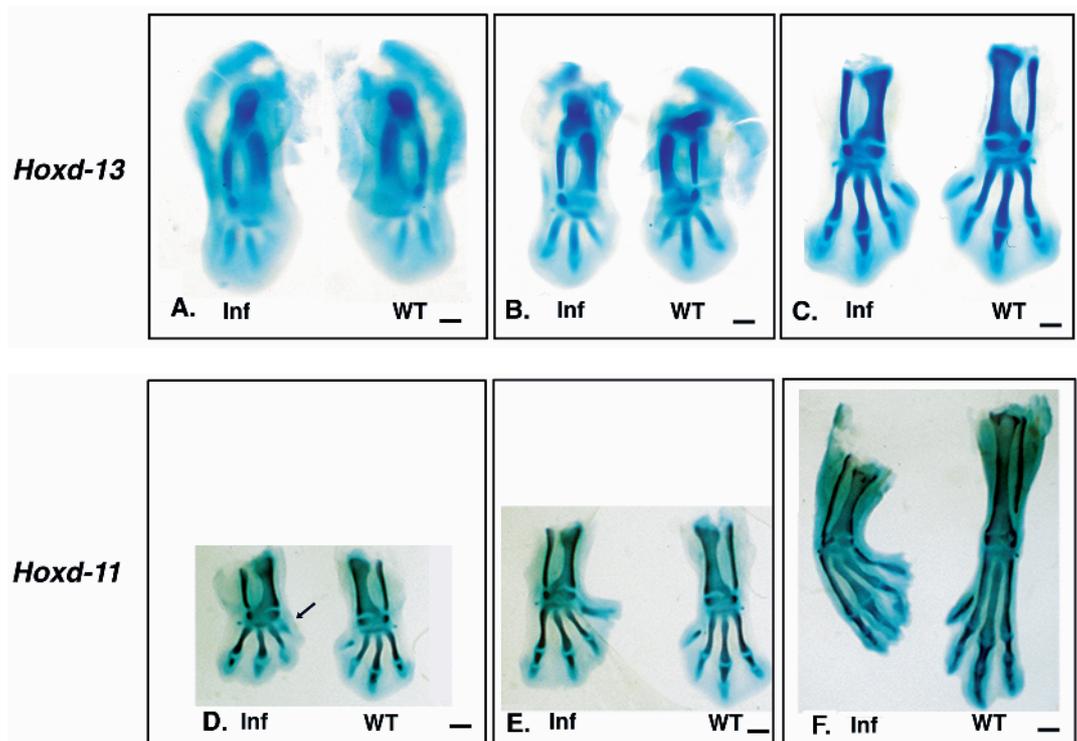
Fig. 6. (A,B) *Hoxd-13* has its first effects during the growth phase of bone development when the tibia and fibula normally begin growing at different rates. Stage 10 embryos were injected into the right presumptive hindlimb with the full-length X *Hoxd-13* virus and harvested at successive days after infection. The skeletons were stained with Alcian blue and cleared in KOH-glycerol. The lengths of the limb bones were measured, and the lengths of the infected tibias (A) and fibulae (B) are expressed as a function of the length of the corresponding contralateral bone. (C,D) *Hoxd-11* misexpression also has late effects on the lengths of the tibia and fibula. Stage 10 embryos were injected into the right presumptive hindlimb with the *Hoxd-11* virus and harvested at successive days after infection. The skeletons were stained with Alcian Blue and cleared in KOH-glycerol. The lengths of the limb bones were measured, and the lengths of the infected tibias (C) and fibulae (D) are expressed as a function of the length of the corresponding contralateral bone. In each plot, the diagonal line with a slope of one represents where the points would lie for wild-type embryos whose corresponding limb bones are the same length.
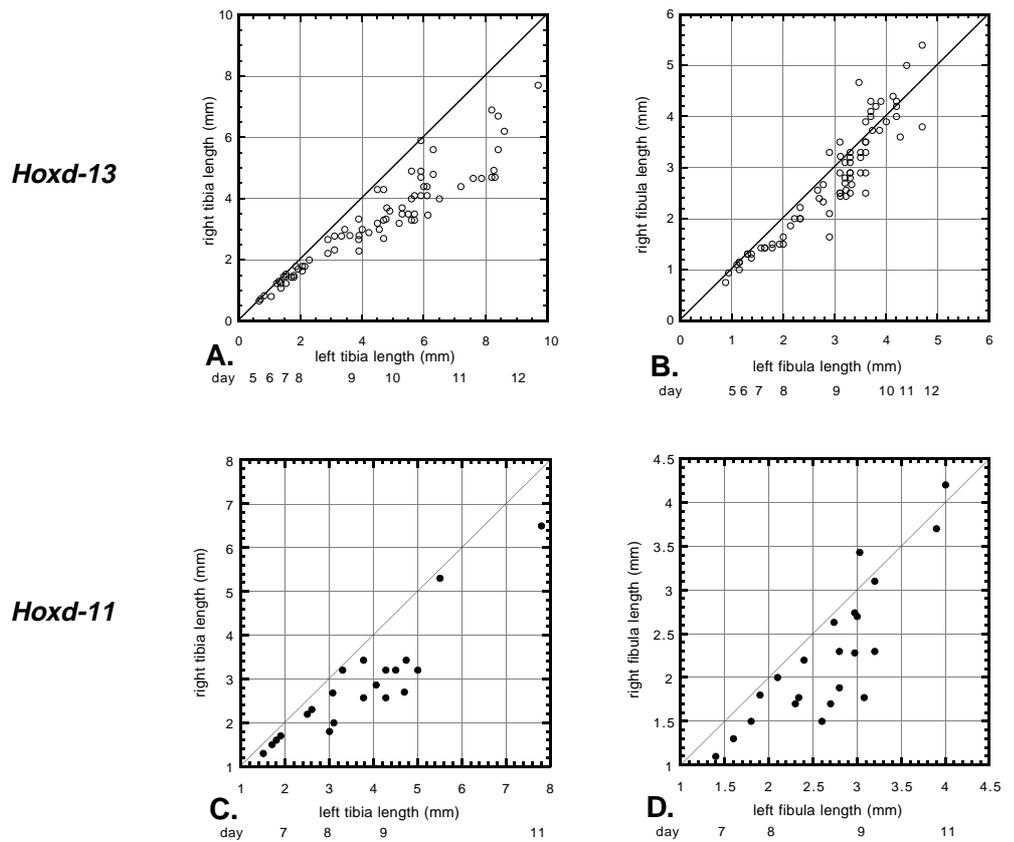
Hoxd-11 also has later effects on the morphology of the limb

