Analysis of Hox gene expression in the chick limb bud

Craig E. Nelson1,*, Bruce A. Morgan2,*, Ann C. Burke1, Ed Lauffer1, Enrico DiMambro1, L. Charles Murtaugh1, Ellen Gonzales2, Lino Tessarollo2, Luis F. Parada4 and Cliff Tabin1,†

1Department of Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115, USA
2Cutaneous Biology Research Center, Massachusetts General Hospital, Harvard Medical School, MGH East, Building 149, Charlestown, MA 02129, USA
3Molecular Embryology Section, Building 539, ABL-BRP, NCI-FCRDC, Frederick, MD 21702, USA
4Center for Developmental Biology, University of Texas South West Medical Center, 6000 Harry Hine Boulevard, Dallas, TX 75235-9133, USA

*These authors contributed equally to this manuscript
†Author for correspondence

SUMMARY

The vertebrate Hox genes have been shown to be important for patterning the primary and secondary axes of the developing vertebrate embryo. The function of these genes along the primary axis of the embryo has been generally interpreted in the context of positional specification and homeotic transformation of axial structures. The way in which these genes are expressed and function during the development of the secondary axes, particularly the limb, is less clear. In order to provide a reference for understanding the role of the Hox genes in limb patterning, we isolated clones of 23 Hox genes expressed during limb development, characterized their expression patterns and analyzed their regulation by the signalling centers which pattern the limb. The expression patterns of the Abd-B-related Hoxa and Hoxd genes have previously been partially characterized; however, our study reveals that these genes are expressed in patterns more dynamic and complex than generally appreciated, only transiently approximating simple, concentric, nested domains. Detailed analysis of these patterns suggests that the expression of each of the Hoxa and Hoxd genes is regulated in up to three independent phases. Each of these phases appears to be associated with the specification and patterning of one of the proximodistal segments of the limb (upper arm, lower arm and hand). Interestingly, in the last of these phases, the expression of the Hoxd genes violates the general rule of spatial and temporal colinearity of Hox gene expression with gene order along the chromosome.

In contrast to the Abd-B-related Hoxa and Hoxd genes, which are expressed in both the fore and hind limbs, different sets of Hoxc genes are expressed in the two limbs. There is a correlation between the relative position of these genes along the chromosome and the axial level of the limb bud in which they are expressed. The more 3′ genes are expressed in the fore limb bud while the 5′ genes are expressed in the hind limb bud; intermediate genes are transcribed in both limbs. However, there is no clear correlation between the relative position of the genes along the chromosome and their expression domains within the limb. With the exception of Hoxc-11, which is transcribed in a posterior portion of the hind limb, Hoxc gene expression is restricted to the anterior/proximal portion of the limb bud. Importantly, comparison of the distributions of Hoxc-6 RNA and protein products reveals posttranscriptional regulation of this gene, suggesting that caution must be exercised in interpreting the functional significance of the RNA distribution of any of the vertebrate Hox genes.

To understand the genesis of the complex patterns of Hox gene expression in the limb bud, we examined the propagation of Hox gene expression relative to cell proliferation. We find that shifts in Hox gene expression cannot be attributed to passive expansion due to cell proliferation. Rather, phase-specific Hox gene expression patterns appear to result from a context-dependent response of the limb mesoderm to Sonic hedgehog. Sonic hedgehog (the patterning signal from the Zone of Polarizing Activity) is known to be able to activate Hoxd gene expression in the limb. Although we find that Sonic hedgehog is capable of initiating and polarizing Hoxd gene expression during both of the latter two phases of Hox gene expression, the specific patterns induced are not determined by the signal, but depend upon the temporal context of the mesoderm receiving the signal. Misexpression of Sonic hedgehog also reveals that Hoxb-9, which is normally excluded from the posterior mesenchyme of the leg, is negatively regulated by Sonic hedgehog and that Hoxc-11, which is expressed in the posterior portion of the leg, is not affected by Sonic hedgehog and hence is not required to pattern the skeletal elements of the lower leg.

Key words: Hox, limb, pattern, chick, Sonic hedgehog
INTRODUCTION

The vertebrate Hox genes are a highly related subset of the homeobox containing transcription factors that are physically linked in four chromosomal clusters (Hoxa, Hoxb, Hoxc, Hoxd). Sequence comparison between members of the four clusters suggests that they evolved from a single ancestral cluster of genes. Therefore, individual Hox genes within each cluster have direct homologs in the other three clusters. There are 13 such sets of ancestrally related homologs, referred to as paralog groups. To display the relationships between the members of the Hox gene family, they are often represented as a 4 by 13 array, in which the horizontal axis represents physical linkage and relative chromosomal position while the vertical axis represents gene similarity and, presumably, common ancestry. Any gene in this array is specified by a letter denoting its chromosomal location and a number conveying its homology and ancestral relationship to paralogs within other clusters (Krumlauf, 1992; Scott, 1992).

All of the Hox genes have specific domains of expression along the anterior/posterior (primary) axis of the embryo (Kessel and Gruss, 1990). In species which have markedly different body plans, the boundaries of these axial expression domains are shifted along the axis to positions that reflect the shifts in morphological characters (Burke et al., 1995). Furthermore, both loss- and gain-of-function experiments have demonstrated the important role of these genes in specifying morphology within different regions of the primary axis. Studies of the role of Hox genes in the specification of vertebral morphologies have indicated that, in many cases, paralogous genes have similar domains of expression and cooperate in the morphogenesis of specific vertebral types (Krumlauf, 1993).

Hox genes are also expressed in restricted domains along the secondary axes of the embryo such as the genital ridge and the limb buds (Dolle et al., 1989, 1991; Izpisua-Belmonte et al., 1991). In contrast to the primary axis, paralogous genes are not generally expressed in similar domains in the limb bud. Gain- and loss-of-function experiments have demonstrated the importance of Hox genes in the specification of limb morphology (Morgan et al., 1992; Dolle et al., 1993; Davis and Capecchi, 1994; Davis et al., 1995; Favier et al., 1995). The results of these experiments, however, cannot be readily accommodated by simple homeotic models.

Accurate interpretation of genetic studies depends on understanding the normal expression and regulation of the genes in question. In the developing limb, the expression patterns of the Hox genes have been partially characterized and some of the signals responsible for the generation of these patterns have been identified. For example, expression studies have established that the Hoxd genes are expressed in a nested set centered on the Zone of Polarizing Activity (ZPA) at the posterior/distal tip of the developing limb bud (Dolle et al., 1989; Izpisua-Belmonte et al., 1991). The relative anterior boundaries of expression of the Hoxd genes in the early limb bud are consistent with their order along the chromosome. This phenomenon, observed along both the primary axis and secondary axes such as limb buds and the genital ridge (Dolle et al., 1991), is known as spatial colinearity. The Hoxa genes have also been shown to be expressed in a spatially collinear manner in the limb bud (Yokouchi et al., 1991). Unlike the Hoxd genes, however, the Hoxa genes are expressed in restricted domains along the proximal/distal axis of the limb and are not polarized along the anterior/posterior axis of the late limb bud. Studies of the normal expression of these genes have established the boundaries of the Hoxa and the Hoxd genes relative to each other and to the condensing cartilage elements of the limb bud (Yokouchi et al., 1991). These reports of Hoxa and Hoxd expression focussed on the fore limb; comprehensive comparison of fore- and hind-limb expression patterns has not been reported (but see Mackem and Mahon, 1991; Mackem et al., 1993).

Schematics of Hox gene expression derived from these studies depict the progression of Hox expression within the limb as a continuous process from early to late limb bud development (Nohno et al., 1991; Tabin, 1991). More recent studies, however, suggest that the expression of the Hox genes in the limb bud might be more complex than originally appreciated (Mackem and Mahon, 1991; Nohno et al., 1991; Tabin, 1991; Mackem et al., 1993). As Hox expression patterns evolve, their relative expression boundaries do not fit the stereotypic view of concentric nested domains. Moreover, in late limb development, some of the Hox expression domains divide into distinct regions (Duboule, 1994). This spatial separation might be indicative of independent regulation of expression within these regions.

