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# *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 facillitate 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.* 1989*b*; 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 $\alpha$-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.* 1989*b*; 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* 1-cell embryos (Wright *et al.* 1989*a*), 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 XlHBox 1* gene (and its murine and chick homologues; Oliver *et al.* 1988, 1989) and the chick *Ghox 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, *XlHBox 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 guanidinium 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 $^{32}$P-labeled using the random primer method (Sambrook *et al.* 1989). Hybridizations were carried out at 42 °C in 50% formamide, 1 M NaCl, 1% SDS, 10% dextran sulphate, and 100 $\mu$g 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 $^{32}$P-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′CGGAATTAACCCTCACTAAAGGGAACAAAAGCTCATTCGTCGGTTCTG3′ and (2) 5′CGCGAAAGAAACGGAAAC3′. Hybridizations were performed using from 1 to 35 $\mu$g of cellular RNA (see text) in 80% formamide, 0.4 M NaCl, 40 mM Pipes pH 6.5, and 2 mM 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 M urea/6% polyacrylamide gels along with size markers produced by *Msp*I cleavage of Bluescript SK$^-$ DNA and $^{32}$P-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-*Sal*I adaptor. After second strand synthesis *Not*I linkers were ligated, cDNAs were digested with *Not*I and *Sal*I, and cloned into $\lambda$*Zap*I (Stratagene). The libraries generated each contained 4–5×10$^6$ 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′GGGCGAATTGGGTACCGGGC3′

and (2) $^{5'}$GGGAACAAAAGCTGGAG$^{3'}$. 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 mM $MgCl_2$, 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: $^{5'}$GGACTAGTGNTNTAYCCNTGGATG$^{3'}$

ELEKEF: $^{5'}$GGACTAGTGGARYTNGARAARGARTT$^{3'}$

(A/E/R/Q)L(E/K)R(A/E/R/K)F:
$^{5'}$GGACTAGTGSVRYTNRARMGNRVRTT$^{3'}$

KIWFQN:
$^{5'}$CGGTCGACGGRTTYTGRAACCADATYTTN$^{3'}$

PCR products were cloned into Bluescript and sequenced as below.

### *Isolation and sequence analysis of cDNA clones*

A 60 base oligonucleotide containing sequences of the appropriate homeobox segment ($^{5'}$CTGGTCGCTTAAATT CAGTCTGTTTGATAGTTCTTTCCTCTTCTGTCGGTTA ATAAACTC$^{3'}$) was $^{32}$P-end-labeled to screen a stage 21–22 wing bud cDNA library using standard methods (Sambrook *et al.* 1989); with hybridizations carried out in 30% formamide, 1 M NaCl, 1% SDS, 10% dextran sulphate and 100 μg ml$^{-1}$ salmon sperm DNA at 42°C and stringent washes performed in 0.1×SSC 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 dideoxy sequencing method of Sanger *et al.* (1977).

### 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, 5×SSC, 0.1 M sodium–potassium phosphate pH 7, 1×Denhardt's, 5% dextran sulphate, 100 mM dithiothreitol (DTT), and 100 μg ml$^{-1}$ *E. coli* tRNA. Sections were washed in 50% formamide, 5×SSC, 10 mM DTT for several hours at 37°C, then for 1 h in 0.1×SSC, 10 mM DTT at 68°C, and then treated with RNAase A. Exposures (using Kodak NTB-2 emulsion) ranged from 3 days up to 21 days (for stage 17–18 embryos).

