Ghox 4.7: a chick homeobox gene expressed primarily in limb buds with limb-type differences in expression

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Summary

Homeobox genes play a key role in specifying the segmented body plan of Drosophila, and recent work suggests that at least several homeobox genes may play a regulatory role during vertebrate limb morphogenesis. We have used degenerate oligonucleotide primers from highly conserved domains in the homeobox motif to amplify homeobox gene segments from chick embryo limb bud cDNAs using the polymerase chain reaction. Expression of a large number of homeobox genes (at least 17) is detected using this approach. One of these genes contains a novel homeobox loosely related to the Drosophila Abdominal B class, and was further analyzed by determining its complete coding sequence and evaluating its expression during embryogenesis by in situ hybridization. Based on sequence and expression patterns, we have designated this gene as Ghox 4.7 and believe that it is the chick homologue of the murine Hox 4.7 gene (formerly Hox 5.6). Ghox 4.7 is expressed primarily in limb buds during development and shows a striking spatial restriction to the posterior zone of the limb bud, suggesting a role in specifying anterior–posterior pattern formation. In chick, this gene also displays differences in expression between wing and leg buds, raising the possibility that it may participate in specifying limb-type identity.

Key words: homeobox genes, chick embryo, limb morphogenesis, pattern formation, in situ hybridization.

Introduction

Limb development is an attractive model for studying the molecular basis of pattern formation in higher vertebrates. The overall structural features of the limb are conserved among vertebrates. Furthermore, the development of the fore- and hindlimb, two related structures that differ primarily in the spatial arrangement of essentially similar tissue components, can be compared. Because of the great facility of various experimental manipulations in the chick embryo, much information concerning limb morphogenesis derives from studies in this organism (reviewed by Amprino, 1984; Brickell and Tickle, 1989; Brockes, 1989; Eichele, 1989; Smith et al. 1989).

Tissue-grafting experiments in the chick have demonstrated regulation of events during pattern formation along three axes of the limb. A specialized apical ectodermal ridge (the AER) is necessary for the continued proximodistal (P–D) outgrowth of the limb (Saunders, 1948; Summerbell, 1974), although it is the underlying mesenchymal component that determines both the identity and temporal pattern of limb formation (Zwilling, 1964; Rubin and Saunders, 1972; Summerbell et al. 1973). The ectoderm also plays a role in determining the dorsoventral (D–V) polarity of the limb bud (MacCabe et al. 1974). A zone of mesenchyme, the zone of polarizing activity (ZPA), located at the posterior margin of the limb bud, is important in specifying the anterior–posterior (A–P, or rostrocaudal) pattern of the limb. Fragments of ZPA transplanted to the anterior margin of the limb bud cause mirror image duplications of digits along the A–P axis (Saunders and Gasseling, 1968; Tickle et al. 1975). There is considerable evidence implicating retinoic acid (RA) as a diffusible ‘morphogen’ which mediates the effects of the ZPA and forms a gradient along the A–P axis (Tickle et al. 1982, 1985; Summerbell, 1983; Thaller and Eichele, 1987, 1988; reviewed by Brickell and Tickle, 1989; Eichele, 1989; Smith et al. 1989). The ability to manipulate the chick limb bud experimentally and modulate the pattern formed with respect to these axes will facilitate the analysis of putative regulators of patterning.

By assuming that factors regulating patterning are widely conserved during evolution, genes that may play key roles in orchestrating vertebrate development, such as the vertebrate homeobox family, have been identified (reviewed by Scott et al. 1989; Wright et al. 1989b; Kessel and Gruss, 1990). Homeobox genes, encoding transcriptional regulators, were first identified genetically in Drosophila where they function in specifying the segmented body plan, and act combinatorially in complex regulatory cascades to determine the pos-
itional identity of cells along the A–P axis of the embryo (reviewed by Akam, 1987, 1989; Gehring, 1987; Hayashi and Scott, 1990). The common feature of this gene family, the homeobox, consists of a 180 bp segment of DNA encoding a highly conserved 60 amino acid domain that contains an α-helix-turn-helix motif important for sequence-specific binding to DNA (reviewed by Scott et al., 1989; Hayashi and Scott, 1990). The restricted temporospatial expression domains of homeobox genes during development (reviewed by Wright et al., 1989b; Kessel and Gruss, 1990), the induction of developmental abnormalities by ectopic- or overexpression of homeobox genes (Balling et al., 1989; Wolgemuth et al., 1989; Kessel et al., 1990), and the inhibition of homeobox gene function by antibody injection into Xenopus X1-cell embryos (Wright et al. 1989a), all indicate that these genes play regulatory roles in vertebrate development.