Experiments addressing the regulation of Hox gene expression during limb development suggest that the product of the Sonic hedgehog gene, expressed in the ZPA (a region of the posterior mesoderm), combined with Fibroblast Growth Factors (FGFs) produced in the overlying Apical Ectodermal Ridge (AER), are responsible for initiating and possibly regulating Hox gene expression (Riddle et al., 1993; Laufer et al., 1994; Niswander et al., 1994). Application of retinoic acid, transplantation of the ZPA and ectopic expression of Sonic hedgehog all elicit ectopic expression of the Hoxd genes (Izpisua-Belmonte et al., 1991; Nohno et al., 1991; Riddle et al., 1993). Induced Hox gene expression mirrors the endogenous expression patterns and precedes the formation of ectopic skeletal elements (Laufer et al., 1994). Coordinated patterning by the ZPA and the AER (Summerbell et al., 1973; Izpisua-Belmonte et al., 1992) and retrovirally mediated expression of Sonic at the anterior margin of a limb bud denuded of its AER, reveal that Sonic requires the influence of the AER to induce the expression of the Hoxd genes (Laufer et al., 1994). It has been further demonstrated that the competence of the denuded mesoderm to respond to the Sonic signal by expressing Hox genes can be rescued by ectopically applied FGF (Laufer et al., 1994). The combined results of these studies suggest that signals from the AER and the ZPA coordinate the Hox gene expression in the developing limb bud.

Considerably less is known about the expression and regulation of the Hoxb and Hoxc genes during limb development. However, it has been reported that expression of individual Hoxb and Hoxc genes is restricted to either the fore or the hind limb bud (Cho et al., 1988; Oliver et al., 1988a,b; Wedden et al., 1989; Ersselius et al., 1990; Molven et al., 1990; Peterson et al., 1992; Wall et al., 1992; Bittner et al., 1993; Charite et al., 1994; Peterson et al., 1994), suggesting that these genes might play distinct roles in patterning the limb from the members of the Hoxa and Hoxd clusters.

In summary, although aspects of the limb expression of many of the Hox genes have been reported in the literature, it
is necessary to have a more comprehensive description of Hox gene expression in the limb to fully understand experimental phenotypes and to understand the mechanisms controlling Hox gene expression, thus patterning the limb. To this end, we undertook the cloning of the Hox genes expressed in the chick limb bud, and here report their expression patterns and aspects of their regulation by the signalling centers of the limb bud.

**MATERIALS AND METHODS**

Unless otherwise noted, all standard cloning techniques were performed according to Ausubel et al. (1989), and all enzymes and molecular biology reagents were obtained from Boehringer Mannheim Biochemicals. Sequences were analyzed using both GCG (Devereux et al., 1984) and DNASTAR software (Madison, WI). Searches for related sequences were done through the BLAST (Altschul et al., 1990) network service provided by the National Center for Biotechnology Information.

**PCR**

Three degenerate upstream primers (AB507, AB5056, AB508) were paired with a single downstream primer (BAM9) to PCR-amplify homeobox-containing sequences from chick genomic DNA. Upstream primers were directed against the following motifs: AB507, RKKRKPY; AB5056, RKKRCPY; AB508, RKKRVPY; BAM9, WFQNRRA. Sequences were as follows: AB507, C/AGIAAA/GAAA/GC/AGIAAA/GGCTTA; AB5056, C/AGIAAA/GAAA/GC/AGITGC/TGCCTA; AB508, C/AGIAAA/GAAA/GC/AGITGC/TGCCTA; BAM9, IGCICG/TICT/GG/ATTT/CCTGG/AAACCA; (I=inosine). PCR was done with Boehringer Mannheim Taq polymerase in 1x Boehringer reaction buffer. Primers were included at 10 μM and annealed at 45°C. 40 rounds of PCR were done on 1 μg of chick genomic DNA in a 50 μl reaction. Product of the expected size (150 bp) was isolated on a 2% agarose gel, re-amplified with kinased primers and cloned into plBluescript (Stratagene, LaJolla, California). 100 clones, representing 23 unique homeobox-containing sequences, were prepared and sequenced. These clones were pooled and used to screen a stage 26 chick wing and leg cDNA library.

**Library construction and screening**

An oligo(dT)-primed cDNA library was constructed from stage 26 wing and leg bud poly(A)-selected RNA. Library construction was essentially as outlined by Ausubel et al. (1989). The library was constructed and packaged in ZAP2 (Stratagene) following the manufacturers recommended protocols. Screening was done based on hybridization conditions outlined by Church and Gilbert (1984). Hybridization was done at 42°C and washes at 50°C. Approximately 250 phage clones were isolated and in vivo excision was performed following manufacturers’ protocols.

**Determination of the identity of the Hox clones**

The isolated clones were initially sequenced with a degenerate

![Fig. 1. Verification of the identity of the cloned chicken Hox genes. (A) Chick (chk) Hox homeobox sequences aligned with orthologs from other vertebrate species (hum, mus). The conceptually translated homeobox sequence of each of the chick Hox genes reported in this paper is displayed aligned with the same region from the nearest ortholog in the database. (B) A dendrogram derived by comparing putative full length protein sequences of the chick genes reported here with the same region of the nearest ortholog and nearest homologue in the database. In this diagram, the relative length of a branch represents the divergence of that sequence from the common sequence represented by the node from which the branch stems. Sequences stemming from a common node are more similar to one another than sequences removed by one or more nodes. The full length coding sequence of Hoxc-11 from a vertebrate species other than chick was not available from the database at the time of writing. These sequence comparisons and diagrams were generated using DNA Star Megalign software employing the Jotun-Hein alignment algorithm and a structural residue weight table.](image-url)
homeobox primer (BAM9). This sequence allowed putative identity assignments to be made, which were subsequently reinforced by extensive sequence analysis and comparison to published chick, human and mouse sequences.

Since none of the genes of the chicken Hoxc cluster have been previously reported, gene identity was based upon sequence similarity to putative orthologs and paralogs from chick, mouse or human. The putative protein sequence of the homeobox of these genes is compared to the homeobox sequence of the nearest mammalian ortholog in Fig. 1A. We observe no amino acid deviations within the homeobox between the chick genes and their nearest ortholog. A tree representing the similarity of putative full length protein sequences of these genes to the nearest reported paralogs and orthologs is shown in Fig. 1B. This tree also illustrates the divergence of these orthologs from each other and the nearest reported paralogs. Both comparisons strongly support the initial identifications made on the basis of nucleotide sequence similarity.

Nucleotide or protein sequence similarity alone, however, left open the possibility that these cDNAs represented highly related homeobox-containing genes not physically linked to the Hoxc cluster. Therefore the assignment of these genes to a common cluster was supported by pulsed field gel linkage analysis. Chick genomic DNA was prepared by protease digestion of CEFs in agarose. This DNA was then subject to digestion by either Sfil or BssHI and separated on

useful for visualization of the posterior extent of the anterior/proximal expression domains of these genes separate from underlying expression in the path of myoblast migration (see Fig. 3). All limb buds are oriented with anterior to the top and distal to the right. All whole mounts are dorsal views. Wing-specific expression is observed for Hoxc-4 and Hoxc-5. Both of these genes are restricted to an anterior/proximal portion of the developing fore limb bud. Whole mount in situ analysis and analysis of late stage section in situ suggests that these genes occupy approximately the same domain of the limb bud. Hoxc-5 is particularly difficult to detect in the limb bud, although a strong signal is observed in the neural tube and reasonable signals are apparent in the axial mesoderm (data not shown, see Burke et al., 1995). Because of this, our attempts to localize the transcripts of Hoxc-5 in the limb met with varying success. Clear signals were obtained most reliably in whole mounts and late stage sections. Hoxc-6 and Hoxc-8 RNAs are detected in an anterior/proximal region of both the developing wing and the leg. In the wing mesoderm both genes appear to be expressed more posteriorly than Hoxc-4. In the wing and leg the anterior/proximal domain of Hoxc-6 appears to be larger than the same domain of Hoxc-8. In the leg, this relationship is somewhat obscured by the additional expression of Hoxc-8 in a pattern that partially overlaps with the dorsal and ventral paths taken by invading myoblasts (see text). Expression of both Hoxc-6 and Hoxc-8 is also observed throughout the fore and hind limb ectoderm in sections. Hoxc-9 and Hoxc-10 are expressed only in the leg, in a proximal anterior portion of the limb bud similar to that observed for the other Hoxc genes. Hoxc-9 expression occupies the same region as that of Hoxc-6. The posterior extent of this domain of Hoxc-10 expression is difficult to discern due to expression along the dorsal and ventral paths followed by myoblasts invading the limb (see text). Under-developed whole-mount in situ selectively reveal the anterior/proximal domain of Hoxc-10. This staining suggests that the extent of the anterior/proximal domain of Hoxc-10 expression is similar to that observed for Hoxc-9. Hoxc-11 displays a distinctly different expression pattern from the other members of the Hoxc cluster. Hoxc-11 is expressed strongly in a well defined proximal, posterior domain of the leg bud reminiscent of the early expression patterns reported for distal members of the Hoxd cluster. Unlike the Hoxd genes, however, Hoxc-11 is expressed in the presumptive upper as well as the lower leg and is not expressed in the distal portion of the limb.
a 1% agarose pulsed field gel on a Bio-Rad CHEF Mapper using the auto algorithm set to separate fragments from 300 to 10 kb. A single genomic band of approximately 156 kb, from an SfiI digest, hybridized to probes from Hoxc-4, Hoxc-6, Hoxc-9 and Hoxc-10, while a single BssHII band, of approximately 35 kb, hybridized to probes from both Hoxc-10 and Hoxc-11 (data not shown). These results are consistent with the reported Hox genes being physically linked and support the putative identity assignments made on the basis of sequence similarity.