Interestingly, we also observe a decrease in the tibia/fibula length after infection with the *Hoxd-11* virus (Figs 5F, 6C,D; see also Morgan et al., 1992). This raised the possibility that *Hoxd-11* might be capable of affecting limb development both at the condensation stage and later during bone elongation. Alternatively, the effects on the lower leg and on the foot could be due to the activity of this *Hox* gene at the same stage of bone formation. We tested these possibilities by infecting limbs at later developmental stages with the *Hoxd-11* virus. We reasoned that if alterations in the foot and lower leg were both due to *Hox* activity at the same stage of bone development, then we would expect later infections to result in foot but not lower leg phenotypes, since the skeleton of the foot forms later than the that of the leg.

The result of injecting *Hoxd-11* virus at stage 22 and harvesting at day 10 argues against this model. While stage 10 injections affected both the leg and foot, stage 22 injections resulted in affected legs, but normal feet (Table 1). The ability of late infections to affect proximal elements, at a time when condensation can no longer be altered even in distal regions, implies that the effect of *Hoxd-11* on proximal bones occurs at a later stage of bone formation.

We conclude from this experiment that *Hox* genes can affect both condensation and growth stages of bone development, therefore contributing in at least two ways to the establishment and elaboration of pattern.

Changes in tibia and fibula lengths are due to changes in cell proliferation

It is important to understand the mechanism by which misexpression of *Hoxd-13* leads to the observed alterations in pattern of the skeletal elements. It has been proposed that *Hox* genes determine pattern by controlling the rates of cell proliferation in regions where they are expressed (Duboule, 1995). While this has been offered as an explanation for the phenotypes observed in various mutant animals, there has been no direct proof that the alteration of *Hox* gene expression results in a change in cell proliferation. Other possibilities exist to explain how the lengths of the bones are altered; for example, a change in the size of the initial condensation or a change in the rate of cell death could also conceivably lead to changes in bone morphology.