The expression patterns of several homeobox genes have been analyzed in the developing vertebrate limb (reviewed by Brockes, 1989; Eichele, 1989; Smith et al., 1989; Kessel and Gruss, 1990), and their restricted, graded expression patterns suggest a regulatory role in transducing morphogen gradients, such as RA, into positional information in the limb bud. These homeobox genes include the Xenopus XIHBox 1 gene (and its murine and chick homologues; Oliver et al., 1988, 1989) and the chick Gbx 2.1 gene (Wedden et al., 1989) which are both expressed in an anterior–proximal domain of developing forelimb mesenchyme; the murine Hox 7.1 (Hill et al. 1989; Robert et al. 1989) which is expressed in a posterior and distal distribution in early stage limb buds; and several members of the murine Hox 4 cluster (previously Hox 5; Dolle et al., 1989; Dolle and Duboule, 1989; Oliver et al., 1989; Duboule et al., 1990; Izpisua-Belmonte et al. 1990) which are expressed in an increasingly posterior and distal zone of the early limb bud according to their chromosomal order. There is evidence that a number of different homeobox genes may be regulated by RA from in vitro studies (reviewed by Brickell and Tickle, 1989; Kessel and Gruss, 1990; Ragsdale and Brockes, 1990) and it has recently been shown that expression of at least one of these genes, XIHBox 1, is modulated in vivo by applied RA in the developing limb bud, although not in the manner that might have been predicted from the pattern of the RA gradient (Oliver et al. 1990). It seems probable that a complex, combinatorial network of factors, including retinoic acid receptors and homeobox genes, orchestrate pattern formation in the limb bud.

Since several homeobox genes have been identified with potential roles in limb morphogenesis, we wished to assess the repertoire of homeobox genes that are expressed in chick limb buds, considering the experimental versatility of the chick embryo for studying limb development. At least 17 different homeobox genes that appear to be expressed in early stage limb buds were identified using polymerase chain reaction (PCR) amplification of limb bud message populations with degenerate oligonucleotide primers. One of these genes was chosen for detailed analysis based on its novel homeobox sequence and its restricted expression in developing limb buds. The spatiotemporal expression pattern of this gene suggests roles in A–P patterning and specification of limb-type identity during morphogenesis.

Materials and methods

Embryos

White Leghorn chick embryos were incubated at 38°C, staged according to criteria established by Hamburger and Hamilton (1951), and dissected in Dulbecco's phosphate-buffered saline and processed as indicated below.

Preparation and analysis of RNA

RNAs were extracted from embryonic tissues using either guanidium thiocyanate or RNAzol (Cinna-biotex), and poly(A)+RNA was purified by oligo(dT)-cellulose chromatography as described (Sambrook et al., 1989).

For northern blots, RNAs were separated on 1.2% agarose/formaldehyde gels (Sambrook et al., 1989), transferred to Gene Screen plus (Dupont-NEN), and hybridized to DNA probes 32P-labeled using the random primer method (Sambrook et al., 1989). Hybridizations were carried out at 42°C in 50% formamide, 1x NaCl, 1% SDS, 10% dextran sulphate, and 100 μg ml−1 salmon sperm DNA and stringent washes were performed in 0.1×SSC, 0.5% SDS at from 45–60°C for probes of base composition from 42% to 56% GC.

RNAase protection analysis was performed as described by Gilman (1987). Briefly, DNA fragments were subcloned into Bluescript (Stratagene) and 32P-labeled riboprobes were prepared (Stratagene T3 or T7 RNA polymerase). One of the riboprobes used was transcribed from a template generated by PCR amplification of the appropriate cDNA under standard reaction conditions (see below) using the following primers to incorporate a T3 phage promoter: (1) 5' CGGAAT- TAAACCCTCAGTAAAGGGAACAAAAGCTCATTCGTC-3' and (2) 5' GCGGAAGAAACGGAACGC3'. Hybridizations were performed using from 1 to 35 μg of cellular RNA (see text) in 80% formamide, 0.4 M NaCl, 40 μm Pipes pH 6.5, and 2 μm EDTA at from 48°C to 62°C for riboprobes of base composition from 40% to 70% GC. Products of ribonuclease digestion were analyzed on 8% urea/6% polyacrylamide gels along with size markers produced by Msp1 cleavage of Bluescript SK+ DNA and 32P-labeling by a Klenow fill-in reaction (Sambrook et al., 1989).

Construction of cDNA libraries

cDNAs were synthesized using the Gubler-Hoffman method (Sambrook et al., 1989), from wing or leg bud poly(A)+ RNAs extracted at early (17–18) or later (21–22) stages. First strand synthesis was primed with an oligo(dT) primer-Sall adaptor. After second strand synthesis Norl linkers were ligated, cDNAs were digested with Norl and SalI, and cloned into XZapl (Stratagene). The libraries generated each contained 4–5×106 independent clones.

PCR amplification and cloning of homeobox sequences

Phage library DNA was isolated from a plate lysate (Sambrook et al., 1989), and cDNAs were first selectively amplified from the pooled phage DNA stock using the following oligonucleotide primers flanking the cDNA cloning sites in the vector: (1) 5' GGGCGAAATTGGGTACCGGGC...
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Isolation and sequence analysis of cDNA clones

A 60 base oligonucleotide containing sequences of the appropriate homeobox domain (5'GGGACTAGTGGTNTAYCCNTTGATG'3') was used to amplify chick limb bud cDNA template by PCR. The products of PCR amplification yielded predominantly DNA fragments of the expected size which were cloned. The DNA sequences of 150 individual clones were determined. Fig. 1B shows the deduced amino acid sequences for the different homeobox domains identified, along with their assignment as probable homologues of homeobox genes characterized in other organisms. Homeodomains are grouped according to their relationship to murine 'subfamilies' that represent related homeobox genes located on different chromosomal clusters of homeobox genes, which are thought to have arisen from evolutionary duplication events (Duboule and Dolle, 1989; Graham et al. 1989; Kappen et al. 1989). The extent of homology at the nucleotide sequence level with 'subfamilies' of chick homeodomains ranged from 68% to 87%. Several of these genes have been detected previously in developing or regenerating limbs as indicated by an asterisk (*) in Fig. 1B. Of those genes detected by PCR that have not yet been analyzed for expression in limb buds, many were very frequently represented among the 150 clones analyzed, suggesting that they will also show bona fide expression during limb development.