For those chick genes with no reported sequence information, but published expression patterns (Yokouchi et al., 1991), identity was also based upon agreement with previously reported chick limb expression patterns. This group includes Hoxa-10, Hoxa-11 and Hoxa-13.

Identity assignments of previously unreported genes of the chicken Hoxa, Hoxb and Hoxd clusters were based upon sequence similarity with cloned mouse and human homologs and comparison with reported limb expression patterns (where available). Genes in this group include Hoxa-9, Hoxb-9 and Hoxd-9.

Chick embryos
Fertilized standard specific white Leghorn chick eggs obtained from SPAFAS (Norwich, Connecticut) were used for all experiments. Eggs were incubated at 37.5°C and staged according to Hamburger and Hamilton (1951).

Radioactive in situ hybridizations
Radioactive in situ hybridizations were performed as described by Tessarollo et al. (1992).

Photography
Radioactive in situ hybridizations were illuminated simultaneously using transmitted blue light to illuminate the tissue and reflected red light to illuminate the silver grains of the emulsion. Slides were photographed on a Nikon Axiophot and scanned using a Kodak RFS 2035 plus film scanner into Adobe Photoshop (Mountainview, CA), where contrast was optimized using the Auto Levels function. Extensive comparison of the resultant images with direct observation of the specimens indicates that this photographic process faithfully recreates the signals seen through the microscope.

Whole-mount in situ hybridizations
Whole-mount in situ hybridizations were carried out as described by Riddle et al. (1993) and Burke et al. (1995). All probes are as indicated in Burke et al. (1995) except for the Sonic probe, which is described by Laufer et al. (1994).

Immunohistochemistry
Whole-mount antibody staining using the Xlhbox-1 antibody (Oliver et al., 1988a,b) (kindly provided by E. De Robertis), was performed as described by Burke et al. (1995).

**Fig. 3.** Expression of Hoxc-8, Hoxc-9 and Hoxc-10 in the path of myoblast migration. Neighboring transverse sections through the leg bud of a stage 23 embryo were hybridized to probes against Pax-3 (A), Hoxc-8 (B), Hoxc-9 (C) and Hoxc-10 (D). The overlap of the Hox gene signal with the Pax-3 signal in the limb indicates that these Hox genes are expressed in the dorsal and ventral paths taken by myoblasts as they invade the limb from the neighboring dermamyotome. Note that the overlap of these expression patterns is not complete and that the Hox genes are expressed not only more dorsally than Pax-3, but also in the Pax-3 negative gap between the dermamyotome and the limb bud. Sections are oriented with dorsal to the top.

**Fig. 4.** Normal and perturbed expression of Hoxb-9 and Hoxc-11. The top two rows of panels (A-H) depict the expression of Hoxb-9 (A-D) and Hoxc-11 (E-H) during normal development. In situ hybridizations to neighboring sections taken from legs at stage 22 (A,E), stage 23 (B,F) and stage 27 (C,G) show signal in red against blue tissue. (D,H) Whole mount in situ hybridizations to Hoxb-9 (D) and Hoxc-11 (H). The bottom set of panels (I-N) depicts the results of experiments testing the effect of ectopic Sonic hedgehog (LL) on the expression of these two genes. In the sectioned material, the domain of ectopic Sonic expression has been outlined in black and overlaid on neighboring sections to correlate ectopic Sonic expression with regulation of the putative target genes. As can be seen in (J), there is a split in the proximal Hoxb-9 expression domain that correlates well with the region of Sonic expression. Down-regulation of the ectopic subapical Hoxb-9 expression is also evident in the regions of highest subapical Sonic expression (J,M), (K,N). The lack of any apparent induction of Hoxc-11 expression by ectopic Sonic hedgehog. All limb buds are oriented with anterior to the top and distal to the right. All whole mounts are dorsal views.
**Vital dye labeling and analysis**

Dil (1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes, Oregon) was prepared as a 0.5% (w/v) stock in 100% ethanol, and diluted 1:9 with 0.3 M sucrose for injection. Injections were made into the progress zones of stage 19-24 chick fore limb buds; the pipette was inserted past the surface ectoderm, and enough Dil injected to produce a small, visible bolus in the mesenchyme. The embryos were photographed during or immediately after injection for a visual record of the injection procedure. Embryos were harvested 24 or 48 hours later; the injected limbs were removed, and viewed and photographed using direct light and rhodamine filters to visualize Dil staining. The figure presented in the paper was generated by photographically superimposing the signal viewed by rhodamine fluorescence onto the image seen in direct light.

**Retroviral misexpression**

*Sonic hedgehog*-carrying retrovirus (Riddle et al., 1993) was used to infect limb mesoderm subapically as described in Laufer et al. (1994). Injections were done just under the AER and embryos were allowed to develop for 48 hours prior to harvest. This protocol results in somewhat variable *Sonic* misexpression, depending on the amount of virus injected and the exact location of the injection.

**RESULTS**

**Cloning and initial characterization of the limb Hox genes**

In order to develop a complete picture of Hox gene expression during chick limb development, we attempted to isolate clones of all the Hox genes expressed in the developing chick limb. We generated chicken homeobox-specific probes, utilizing a panel of degenerate PCR primers designed against conserved regions within the homeobox of known Abd-B-related Hox genes. This primer panel was used at low stringency to PCR-amplify fragments of chick genomic DNA. Bands of the appropriate size were cloned and sequenced. Sequencing revealed 23 distinct homeodomain sequences in the PCR clone pool.

These homeodomain clones were used, as a pool, to screen a stage 27 wing and leg bud cDNA library from which approximately 250 clones were isolated. The identities of the isolated cDNAs were determined by extensive sequence comparison with previously reported chick, human and mouse sequences, as well as physical linkage and expression analysis (see Materials and Methods). Ultimately this screen yielded 23 Hox genes and two non-clustered homeobox-containing genes: Msx-1 and Msx-2. The cloned Hox genes included the Abd-B-related genes of the Hoxa and Hoxd clusters (paralogs 9 through 13) and most of the Hoxc genes (Hoxc-4 through Hoxc-11). Neither Hoxc-12 nor Hoxc-13 were obtained in our screen. This result is consistent with the lack of mesodermal limb expression of these genes observed in mice (Peterson et al., 1994).

In order to examine the expression of the Hox genes during normal limb development, in situ hybridization was carried out against whole-mount and sectioned embryos. Many of the genes examined display dynamic, complex expression patterns. In describing these patterns we make use of the following terms: upper arm/leg refers to the segment encompassing humerus/femur; lower arm/leg refers to the segment including the radius and ulna/tibia and fibula; wrist refers to the proximal carpals/tarsals; and hand/foot refers to the distal carpals and phalanges. Digital arch refers to the distal carpals. The chick fore limb is referred to as the wing or arm while the hind limb is referred to as the leg.

In situ hybridization with digoxigenin-labeled probes in whole mount and with radioactive probes in sections do not always give identical results. Whole mounts are invaluable for generating an accurate understanding of complex three-dimensional expression domains. Signal intensity observed with whole mounts, however, is only proportional to transcript abundance over a narrow range and relative expression levels are often obscured. Therefore, we rely on sectioned material hybridized with radioactive probes to detect variations in the abundance of expression across a domain as well as to define the relative borders of expression domains of different genes. Discrepancies between the results generated by these two methods generally appear as the whole-mount signal is developed beyond the linear range and signal to noise becomes compressed. Thus whole mounts may misrepresent relative levels and borders of expression. For this reason we direct the reader to the radioactive in situ for accurate intragenic comparison of expression levels and intergenic comparison of expression boundaries.

**Expression of the Hoxc genes**

The expression patterns of the Hoxc genes have not been previously reported in the chick limb bud. Expression patterns of the Hoxc genes were examined by in situ hybridization to both whole-mount and sectioned embryos at several stages of limb development (Fig. 2). With the exception of Hoxc-11, the members of the Hoxc cluster are expressed in the anterior/proximal portion of either the wing or the leg or both.