**Table 1. *Hoxd-11* infection has both late and early effects on the limb**

Stage infected	No. of limbs affected	
	Lower leg	Foot
10	35/40	35/40
22	25/28	0/28

Embryos were injected at either stage 10 or stage 22 in the right hindlimb with a *Hoxd-11* virus and allowed to develop to day 10. The skeletons were stained with Alcian blue and cleared with KOH-glycerol, and the phenotypes in the leg were assessed for changes in the lower leg (tibia/fibula) and the foot. 40 embryos were injected at stage 10, and 28 embryos were injected at stage 22.
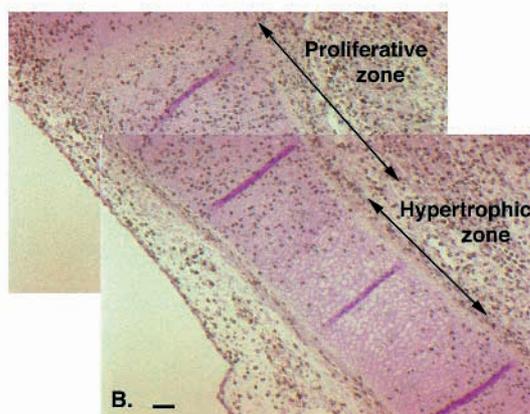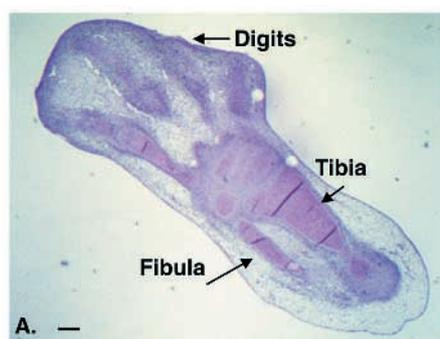
Fig. 7. [³H]Thymidine labeling of day 5.5 hindlimbs. Embryos were injected in the presumptive right hindlimb region at stage 10 with the M2 truncated *Hoxd-13* virus, and then labeled in ovo with [³H]thymidine 4 days later for 8 hours. The hindlimbs were embedded next to each other in paraffin, sectioned in the frontal plane, and the sections were exposed to photographic emulsion to detect labeled cells. (A) Low power (5× objective) view of a representative section through a contralateral hindlimb. Scale bar: 0.09 mm. (B) High power (20× objective) view of a section through the distal end of the tibia of an injected hindlimb. The black dots in the proliferative zone indicate reduction of silver grains over nuclei labeled with [³H]thymidine; note the largely unlabeled hypertrophic cartilage in the middle of the tibia. To determine the number of labeled cells in the tibia for this section, labeled cells were counted within the tibia from the midpoint in the hypertrophic cartilage to the distal end of the tibia. Scale bar, 0.019 mm. (C) Results of [³H]thymidine labeling experiment. For the tibias and fibulae of each embryo, the total number of labeled cells was derived by summing the number of labeled cells for every fifth section through that bone.
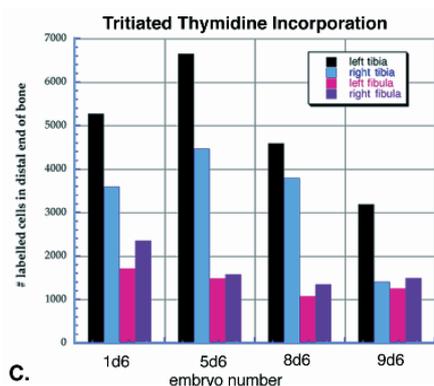
We tested whether the misexpression of *Hoxd-13* causes changes in the number of dividing cells in the proliferative zones of the growing cartilage. The M2 truncated form of *Hoxd-13* was of particular interest in this regard since its misexpression results in lengthening of the fibula and shortening of the tibia, allowing us to determine if these opposite effects are both a result of altered proliferation rates. Since we expected that changes on a cellular level should be detectable prior to gross morphological changes, we examined the effects of the M2 *Hoxd-13* virus on day 6, just prior to the earliest observed changes in overall bone length. We infected the right hindlimb at stage 10, allowed the embryos to develop 4 days, then injected the yolks with [³H]thymidine at day 5.5 to label dividing cells in the embryo. After 8 hours of exposure to [³H]thymidine, the embryos were killed, the hindlimbs were sectioned and the slides were dipped in radiographic emulsion to determine the location of labeled cells (Fig. 7A,B). We counted labeled cells in the proliferative zones at the distal ends of the tibia and fibula and compared the numbers for injected and contralateral limbs. We found a decrease in the number of labeled cells in the tibia of the injected limb, and a small increase in the number of labeled cells in the fibula of the injected limb (Fig. 7C). We therefore conclude that the mechanism by which *Hoxd-13* causes an alteration in the lengths of these bones is by changing the rates of their proliferation.

DISCUSSION

In this paper we report the misexpression phenotype of *Hoxd-13* in chick limbs and compare it to the misexpression phenotype of *Hoxd-11*. We find that *Hox* genes can affect two different stages of skeletal development, *Hoxd-11* affecting both the early condensation stage in the foot and the later growth phase in the lower leg, and *Hoxd-13* acting exclusively during the late growth phase of bone development.