Cloning and cDNA sequence of the chick Ghox 4.7 gene

A 60 base long oligonucleotide was generated from the unique sequences between the PCR primers of the LAn 19 homeobox clone (see Materials and methods) and used to screen a AZap cDNA library prepared from stage 21-22 chick wing bud poly(A)+ RNA at high stringency. A series of overlapping clones differing in the positions of their 5' termini were isolated (Fig. 2A), and the nucleotide sequences of both strands were determined using the dideoxy sequencing method of Sanger (1977).

Results

Detection of candidate homeobox genes expressed in limb buds by PCR amplification

To identify homeobox genes that are expressed during limb development, several degenerate oligonucleotide primers were constructed from highly conserved domains within, and just upstream of the homeobox (amino acid consensus sequences shown in Fig. 1A) and

and (2) 5'GGGAAACAAAGCTGGAG'3'. The PCR reactions were modified from standard conditions (Perkin-Elmer Cetus) to give more uniform amplification of longer (1 to 3 kb) sequences, by including 800 µM dNTPs, 7.5 µM MgCl2, and performing a total of 11 cycles with the 72°C extension step for 5 min, followed by one cycle with extension for 10 min, and one cycle with extension for 20 min. These conditions yielded amplified products ranging in size from 0.3-4 kb (a distribution similar to the library cDNAs). These amplified cDNA 'inserts' served as template for a second PCR amplification using 4 µM concentrations of degenerate oligonucleotide primers and standard reaction conditions, except that for the first five cycles, annealing was at 45°C instead of 55°C, and a two minute ramp was included to reach the 72°C extension step. The sequences of degenerate primers used were as follows (IUPAC code used):

(I/L/V)YPWM: 5'GGGACTAGTGGTNTAYCCNTTGATG'3'

ELEKEF: 5'GGGACTAGTGGARYTNGARAARGARTT'3'


KlKFQN: 5'GGGACTAGTGGRTYTTGRAACADATYTT'3'

PCR products were cloned into Bluescript and sequenced as described below.

In Situ Hybridization

Embryonic sections and riboprobes were prepared for in situ hybridization as previously described (Nakamura et al. 1989). Hybridization was carried out at 45°C in 50% formamide, 5xSSC, 0.1 M sodium-potassium phosphate pH 7, 1x Denhardt's, 5% dextran sulphate, 100 mM dithiothreitol (DTT), and 100 µg ml⁻¹ salmon sperm DNA at 42°C and stringent washes performed in 0.1xSSC at 45°C. For further analysis, clones were converted from phage to plasmids using the automatic excision protocol (Short et al. 1988). The complete sequence of both strands of cDNA clones were determined using the dyeoxy sequencing method of Sanger et al. (1977).

Cloning and cDNA sequence of the chick Ghox 4.7 gene

A 60 base long oligonucleotide was generated from the unique sequences between the PCR primers of the LAn 19 homeobox clone (see Materials and methods) and used to screen a AZap cDNA library prepared from stage 21-22 chick wing bud poly(A)+ RNA at high stringency. A series of overlapping clones differing in the positions of their 5' termini were isolated (Fig. 2A), and the nucleotide sequences of both strands were determined. Since most of the clones were primed by oligo(dT) at an internal poly(A) stretch within the middle of the 3' untranslated region, an additional shorter clone extending from the homeobox to the true 3' end was also analyzed to obtain the complete 3' untranslated sequence (L19-6 in Fig. 2A). Fig. 2B shows the complete cDNA sequence and the deduced amino acid sequence for Ghox 4.7 (derived from L19-21 and L19-6). This sequence is likely to be full length and to include the complete protein coding sequence for the following reasons: (1) The length agrees with the size of the single transcript seen on northern blot analysis (about 1.6 kilobases long, see below); (2) there are two inframe stop codons upstream of the initiator methionine; and (3) in vitro translation
of RNA transcribed from the phage T3 promoter of clones, including either the entire predicted coding sequences or 3' truncations, efficiently yielded proteins of the expected sizes (despite a poor translational initiation consensus sequence; Kozak, 1986); while clones beginning just 3' of the initiator methionine yielded no protein product (data not shown). The relative molecular mass of the full-length polypeptide product was about 34×10^3 on denaturing gels, close to the predicted size of 30×10^3, considering that the apparent size of homeodomain proteins as judged by denaturing gel electrophoresis tends to be somewhat higher than predicted (eg. Kessel et al. 1987; Erselius et al. 1990).