Expression in fore or hind limb begins at the earliest stages of limb bud outgrowth and varies with gene order along the chromosome. The most 3’ members of the cluster, Hoxc-4 and Hoxc-5, are expressed only in the wing. The next two members of the cluster, Hoxc-6 and Hoxc-8, are transcribed in both the wing and the leg while the more 5’ members of the cluster, Hoxc-9, Hoxc-10 and Hoxc-11, are expressed exclusively in the leg. Despite the colinearity of limb-specific expression, there is no apparent colinearity of expression borders within the limb bud. Furthermore, in contrast to the dynamic limb expression patterns of the Hoxa and Hoxd genes described below, the anterior/proximal Hoxc genes maintain the same relative domains of expression as the limb bud grows, until at least stage 27 (data not shown).

**Expression of the Hoxc genes along the path of myoblast invasion**

Hoxc-8, Hoxc-9 and Hoxc-10 were observed in transverse section to be expressed in dorsally and ventrally restricted domains of the hind limb (Fig. 3). Cursory analysis of these domains suggested a resemblance to the dorsal and ventral paths taken by myoblasts invading the limb bud. Pax-3 expression has been shown to mark these migrating myoblasts (Williams and Ordahl, 1994). Expression of Hoxc-8, Hoxc-9 and Hoxc-10 in the path of migrating myoblasts was confirmed by in situ hybridization of adjacent sections to a probe for Pax-3. In the leg, the dorsal and ventral expression patterns of Pax-3 and Hoxc-8, Hoxc-9 and Hoxc-10 show significant overlap, with two notable exceptions. The region between the somitic dermatomyotome and the pre-
sumptive muscle mass of the limb is negative for Pax-3 expression at this stage while Hox gene expression is continuous from the somite into the limb. The same relationship is true for the dorsal-most peripheral mesoderm of the limb. Both of these regions are negative for Pax-3 expression but positive for expression of the Hox genes. Analysis of this relationship over the time course of myoblast invasion of the limb (stage 18 through stage 25; Williams and Ordahl, 1994) revealed that the dorsal and ventral expression of the Hox genes is set up during the course of myoblast migration with roughly the same spatio/temporal expression profile as Pax-3 (data not shown). Expression of the Hox genes along the path of myoblast migration is not very robust during early stages of limb development and is easily discernible only after stage 22.

**Expression of the Hoxb genes**

Although it was not isolated in our screen, Hoxb-8 is expressed transiently in a posterior domain of the early mouse limb bud and has been implicated in the regulation of Sonic hedgehog expression (Charité et al., 1994). Because of this potentially important role, when Hoxb-8 was not initially identified in our screen, we used PCR to isolate a probe corresponding to the published chicken Hoxb-8 sequence (Scotting et al., 1990). This product was then used to identify larger cDNA clones of the Hoxb-8 gene. These cDNA clones were used whole, and in parts, to generate probes for whole-mount in situ hybridization. Although these hybridizations showed good signal to noise levels, we observed no limb bud expression of this gene in the chick embryo over the time course evaluated (stage 14 to stage 24) at the levels of detection of our assay (data not shown).

The only Hoxb gene isolated in our original screen was Hoxb-9. Hoxb-9 is expressed specifically in the hind limb, where it is observed in the anterior portion of the developing upper and lower leg (Fig. 4A-D). Hoxb-9 has an additional domain of expression in the mesoderm directly subjacent to the AER. This expression tapers off at the anterior limit of the AER and is excluded from the most posterior mesoderm underlying the AER. This region of exclusion coincides with the location of the ZPA and Sonic hedgehog expression.

**Expression of the Hoxa and Hoxd genes during normal limb development**

Previous reports suggested that the genes of these two clusters are expressed in nested sets significantly different from the patterns we observe for the Hox genes. Because of the reported parallels between these two clusters, we analyzed them together, and because their expression patterns turned out to be extremely dynamic (see below), we had to examine their patterns at more developmental stages than was required for the Hox genes.

Whole mount in situ hybridization demonstrates that the relative timing of activation of these genes appears to be the same in both the wing and the leg buds (Figs 5 and 6 and data not shown). The earliest Abd-B-like Hoxd gene expression observed during limb bud outgrowth is the uniform activation of Hoxd-9 and Hoxd-10 along the entire anterior/posterior extent of the early limb bud (Fig. 6, and Laufer et al., 1994). Subsequently, Hoxd-11, Hoxd-12 and Hoxd-13 are activated sequentially at the posterior border of the limb bud (Fig. 6, and Laufer et al., 1994). Hoxa gene activation proceeds from Hoxa-9 and Hoxa-10 through Hoxa-11 and Hoxa-13 (Fig. 5). With the exception of Hoxa-13, the Hoxa genes appear to be activated uniformly along the anterior/posterior extent of the limb bud (Fig. 5). Hoxa-13 is activated at the posterior/distal tip of the bud after Hoxd-13 activation (Figs 5D and 6E).

Radioactive in situ hybridization analysis of sectioned limbs allows for direct temporal and spatial comparisons between the expression domains of the genes examined. The relative spatial distribution of the Hox gene transcripts in the limb bud depends upon the region of the limb bud in question, the time of analysis and the limb identity (wing versus leg). The first observed expression of Hoxd-9 and Hoxd-10 appear to occupy the same domain in the early limb (Fig. 6, and Laufer et al., 1994). Both genes are expressed along the entire early limb and become excluded from the proximal mesoderm as the limb grows out. Marginal expression of Hoxd-9 and Hoxd-10 fades by stage 23 as described above (Figs 6 and 7).

The sequential activation of Hoxd-11, Hoxd-12 and Hoxd-13, coupled with the continued expression of Hoxd-9 and Hoxd-10, creates the familiar concentric nested pattern of Hoxd gene expression that is characterized in many diagrams of Hox gene expression in the limb bud. At stage 23 the Hoxd genes are expressed in a concentric nested set centered around the distal posterior aspect of the wing bud. Hoxd-9 and Hoxd-10 transcripts occupy the largest regions of the wing bud at this stage, while Hoxd-11, Hoxd-12 and Hoxd-13 occupy successively smaller regions of the limb (Fig. 7).

After stage 24 the presumptive upper arm/leg, lower arm/leg and the hand/foot of the limb become easily discernible (Figs 5, 6, 7 and 8). By stage 25 it becomes clear that the Hoxd genes have two persistent, spatially discrete domains of expression, one in the presumptive lower arm/leg and the other in the presumptive hand/foot (Figs 6, 7 and 8). By stage 25, the expression patterns of the Hoxd genes appear to have stabilized into their final distributions as described above (Figs 6 and 7). In the lower leg the expression of all of these genes has faded significantly by stage 25, such that only low levels of expression are detected along the posterior margin of the lower leg (Fig. 7).

At stage 25, within the hand/foot there are spatially distinct domains of expression of Hoxd-10, Hoxd-11, Hoxd-12 and Hoxd-13. Interestingly, the relative boundaries of expression of these genes within the hand/foot are very different from those observed within the lower arm. In the hand/foot, Hoxd-13 has the most anterior border of expression of the Hoxd genes. Hoxd-10, Hoxd-11 and Hoxd-12 transcripts all occupy the same spatial domain within the hand/foot and share an anterior border posterior to that of Hoxd-13 (Fig. 7).

Hoxa-11 is also expressed in the lower arm and leg while Hoxa-13 is expressed in the wrist/ankle and the hand/foot. In situ analysis of adjacent sections shows that the distal border of Hoxa-11 expression matches the proximal border of Hoxa-13 expression (Fig. 9G,H,I). This border also marks the distal border of the proximal domain of Hoxa gene expression (Fig. 9D,E,F). The proximal border of the distal Hoxa expression domain is slightly distal to that of Hoxa-13 (Fig. 9A-C). This is apparent as a clear gap between the proximal and distal domains of Hoxa gene expression, which is located in the region that will give rise to the proximal carpals (Figs 6, 7 and 8).
**Hox gene expression versus cell lineage in the limb bud**

The dynamic expression patterns we observe for the *Hox* genes raises the question of whether the domains represent differential regulation during limb development or a passive consequence of cell proliferation. As a result of differential proliferation rates along the margin of the limb bud, the anterior/distal structures of the limb are derived from cells in the posterior region of the early bud. Thus it is conceivable that the broad domains of *Hoxa* and *Hoxd* gene expression in the late limb bud could be composed of the descendants of cells which expressed these genes in the posterior of the bud at earlier stages. In order to test this idea, we marked cells at various positions along the distal margin of the limb with the lineage tracer DiI and compared the distribution of these cells and their descendants to the pattern of *Hoxa-13* expression from stage 23 to stage 27. We found that cells at the distal tip of the stage 23 limb bud, which do not express *Hoxa-13*, gave rise to cells in the anterior third of the hand which express *Hoxa-13* at stage 27 (Fig. 10). Hence, between these stages, the expansion of the domain of *Hoxa-13* expression reflects both proliferation of previously expressing cells and de novo activation of expression in more anterior cells.