Hoxd-11 misexpression has both early and late effects on limb bones

The early effect of *Hoxd-11* misexpression is to produce a larger than normal digit I condensation which undergoes an additional segmentation event, producing a toe with two phalanges instead of one (Morgan et al., 1992). While the mechanism was not addressed in this study, the larger initial condensation could be caused either by recruitment of more mesenchyme to the condensation or by an increase in proliferation within the early condensation, under the influence of the *Hox* gene expression. The additional segmentation is likely to be controlled by the ectopic expression of signals such as GDF-5 (Storm et al., 1994), and not be a direct consequence of the size of the initial condensation. With regard to this early effect, *Hoxd-11* appears to produce a dominant gain-of-function phenotype in the anterior-most region of the foot, where it is not normally expressed.

The effect of *Hoxd-11* misexpression on the lower leg segment appears to be a consequence of disrupting growth at a later stage of bone development, since the effect on these bones is not apparent at the time of condensation, and since the same phenotype is produced by infection at a later stage of limb development. The strongest late effect of *Hoxd-11* misexpression is on the tibia, which forms in a domain that does not normally express *Hoxd-11*. However, there is a significant effect on the fibula as well, even though the normal domain of *Hoxd-11* expression encompasses the fibula region. This could imply that the level of *Hoxd-11* in the wild-type fibula is not saturating. It is also possible that the length of the fibula is indirectly influenced by the growth of the adjacent

tibia and not directly due to the virus in regions where *Hoxd-11* is already expressed. Alternatively, the ectopic and endogenous proteins could have different properties, since a mouse *Hoxd-11* cDNA was used to construct the virus used in these experiments. Although the homeodomains are identical between chick and mouse *Hoxd-11*, there are differences in their N-termini, which could lead to differences in their growth regulation properties.

Hoxd-13 misexpression causes decreased growth of chondrocytes

The phenotypic consequence of *Hoxd-13* misexpression is to decrease the lengths of the bones in regions of the limb where that gene is not normally expressed. A similar phenotype was observed in the forearms of mice in which ectopic *Hoxd-13* expression was caused by relocation of a *Hoxd-11* transgene in the vicinity of the *Hoxd-13* locus (van der Hoeven et al., 1996). To determine the origin of this phenotype, we examined *Hoxd-13*-infected chick limbs at various times of development. The phenotype is not manifest during the aggregation of pre-cartilagenous mesenchyme, but first becomes apparent during chondrogenic growth. [³H]Thymidine incorporation verified that this alteration is due to a decrease in proliferation in the growth plate of the growing tibia. This mechanism differs from a previous suggestion that the primary role of *Hoxd-13* is to regulate timing of embryonic programs (Dollé et al., 1993).

Mice carrying mutations in *Hox* genes also have decreased growth of long bones in the limb. Therefore, the *Hoxd-13* misexpression phenotype can be interpreted as resulting from a dominant-negative interference with the activity of other *Hox* genes expressed in the proximal limb where *Hoxd-13* is not normally expressed. However, a paradox exists if one considers that a decrease in growth also results from the loss-of-function of *Hoxd-13* itself (Dollé et al., 1993). The decrease in growth in the distal regions of the limb in the *Hoxd-13* loss-of-function, and the proximal decrease in growth in the *Hoxd-13* misexpression can be reconciled by a model in which all *Hox* genes positively regulate growth to varying degrees and compete for regulation of the same growth gene targets. Both the gain- and loss-of-function phenotypes can be explained if one assumes that *Hoxd-13,* normally expressed in the foot and hand, promotes growth less strongly than *Hoxd-11* and *Hoxa-11*, normally expressed in the lower leg and arm. This assumption is consistent with the normal anatomy of the bones in these regions, in which the foot and hand bones are normally shorter than the lower leg and arm bones. The deletion of *Hoxd-13* results in the loss of a growth-promoting gene normally expressed in the hand and foot, and, hence a growth reduction of some bones in that region is observed. In the case of the misexpression of this gene, there is a shortening of bones in the lower leg because the ectopic *Hoxd-13* competes with the normally expressed *Hoxd-11* and *Hoxa-11* for growth-promoting target genes, but does not activate as strongly. This is equivalent to a partial loss of *Hoxd-11/Hoxa-11* activity. van der Hoeven et al. (1996) similarly concluded that the *Hoxd-13* phenotype results from dominant-negative activity in transgenic mouse limbs. It is worth noting that there is a significant difference between *Hoxd-11/Hoxd-12* expression in the wing and leg (Mackem and Mahon, 1991; Nelson et al., 1996). The expression of these genes disappears sooner from the zeugopod region of the leg, hence a dominant competition between *Hoxd-13* misexpression and endogenous *Hoxd-11* could differ in these two contexts, producing differential effects on the fibula and the ulna.