We have designated this gene Ghox 4.7 because of extensive similarity between this sequence and the deduced amino acid sequence for the murine Hox 4.7 gene (Izpisua-Belmonte and Duboule, personal communication). Homology between the two is evenly distributed over their entire length with 66% amino
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Acid identity between the two genes. There are 2 amino acid changes within the homeodomain, resulting in a 96% amino acid identity within this motif. The homeodomain of Ghox 4.7, as well as that of a second chick gene detected by PCR (LUn 2 in Fig. 1B), is more related to the Drosophila Abdominal B than to the Antennapedia homeodomain (Regulski et al. 1985; Scott et al. 1989), as compared in Fig. 3. For Ghox 4.7, there is 36/60 amino acid identity with the Abdominal B homeodomain, compared to 27/60 amino acid identity with Antennapedia. The homeodomain of both these chick genes is also closely related to the human Hox 3F identified by Boncinelli and coworkers (Acampora et al. 1989), with LUn 2 likely representing the chick homologue of Hox 3F. Outside of the homeodomain of Ghox 4.7 there is no region of extended homology other than with the murine Hox 4.7 gene. As frequently seen in homeobox genes, parts of Ghox 4.7 are relatively proline-rich, and the homeodomain is located near the carboxy-terminus (eg. see references in Fig. 1). Other motifs that have been identified in certain subclasses of homeobox genes (Mavilio et al. 1986; Kessel et al. 1987; Schughart et al. 1988), such as a conserved amino terminal domain, a 3' polyacacid residue stretch or other homopolymeric stretches of amino acid residues, and in particular, the conserved pentapeptide motif (shown in Fig. 1A), are not present in this gene.

Northern blot and RNAase protection analyses of Ghox 4.7 expression

The temporospatial pattern of expression of Ghox 4.7 during embryogenesis was examined by northern blot hybridization and RNAase protection analysis. Total cellular RNA was extracted from separated wing buds, leg buds, heads and trunks of chick embryos dissected at various stages (stage 17 to stage 30) spanning the period of limb morphogenesis. Northern blots of these RNAs were probed both with sequences spanning the Ghox 4.7 homeodomain (shown in Fig. 4) and with 3' untranslated sequences of Ghox 4.7 (data not shown). Both probes gave qualitatively and quantitatively similar results. A single transcript of about 1.6 kilobases was detected (see Fig. 4). This transcript was readily detected in developing limb buds, but was present only in trace amounts, after very long exposures, in the trunks of stage 21 and later embryos. No signal was detected in RNAs from head tissues. Ghox 4.7 expression was present in leg buds as early as stage 17, at which time the leg bud first appears as a swelling in the lateral plate mesoderm, but was not detected in wing buds until later stages (stage 21) at a time when limb patterning is well underway (reviewed by Smith et al. 1989), as shown in Fig. 4. To determine more precisely the time of onset of Ghox 4.7 expression in developing wing buds, RNAs extracted from wing and leg buds at intervening stages (18–20) were also hybridized to a Ghox 4.7 probe (Fig. 5), showing that low levels of Ghox 4.7 expression were first detectable in wing buds at stage 19, and became comparable to leg bud levels by stage 20.

At later stages from 21 through 30, the level of expression of Ghox 4.7 was 3- to 5-fold higher in wing buds than in leg buds (Fig. 4). The higher levels of expression seen in wing buds compared to leg buds at these later stages cannot simply reflect the earlier appearance of wing buds (stage 16) than leg buds (stage 17) on the embryo, since the onset of Ghox 4.7 expression in leg buds actually precedes wing bud expression by a number of hours (stage 17 versus stage

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Fig. 2. Structure of Ghox 4.7 cDNA. (A) Diagram of Ghox 4.7 (L19) cDNA clones and hybridization probes. Shown is a scaled diagram of Ghox 4.7 full-length cDNA including relevant restriction sites, and the positions of translation initiation (AUG), the homeobox, translation termination (Ter), an internal poly(A) stretch in the 3′ untranslated region (A13GA6), and the true polyadenylation site (poly(A)). Below is shown the extents of overlapping cDNA clones that were isolated and used in sequence determination. At the bottom is shown the extents of the hybridization probes that were used for northern blots, RNAase protection assays, and in situ hybridization, as referred to in the text. The end-points of these probes occur at internal restriction enzyme sites (as shown), or at the terminus of a cDNA clone, except for the homeodomain-spanning PCR probe which was constructed as described in Materials and methods, and has end-points numbered relative to the complete sequence given below in B. (B) Nucleotide sequence and deduced amino acid sequence of Ghox 4.7. Upstream, in-frame stop codons occur prior to the initiator ATG, an internal poly(A) stretch, and the polyadenylation signal are all underlined. The position of translation termination is indicated as Ter. The homeodomain is boxed.

Fig. 3. Comparison of the predicted amino acid sequence of the Ghox 4.7 homeodomain with other related homeodomain sequences. The Ghox 4.7 homeodomain is compared to the homeodomain of Drosophila Abdominal B (Abd B; Regulski et al. 1985) and with related vertebrate genes human Hox 3F (Acampora et al. 1989) and chick LUn 2 (from Fig. 1B). Positions of amino acid identity relative to the sequence of Abdominal B are indicated by dashes. For the chick LUn 2, the deduced amino acid sequence of only a part of the homeodomain was determined.

19, about 12 h), as seen in Fig. 4 and 5. These quantitative and temporal differences in expression of Ghox 4.7 in wing and leg buds were highly consistent among multiple independent RNA preparations, using either total or poly(A)+ RNAs (data not shown).