**Response of the *Hoxd* genes to ectopic Sonic hedgehog**

Since lineage alone does not appear to be responsible for the dynamic regulation of the *Hox* expression domains we turned our focus to the regulation of *Hox* gene expression. It has previously been shown that *Sonic hedgehog* can initiate *Hoxd* gene expression (Riddle et al., 1993). To test the specific role...
expression of Hoxd-11. Hoxd-12 transcripts remain restricted to the posterior portion of the leg bud. By stage 24 the proximal Hoxd-12 expression has largely faded from the leg while the distal expression remains strong. In the late leg bud, Hoxd-12 transcripts occupy the posterior of the foot from the posterior margin anteriorly to the condensation of metatarsal 2. Little or no expression is seen in the rest of the leg. Early wing expression of Hoxd-12 is very similar to early leg expression. Initially posterior expression appears to extend anteriorly followed by an anterior deflection of the distal border of expression. Eventually, the anterior deflection resolves into a spatially discrete domain within the hand extending from the posterior border of the hand to condensing metacarpal 2. Unlike the leg bud, the wing bud maintains expression of Hoxd-12 in the lower arm. Hoxd-12 expression in the stage 26 lower arm extends from the posterior margin of the limb to the posterior aspect of the condensing radius. Hoxd-12 is first expressed at the beginning of limb bud outgrowth around stage 16. At this early stage, Hoxd-9 expression appears uniform throughout the mesoderm of the limb bud. As the leg bud grows out, the expression levels of Hoxd-9 appear to drop and expression becomes restricted to the peripheral mesoderm. By stage 24 we no longer observe Hoxd-9 expression in the leg bud. In contrast, Hoxd-9 is expressed uniformly throughout the wing bud mesoderm until stage 23/24 when it becomes excluded from the distal mesoderm. Hoxd-9 is not expressed in the distal mesoderm for the remainder of wing bud development, resulting in uniform expression of Hoxd-9 in the region of the presumptive lower arm (with the exception of a posterior region of condensing cartilage). Late wing bud expression of Hoxd-9 appears to become restricted to the marginal mesoderm flanking the condensing radius and ulna in a pattern reminiscent of that seen for Hoxa-11. (B) Hoxd-10 is first expressed uniformly in the mesoderm at the earliest stages of limb bud outgrowth. In the leg this early expression quickly becomes restricted to the peripheral mesoderm. This peripheral expression of Hoxd-10 is subsequently lost, first from the distalmost mesoderm and then from the anterior peripheral mesoderm. In contrast, posterior marginal expression of Hoxd-10 is maintained and appears to spread anteriorly from stage 19 through stage 22. At stage 23 two domains of Hoxd-10 expression can be seen in the leg, one which occupies the posterior, proximal region of the bud, and one which occupies the distal, posterior aspect of the bud. As development proceeds, the posterior, marginal expression fades while expression in the distal, posterior region of the leg bud remains strong. In the late leg bud, only one domain of Hoxd-10 expression can be seen in the presumptive foot. Hoxd-10 expression in the wing bud also begins as uniform mesodermal expression. This early uniform expression appears to become biased posteriorly between stages 20 and 22. By stage 23, Hoxd-10 expression appears posteriorly biased in the proximal mesoderm but appears uniform in the more distal mesoderm. In the late wing bud two discrete domains of expression can be observed, one domain of uniform expression within the presumptive lower arm/leg and a separate domain of posteriorly limited expression within the presumptive hand/foot. (C) Hoxd-11 is first expressed slightly later in limb bud outgrowth than Hoxd-9 or Hoxd-10. Hoxd-11 expression is first observed at the posterior margin of the early stage 18 leg bud. Expression remains restricted posteriorly as the leg bud grows out. By stage 23 an anterior deflection of the distal border of expression is observed. By stage 24 the proximal expression has faded while distal expression remains strong. The late leg bud displays strong posterior expression in the foot but little to no expression in the lower leg. We first observe Hoxd-11 expression in the posterior mesoderm of the wing bud around stage 18. The expression of Hoxd-11 remains posterior as the wing bud develops occupying about half of the anterior/posterior extent of the wing bud mesoderm. Expression of Hoxd-11 in the late wing bud occupies two discrete domains. Expression within the presumptive lower arm is strongest posterior to the condensing radius although transcripts are detected just distal and anterior to the distal end of the radius. Expression in the presumptive hand extends from the posterior margin to the condensation cartilage of metacarpal two. (D) Hoxd-12 expression is first detected at the posterior margin of the limb bud closely following the initial
of Sonic hedgehog in establishing the dynamic expression patterns of the Hoxd genes we observe in the limb, we introduced Sonic to the anterior border of the leg bud at various stages of development. Early injection of Sonic-expressing retrovirus into the anterior of the early wing (stage 19-22) or leg (stage 17-19) reveals that Sonic is able to initiate and polarize Hox gene expression in both the lower arm/leg and the hand/foot (Fig. 11A-E; data not shown). Ectopically induced Hox gene expression displays temporal and spatial profiles similar to the normal expression of these genes (see also Laufert et al., 1994). In the lower arm, ectopically induced Hoxd-10 and Hoxd-11 are activated prior to and occupy a larger domain than does Hoxd-13, reminiscent of their endogenous expression within this region (Fig. 11A-E; data not shown). Conversely, in late injections, where Sonic is expressed in the region of the limb forming the hand, induction of Hoxd-13 expression precedes activation of Hoxd-10 and Hoxd-11 (Fig. 11F-J; data not shown). After both genes have been induced, Hoxd-13 transcripts occupy a larger portion of the hand than do those of Hoxd-11 (Fig. 11K-O), again mirroring the normal distribution of these transcripts within this portion of the limb. The differences seen between early and late injections cannot be attributed to relative amounts of Sonic hedgehog produced; in early limbs the induced Hoxd-11 domain is larger than the Hoxd-13 domain, indicating that Hoxd-11 is more sensitive to Sonic, while in the late limbs the induced Hoxd-13 domain is larger than the Hoxd-11 domain, indicating that at this time Hoxd-13 is more sensitive to Sonic signalling. The induced expression of the Hox genes in response to Sonic reflects normal wing/leg differences in expression (data not shown). That is, if Sonic is ectopically expressed in the anterior of the early wing bud, the induced Hoxd-11 transcripts occupy a large portion of the duplicating lower arm. If, however, Sonic is introduced to the early leg bud, only very faint Hoxd-11 expression is observed in the duplicating lower leg, as seen in the normally developing lower leg (data not shown).

Testing the regulation of Hoxc-11 and Hoxb-9 by Sonic hedgehog

The posteriorly restricted expression pattern of Hoxc-11 (Figs 2, 3E-H) is reminiscent of the early expression of some of the Hoxd genes (Fig. 6B-E). To test the influence of Sonic on Hoxc-11 expression we introduced a Sonic-expressing retrovirus subjacent to the anterior AER of the early leg bud. This protocol results in the infection of a wedge-shaped region of mesoderm within the presumptive lower leg and foot of the late limb bud (Fig. 4L,L). These infected cells express the Sonic transgene. However, analysis of infected limbs by whole-mount in situ hybridization revealed no obvious effects on the expression of Hoxc-11 (Fig. 4N). Hybridization to adjacent sections confirmed that Hoxc-11 is not induced in regions expressing Sonic hedgehog (Fig. 4K).

The expression of Hoxb-9 bears the reverse spatial relationship to Sonic hedgehog to that of the Hox genes; its expression is excluded from the region of the distal, subapical mesoderm that expresses Sonic. We therefore tested whether expression of Hoxb-9 is negatively regulated by Sonic hedgehog by retroviral misexpression. In whole-mount in situ hybridization we observe that as the anterior limb bud forms an ectopic outgrowth, Hoxb-9 is induced under the ectopic AER (Fig. 4M). Interestingly, this induced subapical expression of Hoxb-9 was not evenly distributed (Fig. 4M). Closer analysis of infected limbs by in situ hybridization to neighboring sections revealed that Hoxb-9 was induced under the ectopic AER but appeared to be induced to a lesser extent in the cells which themselves express Sonic (Fig. 4J). Careful alignment of adjacent sections revealed a similar repression of Hoxb-9 expression in the proximal mesoderm expressing Sonic (Fig. 4J).