## Results

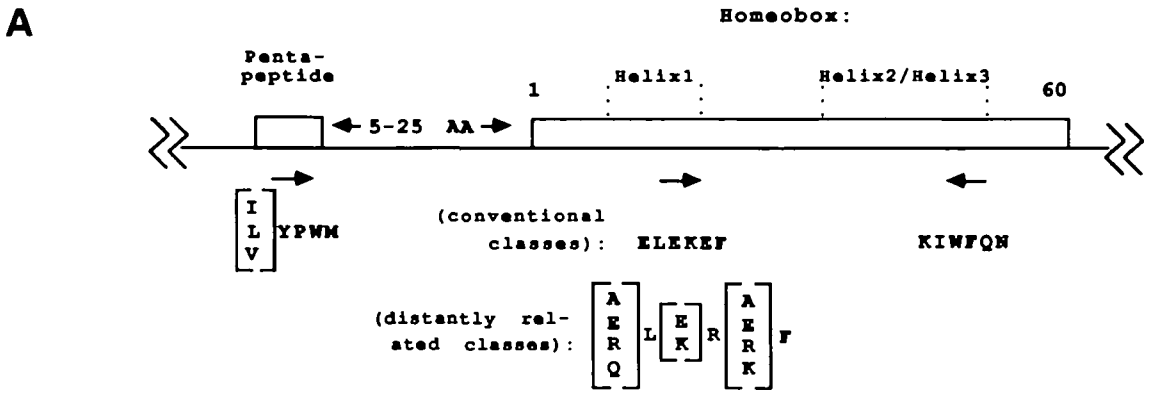
### *Detection of candidate homeobox genes expressed in limb buds by PCR amplification*

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 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 within '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.

Two of the clones obtained by PCR amplification of limb bud cDNA contained an unusual homeobox sequence (LUn 2 and LAn 19 in Fig. 1B) that was somewhat related to *Drosophila Abdominal B* (Regulski *et al.* 1985). One of these (LAn 19) was chosen for further study because preliminary analysis indicated that it was expressed at high levels predominantly in developing limb buds (see below), suggesting that its primary function is in some aspect of limb morphogenesis.

### *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 λZap 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




**B**

| | Clone #: | Sequence (PCR primers underlined): | Probable Homologue (amino acid identity with nearest homologue): |
|---|---|---|---|
| | | Homeodomain | |
| Hox 1.3 subfamily | LPa 25 | LYPWMRnvHISHDnigGPeGKRARTAYTRYQTLELEKEFHFNRYLTRRRRIEIAHALCLSERQIKIWFQN | Hox 1.3 (57/59) |
| | LPa 6 | LYPWMRKLHISHDmt-GPDGKRARTAYTRYQTLELEKEFHFNRYLTRRRRIEIAHALCLSERQIKIWFQN | Hox 2.1, Ghox 2.1 (58/58) * |
| | LPa 33 | VYPWMtKLHmSHe----tDGKRsRTsYTRYQTLELEKEFHFNRYLTRRRRIEIAnnLCLnERQIKIWFQN | Hox 3.4, XlHbox 5 (55/55) |
| Hox 1.2 subfamily | LPa 1 | LYPWMQRMNScaGtvYGAhgRRGRQtYtRYQTLELEKEFHFNRYLTRRRRIEIANALCLTERQIKIWFQN | Hox 1.2 (50/52) |
| | LAn 56 | ELEKEFHFNRYLTRRRRIEIAhsLCLTERQIKIWFQN | Hox 2.2, Ghox 2.2 (25/25) * |
| | LPa 24 | LYPWMQRMNShsGvgYGAdrRRGRQiYsRYQTLELEKEFHFNRYLTRRRRIEIANALCLTERQIKIWFQN | Hox 3.3, XlHbox 1, NvHbox1 (58/59) * |
| Hox 1.1 subfamily | LPa 19 | LYPWMRSsGpDRKRGRQTYTRYQTLELEKEFHfNRYLTRRRRIEIAHALCLTERQIKIWFQN | Hox 1.1, Xhox 36 (51/51) * |
| | LPb 2 | VYPWMRStGtDRKRGRQTYTRYQTLELEKEFHyNRYLTRRRRIEIAHALCLTERQIKIWFQN | Hox 2.3, Ghox 2.3 (48/51) * |
| Hox 2.4 subfamily | LPb 33 | IYPWMRPQAAGRRRGRQTYSRYQTLELEKEFLFNPYLTRKRRIEVSHALGLTERQVKIWFQN | Hox 2.4, Chox 2.4 (51/51) |
| | LAn 37 | ELEKEFLFNPYLTRKRRIEVSHALGLTERQVKIWFQN | Hox 3.1 (25/25) |
| Hox 1.7 subfamily | LAn 2 | ELEKEFLFNMYLTRDRRYEVARlLNLTERQVKIWFQN | Hox 1.7 (25/25) |
| | LAn 10 | ELEKEFLFNMYLTRDRRYEVARvLNLTERQVKIWFQN | Hox 3.2 (25/25) * |
| | LAn 58 | ELEKEFLFNMYLTRDRRYEVARiLNLTERQVKIWFQN | Hox 4.4 (previously 5.2) (25/25) * |
| | LAn 6 | ELEKEFLFNMYLTRERRLEISKSINLTDRQVKIWFQN | Hox 4.5 (previously 5.3) (24/25) * |
| | LUn 2 | ELERGFmdNEFItRQrRrELSdRLNLSDQQVKIWFQN | Human Hox 3F (23/25) |
| | LAn 19 | ELEKEFllNEFInRQkRkELSnRLNLSDQQVKIWFQN | Hox 4.7 (previously 5.6) (24/25) * |
| | LUn 3 | ALERKFRQKQYLSIAERAEFSSSLNLTETQVKIWFQN | Hox 7.1 (24/25) * |