Total cellular RNAs extracted from wing buds, leg buds, heads and trunks were also analyzed for Ghox 4.7 expression using the RNAase protection assay to verify that the temporospatial expression patterns seen on northern blots reflect expression of a single gene. As shown in Fig. 6, the expression of Ghox 4.7 RNA in wing and leg buds was assessed at several different stages ranging from stage 17 to stage 28 using a 3′ untranslated region probe (Rsal–SacI, as in Fig. 2A, 110nt protected) which would be unlikely to show substantial conservation even between related genes. The results of this analysis were both qualitatively and quantitatively similar to the expression pattern revealed on probing northern blots; at very early stages (stage 17), as seen in Fig. 6, expression of Ghox 4.7 was seen in leg but not in wing buds, whereas at the later stages, the message was expressed at 3- to 5-fold higher levels in wing than in leg buds. An RNAase protection assay performed using a homeobox-derived probe (PCR-generated probe, as in Fig. 2A, 160nt protected), gave essentially the same result (Fig. 6, for stage 21, right panel). The temporospatial differences observed in RNAase protection experiments were obtained with several independent RNA preparations that were all normalized for amount by hybridization of control cytoskeletal actin probes to northern blots. For both the 3′ untranslated and the homeobox probes, the protected fragments observed using cellular RNAs comigrated exactly with those generated using in vitro transcribed sense-strand RNAs derived from Ghox 4.7 cDNA clones as a control (data not shown). In particular, the products of protection using the 3′ untranslated probe and control sense-strand RNA also consisted of a doublet as was seen for the cellular RNAs in Fig. 6 using this probe.

The results of the RNAase protection assays also illustrate the relatively high levels of expression of Ghox 4.7 in limb buds and trace levels of expression elsewhere in the embryo. For the experiments shown in Fig. 6, 3 μg of total cellular limb bud RNA was used, and as little as 1 μg of RNA also gave a detectable signal (data not shown). More than ten times this amount of RNA (about 35 μg) was required to detect expression of Ghox 4.7 in trunk RNA from stage 21/24 embryos; and even at this level of sensitivity, no expression was detected in RNA extracted from head tissues, as shown in Fig. 6.

The temporospatial pattern of expression of Ghox 4.7 was examined by in situ hybridization to sections of chick embryos, using an antisense probe spanning the homeobox (SacI fragment, as in Fig. 2A). Since both homeobox and 3′ untranslated probes from this gene gave identical results in hybridizations to northern blots and in RNAase protection analyses (see above), and since hybridization of the homeobox sequence of Ghox 4.7 to chick genomic southern blots detected a single band under conditions of moderate to low stringency (data not shown), the probe used for in situ hybridization should detect exclusively Ghox 4.7. In situ hybridizations were performed with both sense and antisense probes in parallel and at high stringency; sense probes did not show detectable hybridization to any embryonic tissues (data not shown). Serial sections from at least two perpendicular orientations of multiple embryos were analyzed for the stages examined by in situ hybridization.

The overall expression pattern of Ghox 4.7 as analyzed by in situ hybridization to embryonic sections (Figs 7 to 9) showed the predominant site of expression...
Fig. 4. Northern blot analysis of Ghox 4.7 expression in chick embryos during the period spanning limb morphogenesis. Embryos at the stages (st) indicated were dissected into separated wing buds (W), leg buds (L), heads (H), and trunks (T) and 15 μg of total cellular RNA from each source was loaded per lane. The hybridization shown was performed with a homeobox-containing Ghox 4.7 probe (SalI fragment in Fig. 2A). Hybridization with a 3′ untranslated region Ghox 4.7 probe (RsaI-3′ end fragment in Fig. 2A) gave identical results (data not shown). The single major transcript visualized with Ghox 4.7 probes is indicated by the arrow at 1.6 kb, and the relative positions of 28S and 18S ribosomal RNAs is also indicated. Control hybridizations were performed using a chick β-actin cDNA probe (Cleveland et al. 1980) as shown.

Fig. 5. Northern blot analysis demonstrating the delayed onset of Ghox 4.7 expression in wing buds compared to leg buds. Conditions, probes, and abbreviations used are identical to those in Fig. 4.

from stages 18 to 28 to be in the developing limb bud mesenchyme (Figs 7–9; stage 18 not shown), with a relatively lower level of expression also seen in the mesenchyme surrounding the mesonephric ducts, and the most posterior portions of the hindgut mesenchyme (see for eg. Fig. 8G,H). Expression was entirely restricted to the mesodermal component of these tissues with no evidence of hybridization in the epithelium. Particularly striking was the lack of expression within the specialized limb bud epithelium of the apical ectodermal ridge (AER) (Fig. 7E–H). Most of the other tissues of the trunk at these stages showed only background labeling, although a variable, trace level of hybridization was sometimes seen in the most posterior parts of the neural tube. Hybridization to somitic mesoderm was notably absent at all stages evaluated (eg. Fig. 8). No detectable hybridization was evident in head tissues (not shown).