Post-transcriptional regulation of Hoxc-6

Our analysis of the chick Hoxc-6 expression clearly revealed hind limb expression of transcripts of this gene (Figs 2 and 12). Earlier reports demonstrated that Hoxc-6 protein was detected exclusively in the fore limb (or fin) of mouse, frog and zebrafish embryos stained with an antibody raised against Xenopus Hoxc-6 (Xlhbox-1; Oliver et al., 1988a,b; Molven et al., 1990). We therefore analyzed the distribution of the mouse Hoxc-6 transcripts for direct comparison to our chick data (using a probe provided by Kevin Bentley; Schughart et al., 1989). Whole-mount in situ hybridization analysis reveals Hoxc-6 transcripts in the fore and hind limb buds of the mouse embryo, analogous to those observed in the chick (Fig. 12F,F, arrows).

In order to compare the transcription of the Hoxc-6 gene with the production of Hoxc-6 protein, we stained chick embryos with the Xlhbox-1 antibody (kindly provided by E. De Robertis) against the Hoxc-6 protein (Oliver et al., 1988a,b). Whole-mount staining reveals protein expression in the region of the fore limb expressing Hoxc-6 transcript (Fig. 12A,B,C). In contrast, no protein was detected in the region of the hind limb expressing the Hoxc-6 message (Fig. 12B,D).

DISCUSSION

We set out to perform a comprehensive analysis of the expression and regulation of the Hox genes during limb development. A large scale, low stringency screen for homeobox-containing genes expressed in the mesoderm of the developing chick limb bud yielded 23 different Hox genes representing all four Hox clusters. This includes all the members of the Hoxa and the Hoxd clusters previously reported to be expressed in the developing limb, all members of the Hoxc cluster from paralog 4 through paralog 11 and one member of the Hoxb cluster.

Our examination of the expression of these Hox genes during limb development, while not in conflict with earlier descriptions, revealed essential aspects of their expression patterns that have not been previously appreciated. The Hoxa and Hoxd genes have been described as forming concentric nested sets within the limb bud. While this picture is accurate at an early stage of limb development, the expression of these genes is extremely dynamic and the data demands reinterpretation of previous models. A prior report of the expression of Hoxc paralogs 9 through 13 in the embryonic mouse limb suggested that Hoxc-9, Hoxc-10 and Hoxc-11 were expressed in a nested set within the developing hind limb (Peterson et al., 1994). The data shown in that report is consistent in some respects with the data reported here; for instance the expression of Hoxc-11 in the posterior of the hind limb and the absence of Hoxc-12 and Hoxc-13 from the limb.
mesoderm. We do not, however, observe nested expression domains of the other Hoxc genes in the chick. The differences in relative expression domains could be attributable to interspecies differences in expression between the mouse and the chick, or to a difference in the interpretation of the data yielded by the two studies.

**Hoxc genes and the anterior/proximal limb**

The most prominent domain of Hoxc expression in the limb bud is a wedge-shaped zone restricted to the anterior/proximal region of the limb. This general pattern of expression within the limb bud is unlike that of the other Hox clusters expressed in the limb buds. It is neither oriented around a known signaling center (as in the Hoxd genes around the Zone of Polarizing Activity or the Hoxa genes around the Apical Ectodermal Ridge), nor does it display the overlapping colinearity characteristic of Hox gene expression along the primary axis.

Limb-specific Hoxc gene expression resembles the primary axial expression of Hox genes in that fore- and hind-limb specific expression is collinear with gene order along the chromosome. The most’ members of the cluster (Hoxc-4 and Hoxc-5) are restricted to the fore limb while the most 5’ members (Hoxc-9, Hoxc-10, and Hoxc-11) are expressed only in the hind limb. Intermediate genes (Hoxc-6 and Hoxc-8) are expressed in both limbs. This trend suggests that limb expression of the Hoxc genes might be controlled by the same signals that regulate the axial expression of the Hox genes. This mode of regulation does not seem likely, however, due to the discontinuities observed between the anterior limits of expression in the limb mesoderm and of axial expression in the neighboring lateral plate mesoderm. Hoxc-8, for instance, is expressed more anteriorly in the limb mesoderm than in the flank or somitic mesoderm. Thus, the limb expression of the Hoxc genes appears to be regulated by some, as yet unidentified, signal patterning the anterior/proximal region of the limb.

It has been demonstrated that at least one of these anteriorly expressed genes, Hoxc-6, is responsive to retinoic acid (RA). Unlike the induction of the Hoxd genes by RA, the induction of Hoxc-6 by RA is not restricted to the region beneath the apical ectodermal ridge (Oliver et al., 1990). Rather, Hoxc-6 is induced diffusely surrounding the source of RA. This suggests that while the induction of the Hoxd genes by RA is likely to be indirectly mediated by Sonic and Fgf-4 (Riddle et al., 1993; Laufer et al., 1994; Niswander et al., 1994), the induction of Hoxc-6 by RA may involve a different mechanism. The direct induction of Hoxc gene transcription by RA has been demonstrated in vitro (Simeone et al., 1991) and may play a role in regulating Hoxc gene expression in the embryo.

It has been shown that the anterior/proximal region of the fore limb is fated to give rise to portions of the pectoral girdle and the glenoid (Saunders, 1948). By analogy, the anterior/proximal region of the hind limb may contribute to the formation of the pelvic girdle. The established role of Hox gene expression in the specification of morphology suggests that the robust Hoxc gene expression seen in these regions of the limb might effect the patterning of these structures. Such a role has been proposed by Oliver et al. (1990) as an explanation for the effects of RA on the morphology of the pectoral girdle. RA application to the anterior of the early limb bud results in both the expansion of Hoxc-6 expression and pectoral malformations. RA, however, can also induce the expression of many other Hox genes in vitro (Simeone et al., 1991), and it is not clear that there is a causal relationship between the induced expansion of the Hoxc-6 expression and the observed pectoral phenotype. Other experiments that cause misexpression or null mutations of Hoxc genes have not shown any significant or consistent malformations of the pectoral or pelvic girdles (Hoxc-8, Le Mouellec et al., 1992; Pollock et al., 1992; Hoxc-9, Suemori et al., 1995). These data do not support a critical functional role for the Hoxc genes in the morphogenesis of this region of the limb bud. It is possible, however, that the partially overlapping expression of the Hoxc genes in this region regulates the morphology of the pectoral and pelvic girdles in a fashion similar to the regulation of a given vertebral type by multiple Hox genes. If so, observable morphological effects on these elements would only be expected after the expression of several of the Hoxc genes had been removed. This possibility has not yet been addressed by multiple knockouts of these genes but may provide an explanation for the pectoral malformations observed after the application of RA (which is likely to affect multiple Hox genes) (Oliver et al., 1990), and the subtle phenotypes sometimes displayed in the pectoral and pelvic girdles of animals carrying null alleles of, or misexpressing, the Hoxc genes. If girdle morphology were coordinately regulated by the Hoxc cluster, it would be an interesting contrast to the regulation of vertebral morphology by neighboring groups of paralogs rather than a given Hox cluster.

**Hoxc genes and myoblast migration**

In situ hybridization of transverse sections revealed that the broad anterior/posterior domain of expression of Hoxc-8, Hoxc-9 and Hoxc-10 in the hind limb actually comprises two distinct regions of expression, one restricted to a subset of the dorsal mesoderm and the other to a subset of the ventral mesoderm. These regions superficially resemble the routes taken by muscle precursor cells invading the limb bud. Close examination of adjacent sections hybridized with Pax-3 (a marker of the myoblast lineage; Williams and Ordahl, 1994), however, reveals that the dorsal and ventral domains of Hoxc gene expression are broader than the region occupied by invading myoblasts. Specifically, Hoxc gene expression extends more dorsally than Pax-3 expression and is continuous between the dermamyotome and the limb mesoderm (a region that is Pax-3 negative by stage 23). Thus despite the notable spatial and temporal overlap between these expression patterns it is likely that the Hoxc genes and Pax-3 are expressed in different cell populations.

The patterning of the myotomal cell migration within the limb bud is known to be controlled by lateral plate cells (Gumpel-Pinot, 1984). The Hoxc-positive cells within the limb bud may be a lateral plate population into which the Pax-3-positive somitic cells migrate. There has been no report of inappropriate limb muscle formation or vascularization in embryos with perturbed Hoxc gene expression (Jegalan and De Robertis, 1992; Le Mouellec et al., 1992; Pollock et al., 1992; Suemori et al., 1995). Extensive overlap of Hoxc-8, Hoxc-9 and Hoxc-10 dorsal and ventral expression domains and possible functional redundancy may be the
underlying reason for the lack of an obvious muscle phenotype in these animals. Detailed analysis of muscle and neural crest cell migration into the limb in compound Hoxc null mice may reveal the functional significance of these expression patterns.