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 concensus 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 $34\times10^3$ on denaturing gels, close to the predicted size of $30\times10^3$, 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

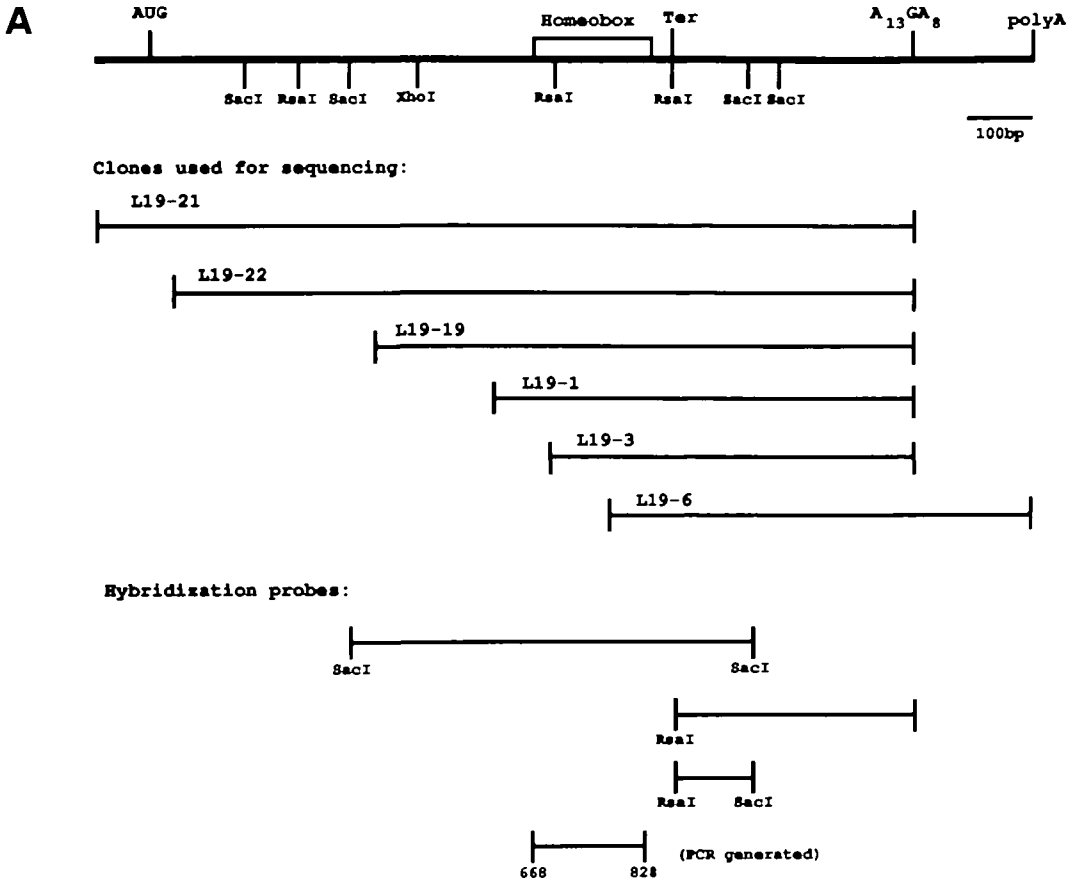
**Fig. 1.** Identification of homeobox genes expressed in chick embryo limb buds by PCR amplification.
(A) Schematic of highly conserved domains present in homeobox genes and consensus amino acid sequences that were used to make degenerate oligonucleotide primers for PCR amplification. The diagram indicates the approximate relative positions of the conserved pentapeptide present upstream of some homeobox genes (Mavilio *et al.* 1986; Kessel *et al.* 1987; Schughart *et al.* 1988;) and the highly conserved residues present within the α-helix 1 and helix 2 and 3 regions of the homeodomain proper (reviewed by Scott *et al.* 1989). The amino acid consensus sequences which were chosen for generating oligonucleotide primers are also shown. For the α-helix 1 region, two different primers were made based on two amino acid consensus sequences as shown. The nucleotide sequences of the PCR primers used are given in Materials and methods.
(B) Deduced amino acid sequences of the different homeodomain segments amplified from chick embryo limb bud cDNAs. The different clones that were obtained are designated according to which 5′ oligonucleotide primer was used; pentapeptide (LPa or LPb), conventional helix 1 (LAn), or distantly related helix 1 (LUn). The homeobox segments are grouped according to murine subfamilies (Duboule and Dolle, 1989; Graham *et al.