At very early stages during limb development shortly after both limb buds have formed (stages 17–18), low levels of uniform expression of Ghox 4.7 were detectable in the mesenchyme of leg buds (data not shown), but no expression was seen in wing buds (or in adjacent somitic mesoderm), in agreement with the results seen on northern blots and RNAase protection analyses. At later stages, the expression of Ghox 4.7 in developing limb buds showed a striking restriction to the posterior zone of the limb bud. This pattern was best appreciated on coronal (or frontal) sections through flattened segments of trunk oriented parallel to the long axis (Fig. 7A–D). At stage 21, a time when patterning is still actively ongoing and prior to the onset of histologically evident differentiation in the limb bud mesenchyme, this restricted expression along the A–P axis was pronounced in both wing and leg buds (Figs 7A,B; 8A–H), ranging from very high levels of expression near the posterior border of the bud (eg. Fig. 8C,D,G,H) to background levels near the anterior border (eg. Fig. 8A,B,E,F). On transverse sections taken at various different levels along the A–P axis of the limb bud, gradations in the level of expression of Ghox 4.7 along the dorsoventral (D–V) and proximo-distal (P–D) planes was also appreciated (Fig. 7E–H). In very posterior transverse sections through limb bud, the expression of Ghox 4.7 was very high and uniform (Fig. 7G,H). As the expression level decreased in more anterior transverse sections, the expression of Ghox 4.7 appeared to be more spatially restricted in the D–V and P–D planes (Fig. 7E,F), becoming progressively more dorsal and also somewhat more distal in its distribution at the anterior limit of expression. Expression in a restricted posterior zone was still maintained at somewhat later stages when foci of precartilaginous condensations of the mesenchyme have formed in the limb buds (stage 24, eg. Fig. 7C,D). Although the qualitative spatial pattern of expression seen in wing and leg buds was very similar at stages 21–24 (particularly the restricted domain of expression along the A–P axis), the density of grains in comparable sections of wing buds and leg buds was seen to be consistently higher for wing than leg buds at stage 21–24 (eg. Fig. 8D compared to 8H), consistent with northern blot and RNAase protection analyses.

As the period of patterning is ending, and differentiation processes are underway (stage 24, and more so stage 28), persistent expression of Ghox 4.7 was seen in
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**Fig. 6.** Ribonuclease protection analysis of Ghox 4.7 expression during limb morphogenesis in chick embryos using 3' untranslated and homeobox-spanning antisense RNA probes. Total cellular RNAs extracted from dissected wing buds (W), leg buds (L), heads (H), and trunks (T) at the stages (st) indicated were hybridized with riboprobes transcribed from either 3' untranslated sequences (RsaI–SacI as shown in Fig. 2A; 110 nucleotides (nt) protected) or homeobox-containing sequences (PCR generated template as shown in Fig. 2A; 160 nucleotides protected). For wing and leg buds, 3 μg of total cellular RNA was used, and for heads and trunks, 35 μg of a 1:1 mixture of stage 21 and 24 total cellular RNA was used. Control hybridizations with equivalent amounts of *E. coli* tRNA were performed in parallel. Undigested riboprobe and labeled MspI digested Bluescript SK− markers were run alongside the products of RNAase digestion on denaturing 6% polyacrylamide–urea gels. The specific protected bands (not present in tRNA lanes) seen with each of the probes used are indicated by arrows, and represent full-length protection of the Ghox 4.7-specific sequences, as determined in control hybridizations with the same antisense probes and sense RNA made from cDNA clones.

Discussion

In this paper, we report the complete coding sequence and embryonic expression pattern of a new chick homeobox gene, Ghox 4.7, which is the probable chick homologue of the murine Hox 4.7. Using PCR amplification with degenerate oligonucleotide primers to isolate different homeobox segments from limb bud cDNAs, it also became evident that at least 17 different homeobox genes are expressed during limb development, including multiple members from each of the four major homeobox gene clusters and multiple members within a given subfamily, as defined in mouse (e.g. Duboule and Dolle, 1989; Graham et al. 1989). This probably represents a minimum estimate of the number of homeobox genes actually expressed during limb development, since the PCR screen was not designed to be exhaustive. In fact, several homeobox genes that have been reported to be expressed during limb development in mice were not detected (En-1: Davis and Joyner, 1988; Hox 1.4: Galliot et al. 1989; Hox 5.1 and 5.5: Dolle et al. 1989; Hox 4.3: Izpisua-Belmonte et al. 1990). This raises the question of why such a large number of the known vertebrate homeobox genes (about 50% to date) are expressed in developing limb buds. Certainly there is evidence for active regulation of pattern along at least 3 axes (A–P, P–D, D–V) of the limb, and several homeobox genes have been identified that show graded or restricted expression patterns along one or more of these axes (Dolle and Duboule, 1989; Dolle et al. 1989; Oliver et al. 1988, 1989; Wedden et al. 1989; Hill et al. 1989;
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The expression pattern of Ghox 4.7 displays several notable features. First, the steeply graded posterior domain of expression along the A-P axis is interesting in relation to the RA gradient regulating A-P patterning (Tickle et al. 1982, 1985; Summerbell, 1983; Thaller and Eichele, 1987, 1988; reviewed by Brickell and Tickle, 1989; Eichele, 1989; Smith et al. 1989). Ghox 4.7 may thus be a candidate for one of the genes that is regulated by RA levels via one of the nuclear retinoic acid receptors (reviewed by Brickell and Tickle, 1989; Eichele, 1989; Smith et al. 1989; Ragsdale and Brockes, 1990), thereby transducing the information present in a morphogen gradient into positional information. Both in vivo and in vitro analyses of the modulation of Ghox 4.7 expression by RA levels using various techniques such as limb bud manipulations will be necessary to evaluate this possibility.