**Triphasic expression of the Hoxa and Hoxd genes during normal limb development**

The dynamic expression patterns of the Hoxa and Hoxd genes during limb development are complex but can be modeled as the sum of three independently regulated phases of gene expression. This is most clearly seen with the Hoxd genes. The first phase of Hoxd gene expression (phase one) begins around stage 16, concomitant with the initial outgrowth of the limb. During phase one Hoxd-9 and Hoxd-10 are expressed uniformly throughout the early mesoderm without apparent anterior/posterior bias. Marginal expression of these genes is maintained until stage 22/23, when expression fades from distal to proximal. Hoxd-11 through Hoxd-13 are not expressed during phase one. Beginning at stage 18, phase two expression of Hoxd-9 through Hoxd-13 (Hoxd-10 through Hoxd-13 in the leg) is sequentially initiated at the posterior/distal margin of the limb. Around stage 22/23 phase three begins with the sequential initiation of transcription of Hoxd-13 through Hoxd-10 in an inverted temporal sequence from that observed during phase two.

Global limb expression is the sum of expression driven during all three phases. The discrete contributions of each phase to the total pattern of Hoxd gene expression are shown in Fig. 13. For example, expression of Hoxd-11 in the wing bud from stage 23 to stage 25 is shaped like an ‘L’ lying on its side, with a posterior and a distal segment. That this shape is a consequence of two overlapping domains can be confirmed by examination at stage 27, by which time the two domains have separated revealing the discrete nature of the gene expression generated during each phase.
Fig. 9. Comparison of the proximal/distal boundaries of Hoxa and Hoxd gene expression in the wing. Signal appears as red (A,D,E) or purple (B,E,H). In (C,F,I) one signal has been photographically superimposed upon the other and the overlap appears green. Note the proximal boundary of Hoxa-13 expression is slightly proximal to that of Hoxd-13 (C). (D-F) Illustrate the shared distal boundary of phase two Hoxd-11 expression (D) and proximal boundary of Hoxa-13 expression (E). Note that in the anterior half of the limb, where there is no phase three expression of Hoxd-11 at this stage, the boundaries of phase 2 Hoxd-11 and Hoxa-13 expression are precisely aligned. (G-I) High level Hoxa-11 expression (G) also respects this boundary. The domain of high level Hoxa-11 expression is directly apposed to the domain of Hoxa-13 expression (I). Exclusion of Hoxa-13 expression from condensing cartilage is evident in panels H and I.

Fig. 10. Relative progression of Hox gene expression and cell lineage in the chick wing. DiI was used to label cells within the progress zone of the wing bud at stage 23 (B). The descendents of these cells were then followed out to stage 27/28 (D) and compared to the expression of Hoxa-13 (A,C) at the same stages. X marks the original DiI injection and X' the location of the descendents of these cells. Likewise, the anterior limit of Hoxa-13 expression is marked by Y at the earlier stage and Y' at the later stage. The anterior limit of Hoxa-13 expression begins posterior to the labelled cells (A,B) but expands until it is anterior to the distal descendents of the labelled cells.

Fig. 11. Context-dependent response of the mesoderm to the inductive influence of Sonic hedgehog.
(D,E,I,J,N,O) Whole mount in situ hybridizations to representative limbs from the experimental protocols used to generate the sections to the left. These whole mounts are provided to orient the viewer and should not be used for direct spatial or temporal comparison of gene expression patterns. Sonic hedgehog was introduced to the anterior wing bud via retrovirally mediated misexpression. Top row: phase two and phase three Hoxd gene expression (B,C,D,E) can be generated by introducing Sonic (A) to the anterior of the wing prior to stage 23. Middle and bottom row: if Sonic is introduced after stage 23, only phase three Hox expression is induced. Middle row: early phase three response to Sonic (F); Hoxd-11 (G,I) is not yet induced but clear Hoxd-13 transcription(H,J) is apparent. Bottom row: Later phase three response to Sonic (K); at this time both Hoxd-11 (L,O) and Hoxd-13 expression have been induced but Hoxd-13 (M) clearly occupies a larger domain than Hoxd-11 (L). All embryos in the bottom two rows were injected and harvested at approximately the same stage. The exact age of the embryo and variation in the infection efficiency results in differences in the time to onset of Hox gene induction in different embryos. All embryos thus injected ultimately display very similar molecular and morphological phenotypes (data not shown).
The *Hox* gene expression patterns can similarly be interpreted in terms of overlapping phases of expression, although the coexpression of the phases is not maintained long enough to separate into discrete regions. Hence the lack of expression of *Hox*-9 and *Hox*-10 in the anterior/proximal fore limb and of *Hox*-11 in the anterior/proximal hind limb can be viewed as persistent phase one expression across the entire distal limb overlapping with posteriorly biased phase two expression. *Hox*-13 is the only gene of the *Hox* cluster to be expressed during phase three.

The apparently independent expression of the *Hox* genes during these three phases suggests that they might be regulated by discrete enhancers driving phase-specific expression in the limb. This hypothesis is supported by transgenic analysis of the mouse *Hoxd-11* promoter, which suggests that separate cis-regulatory elements are used to drive expression in the lower arm and the hand (Gerard et al., 1993).

**Phases of *Hox* gene expression and specification of the segments of the limb**

AER extirpation studies of the developing limb bud reveal that each of the three proximodistal segments of the limb (upper arm/leg, lower arm/leg and hand/foot) is determined sequentially from proximal to distal (Saunders, 1948; Summerbell et al., 1973). There is excellent correlation between these temporal maps and the onset of phase-specific *Hox* gene expression. During the specification of the upper arm/leg we observe the non-polar expression of *Hoxd*-9, *Hoxd*-10, *Hoxd*-9 and *Hoxa*-10. During the specification of the lower arm/leg we observe the sequential activation and posteriorly polarized expression of *Hoxd*-9 through *Hoxd*-13 and uniform expression of *Hoxa*-11. Specification of the hand/foot begins after stage 23 and coincides with the onset of phase three *Hox* gene expression. During phase three, *Hoxa*-13 transcription is activated at the posterior border of the limb bud followed by *Hoxd*-13 and subsequently *Hoxd*-12, *Hoxd*-11 and *Hoxd*-10.

The temporal correlation between the time of specification of each of the segments of the limb and the onset of phase-specific *Hox* gene expression is strongly supported by the ultimate spatial correlation of each of these segments of the limb with the spatially discrete domains of *Hox* gene expression generated during each phase. An implication of this correlation is that the initiation of phase-specific expression of the *Hox* genes marks key boundaries in the proximodistal development of the limb.

**Role of sequential induction in establishing *Hox* expression borders**

The way in which the nested patterns of *Hox* gene expression are established is unknown. One possible way to generate these patterns in the limb would be to sequentially activate the *Hox* genes at the posterior distal tip of the growing limb bud and simply leave behind a nested set of activated genes within each region of the limb. Sequential activation coupled with growth at the distal tip and subsequent cell division proximally (to amplify the distance between activation borders) could generate the final patterns observed in the limb bud.

In order to test this possibility more definitively, we mapped the anterior border of *Hoxa*-13 expression relative to the fate of cells where it is initially activated at the posterior/distal tip of the limb bud. If sequential activation were solely responsible for generating the distribution of *Hoxa*-13 expression observed in the hand, cells marked anterior to the site of initial activation of this gene should remain anterior to its expression throughout limb development. Contrary to this expectation, we find that cells labeled well anterior to the site of initiation of *Hoxa*-13 expression give rise to progenitors that lie within the lateral domain of expression of this gene. This observation implies that the boundary of *Hoxa*-13 expression moves anteriorly during limb development in a manner that is not attributable to differential cell proliferation. Therefore, sequential activation of *Hox* gene expression may contribute to the ultimate nested patterns of expression, but it is not solely responsible for establishing *Hox* gene expression boundaries within the limb bud.

**Role of Sonic hedgehog in phase-specific *Hox* gene expression**

*Sonic hedgehog* has been identified as a signal that can influence the expression of *Hox* genes in the limb (Riddle et al., 1993). The initial phase of limb outgrowth and *Hox* gene expression occurs prior to the onset of *Sonic* expression (Laufer et al., 1994), thus some other signal must be responsible for phase one expression of the *Hox* genes. The onset of phase two expression, however, coincides with the onset of *Sonic hedgehog* expression. During phase two *Sonic hedgehog* appears to sequentially activate *Hoxd*-9 through *Hoxd*-13 (*Hoxd*-10 through *Hoxd*-13 in the leg). *Sonic* also appears to drive phase three *Hox* gene expression. When *Sonic* is introduced to the anterior of the limb bud during the specification of the hand (phase three), ectopic induction of *Hoxd* expression characteristic of the hand is observed; e.g. *Hoxd*-13 is induced prior to, and expressed in a broader domain than *Hoxd*-11.