* 1989), the primer-derived sequences are underlined, and sequences within the homeodomain proper are bracketed above. Amino acid residues that are conserved between chick 'subfamily' members are capitalized; those that are not are shown in lower case. Dashes have been introduced into a sequence where necessary in order to maximize the alignment of separated areas of homology. Probable homologues that have been identified in other vertebrates are listed, and amino acid identities of the chick homeodomain segment with the closest vertebrate homologue are indicated in parentheses to the right. An asterisk (*) indicates homeobox genes which have been shown to be expressed in limb buds in a vertebrate. References for the cited vertebrate homeobox genes and their expression are as follows: *Hox 1.3* (Fibi *et al.* 1988); *Ghox 2.1* and *2.2* (Wedden *et al.* 1989); *Hox 2.1* (Krumlauf *et al.* 1987); *Hox 3.4* (Gaunt *et al.* 1990); *XlHbox 5* and *XlHbox 1* (Fritz and De Robertis, 1988; Oliver *et al.* 1988); *Hox 1.2* (Colberg-Poley *et al.* 1985); *Hox 2.2* (Schughart *et al.* 1988); *Hox 3.3* (previously *6.1*, Sharpe *et al.* 1988); *NvHbox 1* (Savard *et al.* 1988; Tabin, 1989); *Hox 1.1* (Kessel *et al.* 1987; Mahon *et al.* 1988); *Xhox 36* (Condie and Harland, 1987); *Hox 2.3* (Meijlink *et al.* 1987); *Ghox 2.3* (Smith *et al.* 1989); *Hox 2.4* (Blatt *et al.* 1988); *Chox 2.4* (Scotting *et al.* 1990); *Hox 3.1* (Breier *et al.* 1988); *Hox 1.7* (Rubin *et al.* 1987); *Hox 3.2* (Erselius *et al.* 1990); *Hox 4.4* and *4.5* (Dolle and Duboule, 1989); Human *Hox 3F* (Acampora *et al.* 1989); *Hox 4.7* (Dolle *et al.* 1989; Izpisua-Belmonte and Duboule, personal communication); *Hox 7.1* (Hill *et al.* 1989; Robert *et al.* 1989).