It is somewhat unusual that Ghox 4.7 is expressed very weakly or not at all in neural tube and somites, which is a characteristic site of expression for many homeobox genes in vertebrates (reviewed by Kessel and Gruss, 1990). As pointed out by several investigators (Acampora et al. 1989; Erselius et al. 1990), there are several Abdominal B-related homeobox genes recently identified in vertebrates that may play a greater role in specifying 'abdominal' structures such as abdominal hindlimbs, kidneys, genitalia, etc. than structures along the A-P axis of the trunk. The expression of Ghox 4.7 (which belongs to one of the subfamilies within this Abdominal B-like group; Fig. 3) seen in the mesenchyme of the urinary ducts and of hindgut may also indicate a role in morphogenesis of abdominal structures. The absence of Ghox 4.7 expression in somites at times when somitic cells are migrating into the limb bud (Chevallier et al. 1977; Christ et al. 1977) is not very surprising; expression may arise in cells derived from the lateral plate mesoderm which give rise to all the nonmyogenic mesodermal elements of the limb (skeletal and connective tissue; Chevallier et al. 1977; Christ et al. 1977). It is these non-somatic mesenchymal elements that account for the primary pattern seen; in fact the skeletal components will form recognizable limb patterns in the total absence of a myogenic component derived from the somites (eg. Chevallier et al. 1977). Alternatively, the somitic components may also begin to express Ghox 4.7 after they have migrated into the limb bud.

Late during limb morphogenesis, when the pattern has already been determined and the process of morphologic differentiation is well underway, Ghox 4.7 continues to be expressed in limbs, particularly in mesenchymal soft tissues of the distal-most part of the limb with a persistent posterior distribution. A similar late expression pattern was also observed by Dolle et al. (1989) in the mouse embryo, although in chick, expression in late stage wing also extended very proximally in the posterior part of the limb. Such late expression may reflect an additional role of Ghox 4.7 during processes of cytodifferentiation. Alternatively, the lingering \textquoteleft remnant\textquoteleft of a steeply graded A-P distribution may indicate that even though the pattern

Fig. 7. In situ localization of Ghox 4.7 transcripts in the posterior mesenchyme of the developing limb bud. Coronal sections (A–D) through stage 21 (A–B) and stage 24 (C–D) wing buds show a distinct posterior domain of expression along the anterior (a) to posterior (p) axis of the limb bud. Transverse sections (E–H) through anterior (E–F) and posterior (G–H) regions of stage 21 wing buds demonstrate the distribution of RNA along the dorsal (d) to ventral (v) plane. A section through the anterior limit of hybridization (E–F) shows a more dorsal and distal distribution of grains while a posterior section (G–H) shows a more uniform distribution. Note that the AER is unlabeled (arrowheads). Bar=500 micrometers (A–D), or 200 micrometers (E–H). Left panels are bright field; right panels are dark field.

Robert et al. 1989; Savard et al. 1988; Tabin, 1989; Izpisua-Belmonte et al. 1990). In addition, it may be necessary to express a very large number of different homeobox genes in distinct but overlapping gradients to specify uniquely all of the different structures of the limb, despite the similarity that some of these structures (eg. bony elements) display. For example, in the case of Drosophila segmentation genes, each repeating stripe of expression in the embryo is apparently regulated by a unique and different combination of factors (reviewed by Akam, 1989). Furthermore, in specifying positional information, homeobox genes may regulate many related processes such as differential growth, programmed cell death and terminal differentiation of tissues which will all contribute to morphogenesis of the limb.

A variety of methods have demonstrated that Ghox 4.7 is expressed primarily in developing limb buds in a striking posteriorly restricted spatial distribution; with quantitative, temporal and lateral spatial features of expression differing between wing and leg buds. Many features of the expression pattern seen with Ghox 4.7 in chick are similar to that reported for the murine Hox 4.7 (Dolle et al. 1989). The murine gene displayed a posterior, distal and dorsal distribution in the developing limb. However, no differences in the level of expression in mouse fore- and hind limb buds was noted, possibly because expression was analyzed only by in situ hybridization. Duboule and coworkers (Dolle et al. 1989) concluded that the genes of the murine Hox 4 cluster show a sequential temporal activation in the developing mouse limb bud; the more 5' the location of a gene in the chromosomal cluster, the more distal and later onset was its expression, correlating with the proximodistal sequence of limb outgrowth. The most 5' member of the cluster, Hox 4.7, showed the most distal and latest onset of expression in mouse embryo limb buds. Although the late onset of Ghox 4.7 expression seen in chick embryo wing buds (stage 19 to 20) is consistent with this notion; the leg bud expression, which begins coincident with appearance of the leg bud in the embryo (stage 17), is not. Likewise, the persistent expression of Ghox 4.7 in a proximal as well as distal posterior distribution in very late stage wing (stage 28) is not consistent with this interpretation.
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Finally, we also noted in analyzing the expression pattern of Ghox 4.7, that the onset of expression was delayed in wing buds compared to leg buds, that the level of expression at later stages was generally several fold higher (3- to 5-fold) in wing buds than leg buds, and that expression at very late stages was much more distally restricted in leg buds than in wing buds. This raises the possibility that Ghox 4.7 also plays some role in the determination of limb-type identity, and/or that the temporal and quantitative differences in the level of its expression in wing versus leg buds reflects in some way the greater divergence in pattern of the avian wing compared to the general tetrapod forelimb. A quantitative comparison of expression of this gene in

has already been determined, the continuing expression of factors regulating positional information may be necessary to ensure the correct program until the entire chondral scaffold is complete, thereby governing the morphology of the bony components of the limb.