These results demonstrate that *Sonic* can initiate *Hox* gene expression during both phase two and phase three and that the response of the mesoderm to *Sonic* is phase-dependent. Thus the transition from phase one to phase two *Hox* gene expression results from the initiation of *Sonic hedgehog* expression, while the transition from phase two to phase three results from a change in the response of the mesoderm to the *Sonic* signal. An important step toward more fully understanding the regulation of *Hox* gene expression by *Sonic* will be the identification of the genetic determinants of the phase-specific responses to the *Sonic* signal.

**Sonic hedgehog and the regulation of *Hoxc*-11 and *Hoxb*-9 expression**

Unlike the other *Hox* genes, the expression pattern of *Hoxc*-11 resembles phase two expression of the *Hoxd* genes, suggesting that it might be regulated by the ZPA. Misexpression of *Sonic hedgehog*, however, does not induce the expression of *Hoxc*-11. This result implies that *Hoxc*-11 is not in the genetic cascade that mediates ZPA-induced morphological duplications. Furthermore, since it is not expressed in these duplications, its expression is not required for the genesis of those structures that are formed in the duplicated region of the limb including the lower leg and the foot (Riddle et al., 1993).

It has been suggested that *Hoxc*-11 might be functionally redundant with *Hoxd*-11 in the leg bud and thus might account for the lack of a strong hind limb phenotype in
animals carrying null alleles of the *Hoxa-11* and *Hoxd-11* genes (Small and Potter, 1993; Davis and Capecchi, 1994; Davis et al., 1995; Favier et al., 1995). The fact that *Hoxc-11* is not induced by Shh demonstrates that it is not required to generate any of the structures induced in a duplicated limb. Furthermore, although *Hoxc-11* could conceivably complement the loss of other parologue 11 proteins in the posterior lower leg, transcripts are not present in either the foot or the anterior half of the lower leg (regions corresponding to forelimb structures that show defects in paralog 11 mutant mice; the anterior lower arm and the hand) and thus is not likely to substitute for the function of these genes in patterning these regions.

Exclusion of distal *Hoxb-9* expression from the region of the ZPA suggested that Sonic might repress the expression of *Hoxb-9* posteriorly. Unlike *Hoxc-11*, *Hoxb-9* expression appears to be influenced by Sonic hedgehog. Regions of the limb showing the highest levels of endogenous and ectopic Sonic expression show a concomitant decrease in the levels of *Hoxb-9* expression. Interestingly, unlike the regulation of other *Hox* genes by Sonic, *Hoxb-9* transcription does not appear to be affected beyond the immediate region of Sonic expression, suggesting that the regulation of *Hoxb-9* transcription by Sonic is mediated by a different mechanism from that effecting the influence of Sonic on the other *Hox* genes in the limb.

**Spatial and temporal colinearity**

Expression of the *Hoxd* genes in the hand and foot reveals an exception to the rule that the relative timing of *Hox* gene activation reflects the relative position of the genes along the chromosome (temporal colinearity). During normal limb development, the time of activation of phase three *Hoxd* gene expression is obscured by the overlapping prior phase two expression of the same genes. Hence the composite expression pattern gives the appearance of overall temporal colinearity. The ectopic expression of Sonic during phase three, however, reveals a reversal in the order of gene activation between phase two and phase three. This exception to the rule of temporal colinearity mirrors the inversion of the spatial nesting of *Hoxd* gene expression observed in the hand, seen both in the endogenous and ectopically induced expression of these genes. Thus both spatial and temporal colinearity are reversed during phase three and the specification of the hand.

A second exception to the general rule of colinearity is seen in the anterior expression of the *Hoxc* genes. While, as discussed above, the 3′ *Hoxc* genes are expressed in the anterior (fore) limb while the 5′ *Hoxc* genes are expressed in the posterior (hind) limb, there is no apparent nesting of this set of *Hoxc* genes within either limb.

**Translational regulation of *Hoxc-6* expression**

One of the most novel and potentially important findings of our study is the discrepancy between the domains of expression of *Hoxc-6*, as assayed at the RNA and protein levels. *Hoxc-6* was one of the first of the vertebrate *Hox* genes to be cloned and as such has been the subject of much analysis. The protein product of this gene has been reported to be expressed specifically in the forelimb (or fin) bud of mouse, zebrafish and *Xenopus* embryos (Oliver et al., 1988a,b; Molven et al., 1990).

In contrast, we observe strong fore and hind limb expression of the *Hoxc-6* message in the chick. There are no previous reports directly addressing the distribution of *Hoxc-6* RNA in the limbs of mouse, *Xenopus* or zebrafish. One study, however, analyzing the promoter of the *Hoxc-6* gene in mice, noted apparently ectopic hind limb expression of a *Hoxc-6* reporter construct (Jegalian et al., 1992), consistent with our endogenous expression data. To verify this interpretation we tested both mouse and chick fore and hind limbs for the presence of *Hoxc-6* message and chick limbs for the presence of *Hoxc-6* protein. These studies revealed that *Hoxc-6* is transcribed in the fore and hind limbs of both animals but translated product is detectable only in the fore limb.

The mouse *Hoxc-6* gene encodes at least two distinct transcripts termed PRI and PRII (Cho et al., 1988). Although these transcripts differ at their 5′ end, our probe should detect both transcripts. Analysis of PRI and PRII transcripts within the developing mouse embryo has not revealed any marked disparity between their distributions (Shimeld et al., 1993). Moreover, both transcripts are translated (Cho et al., 1988). Thus it is unlikely that these alternative transcripts explain the observed discrepancy between RNA and protein distributions. It is possible, however, that we detect an undescribed, untranslatable message in the hind limb of the chick and the mouse.

More likely, we believe that our results reflect the postranscriptional regulation of the *Hoxc-6* gene product. The apparent translational regulation of the chicken and mouse *Hoxc-6* genes described in this study raises the possibility that postranscriptional regulation may be a significant mechanism used to regulate vertebrate *Hox* gene expression. Regardless of whether this discrepancy is due to alternatively spliced messages or postranscriptional regulation of a single message, this data strongly suggests that the interpretation of *Hox* gene function based solely on the distribution of transcripts may be misleading and incomplete. The difference between transcript and protein distribution provides a plausible explanation for the sometimes poor spatial correlation between transcript and phenotypic distribution after the experimental manipulation of *Hox* gene expression. In this regard, monitoring protein as well as transcript distribution represents a significant consideration when interpreting a *Hox* gene’s functional domain within the developing vertebrate embryo.

**Morphogenetic consequences of *Hox* gene expression**

Overwhelming experimental evidence demonstrates a causal link between *Hox* gene expression and morphology. Misexpression data suggests that *Hox* gene expression in the limb bud affects both the condensation of skeletal precursors in the limb bud and the subsequent growth and elongation of these elements (Yokouchi et al., 1995; for a review, see Morgan and Tabin, 1994). This suggests that the dramatic difference in *Hox* gene expression we observe between the wing and the leg might have morphogenetic consequences, consistent with the morphological differences that exist between the lower wing and leg of the chick (see also Mackem and Mahon, 1991; Mackem et al., 1993). On the other hand, there is no obvious correlation between *Hox* gene expression and morphology in the hand and foot.
To the extent that the \textit{Hox} genes do affect morphogenesis of the limb, the existence of three separate phases of their regulation could potentially allow each of the three limb segments to be modified independently during evolution. The transition from phase two to phase three \textit{Hox} gene expression is particularly interesting in that it may reflect the developmental innovation that distinguishes tetrapod limbs from their fin precursor. It has been suggested that the ontogeny of the lower arm is homologous to the ontogeny of primitive and derived fins while the ontogeny of the hand is unique to the tetrapods (Duboule, 1994; Coates, 1995; Sordino et al., 1995). Two dramatic changes in the morphogenetic development of the limb occur between the lower arm and the hand (Shubin and Alberch, 1986): the ‘metaptyriagial axis’ of skeletal condensation changes orientation from proximodistal to anteroposterior at the base of the digital arch, and the side of the axis from which branching originates shifts from anterior (the radius and some carpals) to posterior (the digits) (Shubin and Alberch, 1986; Ahlberg and Milner, 1994). It has previously been suggested that the change in the metaptyriagial axis orientation at the wrist reflects an evolutionary deflection of the \textit{Hox} expression boundary (Coates, 1995). However our data indicate that the distal \textit{Hox} expression represents an independent phase, not a deflection. On the other hand, we point out that the change in skeletal branching pattern from anterior to posterior correlates with the reversal in relative order between the \textit{Hox} genes from phase two to phase three.

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