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′ polyacidic 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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TCGTTTTAGGCTTTCCGCTTATTCCCTGTTGTAACTAAATACTGCTGTAAGAACAGCTGAAGTCCTTTGTTGGAAATGTGTGATCGCAGT   90
                                                                  M  C  D  R  S    5

CTCTACAGATCTGGCTACGTTGGTTCTCTCTTGAACTTGCAATCTCCAGATTCCTTTTATTTTCCCAATTTGCGGGCTAATGGCAGTCAG  180
 L  Y  R  S  G  Y  V  G  S  L  L  N  L  Q  S  P  D  S  F  Y  F  P  N  L  R  A  N  G  S  Q   35

TTGGCGGCTCTGCCCACCATTTCCTACCCCCGGAGCTCGATCCCGTGGACGTGCCCCAGTCCGTGCGCAGCCCAGCCGCAGGGCCACGCG  270
 L  A  A  L  P  T  I  S  Y  P  R  S  S  I  P  W  T  C  P  S  P  C  A  A  Q  P  Q  G  H  A   65

TTCGGCGGCGCGGCGCAGCCGTACCTCCCCGGCTCCGTTCCAATCAGCATCAGCTCCAACAGCAACAAAGAGTGCCTGGAAGAAAACAGT  360
 F  G  G  A  A  Q  P  Y  L  P  G  S  V  P  I  S  I  S  S  N  S  N  K  E  C  L  E  E  N  S   95

AAATATTACGCCCACGACGCGAGCTCCAAACAAGAGGAAAGATGCAGGCAGAGGCAATCTTTCGCAAATGACCCAACTATTACTCACGCA  450
 K  Y  Y  A  H  D  A  S  S  K  Q  E  E  R  C  R  Q  R  Q  S  F  A  N  D  P  T  I  T  H  A  125

GCCAACTTAAAGCCCGCAAAGTATGACCACTCGAGCCTGCCGAGGAGCTTGCACGGCTCGGCCGCTCTTTTTGAAGTGAATTCCTGCACT  540
 A  N  L  K  P  A  K  Y  D  H  S  S  L  P  R  S  L  H  G  S  A  A  L  F  E  V  N  S  C  T  155

TCCAGCCTAAAGGAGGACATCAAGAATTCGGTCAACTTGAACCTGACAGTTCAGCCCGCGGCAGTCCAGTCATGCCTCAGACCTTCAGTG  630
 S  S  L  K  E  D  I  K  N  S  V  N  L  N  L  T  V  Q  P  A  A  V  Q  S  C  L  R  P  S  V  185

CAGGATGGTTTGCCTTGGTGCCCCACCCAGGGGAGATCGCGAAAGAAACGGAAACCGTACACAAAACAGCAAATCGCCGAATTGGAAAAC  720
 Q  D  G  L  P  W  C  P  T  Q  G  R  S  R  K  K  R  K  P  Y  T  K  Q  Q  I  A  E  L  E  N  215

GAGTTTCTCCTCAATGAGTTTATTAACCGACAGAAGAGGAAAGAACTATCAAACAGACTGAATTTAAGCGACCAGCAAGTGAAAATTTGG  810
 E  F  L  L  N  E  F  I  N  R  Q  K  R  K  E  L  S  N  R  L  N  L  S  D  Q  Q  V  K  I  W  245

TTTCAGAACCGACGAATGAAAAAGAAAAAGAGTGGTAATGCGCGAGCAGGCGCTCTCTATGTACTAGCGCGGCGCCCGCCCGTTCCCGCAT  900
 F  Q  N  R  R  M  K  K  K  R  V  V  M  R  E  Q  A  L  S  M  Y  Ter                          266

GAACTTGCGTTCGCCCCGTAGAGTAGCGTGTGGTCGCGGCTGCGAGGCGGGAGCTGGGTGGGTTTTCCCCTTTAAGCCGAGCTCTCGGAA  990

CGCGTAGGATCCCCGCGTGCGTCGGCAACCGCTGAGCTCTTAGGCAGAGCGAAGAAGACGAGCCAGCCCAGCACTGAAACCCGTATTATC 1080

AGCTTTTCCCCCTTTCTTTCTCCTTTTATATAGATCTTCTCCCGCTTCTAACCCCCTCTTTCCCCGGAGAACGTGCGGCGCTAAACTAAA 1170

ATCGATGCCTTTTAACTGTATGTAAACACCACTTTGCAGGAAAAAAAAAAAAAGAAAAAAAAGAATAAAAGAATAATGCCTAGTAAAGAC 1260

GTCTGTAAATGCCGGCTGGTCCCTTCATGTGTTCCCCGCTTCTGAAGGCGAGCATGCGGCGCTCGCGTGTTTTGTGGTTTGGAATAACCT 1350

GTTGGAGCACAGATCGCACAAAGCGGTTACCAAATAAACCTTTTTAGTTTTCAAAAAAAAAAAAAAAAAA                    1419
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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 ($A_{13}GA_8$), and the true polyadenylation site (poly(A)). Below this 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 refered 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 that 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.