Hoxd-13 misexpression phenocopies the result of barrier insertions in the lower leg

Misexpression of *Hoxd-13* results in a decrease in the lengths of the bones in regions where it is not normally expressed. In most cases, the degree to which different bones are affected presumably reflects differences in the complement of other *Hox* genes expressed in each limb segment. An interesting exception is the lower leg; while the tibia is the most severely affected bone in the leg (72% of normal length by day 10), the fibula is far less affected (95% of normal length). We believe this apparent difference in growth control is a secondary consequence of altering interactions between the growing tibia and fibula. In the wild-type hindlimb, the tibia and fibula condense at 4-5 days of development and remain equal in length until about 7 days of development. At that point the tibia normally begins lengthening at a much faster rate. In contrast, in *Hoxd-13* infected limbs these two bones remain approximately equal in length up to at least 12 days of development. This misexpression phenotype is nearly identicle to a phenotype produced by the surgical insertion of a mica barrier between the presumptive tibia and fibula at stage 24 (Hampé, 1960). It is thought that the barrier prevents a late interaction between the tibial growth plate and the fibula, which normally results in the loss of the distal fibular growth plate (Archer et al., 1983; Müller, 1989).

We think that it is likely that the primary effect of *Hoxd-13* misexpression is a significant decrease in growth of both bones in the lower leg. This reduction in growth in the lower leg secondarily disrupts the interaction between the growth plates of the tibia and fibula, allowing the fibula to retain its distal growth plate and grow to greater lengths than it does in wild-type limbs. Thus, in infected limbs, the size of the fibula is modified both by the retention of the growth plate and by the overall reduction in proliferation, resulting in fibulae that are only reduced in length by an average of 5% compared to the contralateral fibulae. It is likely that this 5% decrease in fibular length would be a larger reduction if the lengths of the infected fibulae were compared to the lengths of wild-type fibulae whose interactions with tibias had been prevented (as by the barrier insertions). By this interpretation, the direct effect of *Hoxd-13* on the tibia and fibula may be equivalent, as it is on the radius and ulna.

N-terminal truncations of *Hoxd-13* have milder misexpression phenotypes

Misexpression of weaker alleles of *Hoxd-13*, constructed by making N-terminal truncations, have slightly less effect on bone growth. As a consequence, in many cases (especially with the M2 virus), the infected fibula is longer than the contralateral fibula because the reduction of growth directly caused by *Hoxd-13* misexpression is apparently less than the increase in growth resulting from the blocked growth plate interaction with the tibia.

The truncated forms were designed to test the requirement for the alanine-repeats and the rest of the N terminus for *Hoxd-13* function. This region was suggested to be significant for function because expansions of the alanine repeat result in synpolydactyly in humans (Muragaki et al., 1996). The deletion

of this domain had only a mild effect on the chick misexpression phenotype, suggesting that the N terminus plays only a minimal role in the activity of the protein. It could be argued that this does not reflect the true importance of the N-terminal domain of the protein in its normal expression domains, since the misexpressed gene appears to act as a dominant-negative in regions where it is not normally expressed. However, if the truncated alleles were simply blocking other *Hox* genes one would have expected to see a phenotype in the digit region as well, where *Hoxd-13* is normally expressed. Additional experiments, misexpressing variants with artificially expanded alanine repeats, will provide more information concerning the role of this domain.

Conclusions

Our results indicate that *Hox* genes can affect at least two distinct stages of bone development when misexpressed in the developing limb. The consequence of early misexpression of *Hoxd-11* is to alter the condensation and segmentation of digit I such that it develops identically to digit II, which normally expresses *Hoxd-11*. The simplest interpretation is therefore that *Hoxd-11* ectopically affects digit I by altering the same processes that it normally controls in patterning digit II. The effect of misexpression of *Hoxd-13* is to alter the late growth phase of bones in regions of the limb where it is not normally active. This is likely due to an interference with the late action of other endogenous *Hox* genes expressed in these regions. On a cellular level, our [^{3}H]thymidine experiments directly demonstrate that one of the roles played by *Hox* genes is to regulate the rate of proliferation in the growth plates of growing skeletal elements. Thus, although the phenotypes analyzed for *Hoxd-13* and *Hoxd-11* are the result of ectopic expression, it is likely that they reflect different stages of bone development normally regulated by the *Hox* genes.

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