Fig. 8. Expression domains of Ghox 4.7 in stage 21 limb buds. Transverse sections through anterior (A-B and E-F) and posterior (C-D and G-H) regions of wing buds (A-D) and leg buds (E-H) show expression restricted to the posterior regions of the limb buds. Some labeling can also be seen in the mesenchyme of the mesonephric ducts (m) and the hindgut (h). Bar=500 micrometers. Left panels are bright field; right panels are dark field.

Fig. 9. Expression domains of Ghox 4.7 at a later stage of limb development. Distinctions in limb-type expression of Ghox 4.7 are evident by stage 28 (A-F). Coronal (A–B) and parasagittal (C–F) sections through stage 28 wing (A–D) and stage 28 leg (E–F) illustrate the intense labeling seen in the distal-most (di) part of the limb in the mesenchymal soft tissues surrounding the cartilage anlage, whose central regions are unlabeled. Expression in both types of limb at stage 28 also still shows a posterior restriction along the anterior (a) to posterior (p) axis. However, in sections of leg at stage 28, expression is entirely restricted to the posterior distal tip of the limb, while in wing, a posterior zone of mesenchymal tissue extending proximally (pr) to the body wall also continues to express Ghox 4.7, as indicated by the arrow in panel A. Bar=500 micrometers. Panels A, C, E are bright field; panels B, D, F are dark field.
developing fore- and hindlimbs of other vertebrates will be necessary to address this issue.

Ghx 4.7 is unlikely to be involved in ‘initiating’ the determination of limb-type identity, since this occurs quite early in cells of the lateral plate mesoderm well before a limb bud appears (Rudnick, 1945; Stephens et al. 1989), whereas Ghx 4.7 expression begins subsequent to the formation of the limb bud. However, it seems reasonable to assume that the maintenance of limb-type identity is an active process, since limb-type identity is in fact preserved during tissue grafting experiments using wing and leg bud mesenchyme (eg. Zwilling, 1964; reviewed by Amprino, 1984), and therefore will require the ongoing expression of certain genes. Perhaps the differential pattern of expression of Ghox 4.7 RNA seen in wing and leg buds represents an outcome of other prior wing-leg differences and plays a later role in morphogenesis when these pre-determined limb type differences must be translated into gross morphologic differences.

Whether limb identity is mediated by qualitative and/or quantitative differences in expression levels of regulatory genes is not known. Although very little is known about the molecular mechanisms whereby limb type identity is established, at least one homeobox gene is differentially expressed both in developing limbs in Xenopus embryos (XTHBox 1; Oliver et al. 1988), as well as in regenerating limbs in newt (NvHbox 1; Savard et al. 1988; Tabin, 1989). In Xenopus, hindlimb expression was restricted to the ectoderm compared to strong mesenchymal expression also present in the forelimb, as evaluated at the protein level with antibodies, suggesting a qualitative difference in expression between the two limb types. However, in newt, differences in expression of this gene between the perichondrial blastemas of fore- and hindlimbs were reported to be from 3- to 10-fold as determined at the RNA level, suggesting quantitative differences in expression. If some of the differences in gene expression determining limb type identity are more quantitative than qualitative in nature, it will be necessary to undertake a careful analysis of the relative levels of expression of regulatory genes as they are identified to begin to address the question of how limb identity is determined. One way in which modest quantitative differences in regulatory gene products could be amplified into large differences in expression of their targets is through cooperative interactions, leading to synergistic effects. In fact, such cooperative interactions have been demonstrated for Drosophila homeobox genes (Han et al. 1989). In the case of Ghox 4.7, it may also be interesting to re-evaluate expression at the protein level using antibodies, in the event that a component of differential expression of this gene occurs at the translational level.

In summary, it appears that a large number of homeobox genes are expressed in developing limb buds of chick embryos. Analysis of one of these genes, Ghox 4.7, reveals a strongly posterior spatial restriction within the limb bud and expression differences in wing and leg buds that suggest potential roles in pattern formation and in specifying limb type. As the expression of more of these genes is studied, we may begin to generate a more complete list of the regulatory components and understand their roles in pattern formation. An analysis of the cross-regulation of these genes by each other as well as by other key components such as the retinoic acid receptors, will also be critical in determining how these combinatorial regulatory networks specify positional information.

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References


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Note Added in Proof
After this paper was in press, Nohno et al. (Cell 64, 1197–1205) and Izpisua-Belmonte et al. (Nature 350, 585–589) reported on the expression pattern of several members of the chick Hox4 cluster in developing limb buds.