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 (*Rsa*I–*Sac*I, 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 *as revealed by* in situ *hybridization*

The expression pattern of *Ghox 4.7* was examined by *in situ* hybridization to sections of chick embryos, using an antisense probe spanning the homeobox (*Sac*I 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

```
Abd B:        VRKKRKPYSKFQTLELEKEFLFNAYVSKQKRWELARNLQLTERQVKIWFQNRRMKNKKNS
Ghox 4.7:     S-------T-Q-IA---N---L-EFINR---K--SNR-N-SDQ-----------K-RVV
Human Hox 3F: S---------L-LA---G---V-EFITR-R-R--SDR-N-SDQ-----------K-RLL
Chick LUn2:                       MD-EFITR-R-R--SDR-N-SDQ--
```

**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.

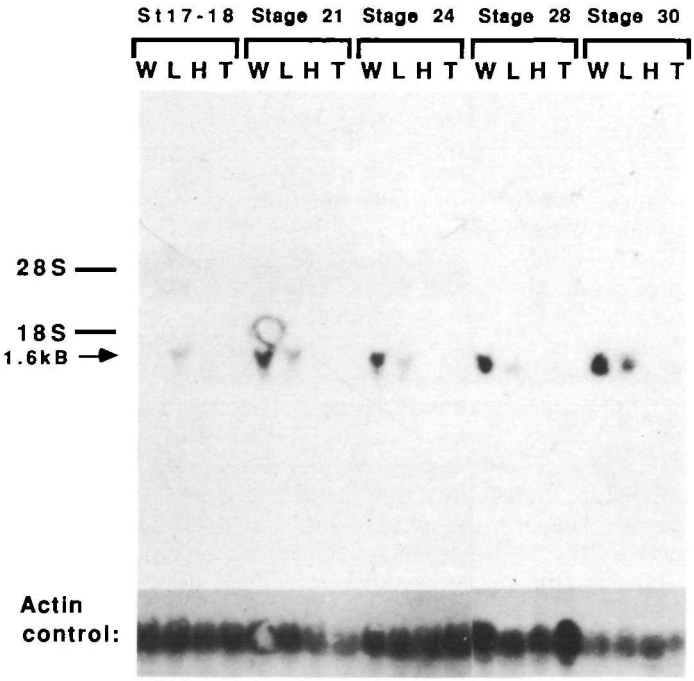



**Fig. 4.** Northern blot analysis of *Ghox 4.7* expression in chick embryos during the period spanning limb morphogenesis. Embryos at the stages (st) indicated were dissected into separated wing buds (W), leg buds (L), heads (H), and trunks (T) and 15 $\mu$g of total cellular RNA from each source was loaded per lane. The hybridization shown was performed with a homeobox-containing *Ghox 4.7* probe (*Sac*I fragment in Fig. 2A). Hybridization with a 3′ untranslated region *Ghox 4.7 probe* (*Rsa*I-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 $\beta$-actin cDNA probe (Cleveland *et al.* 1980) as shown.

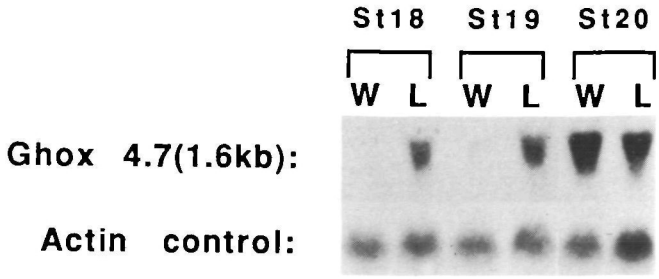



**Fig. 5.** Northern blot analysis demonstrating the delayed onset of *Ghox 4.7* expression in wing buds compared to leg buds. Conditions, probes, and abbreviations used are identical to those in Fig. 4.

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 precartilagenous 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 (e.g. 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

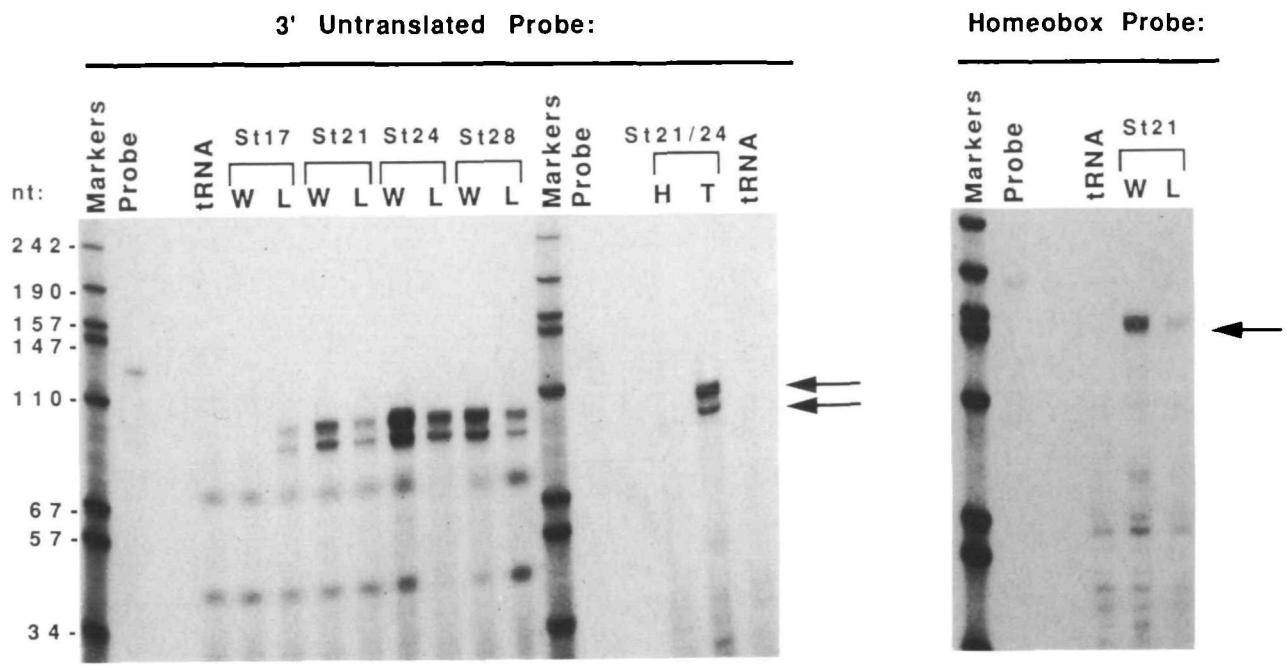



**Fig. 6.** Ribonuclease protection analysis of *Ghox 4.7* expression during limb morphogenesis in chick embryos using 3′ untranslated and homeobox-spanning antisense RNA probes. Total cellular RNAs extracted from dissected wing buds (W), leg buds (L), heads (H), and trunks (T) at the stages (st) indicated were hybridized with riboprobes transcribed from either 3′ untranslated sequences (*Rsa*I–*Sac*I 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 *Msp*I 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.

limb buds at a comparatively high level (Fig. 7C,D, and Fig. 9). At stage 28, a time at which the cartilagenous blastemata corresponding to the future skeletal elements have formed, both spatial as well as quantitative differences in *Ghox 4.7* expression were apparent in wing and leg by *in situ* hybridization. In both types of limb, the hybridization signal was predominantly in the posterior mesenchymal soft tissues of the distal-most part of the limb surrounding the cartilagenous foci, and also along the proliferating edge of the cartilagenous anlage (Fig. 9A–F), while the centers of chondrification were negative. However, in stage 28 wing, the posterior mesenchymal soft tissues also showed persistent expression of *Ghox 4.7* in a zone extending from the distal tip proximally up to the body wall, reminiscent of the expression pattern present at earlier stages (Fig. 9A–D), while in stage 28 leg, expression was entirely confined to the distal-most part of the posterior limb (Fig. 9E,F).

## 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;

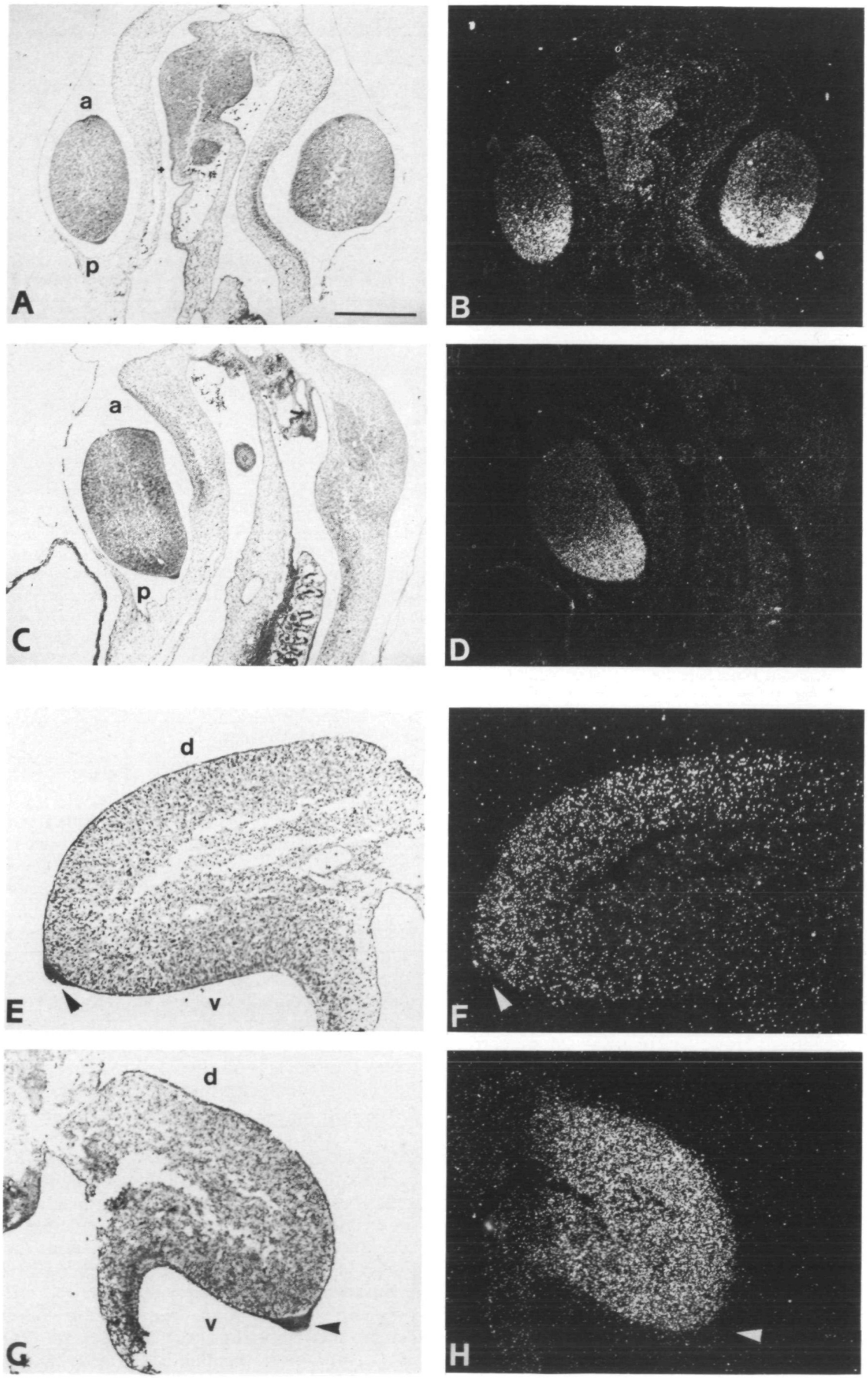

a
p
A
B
a
p
C
D
d
v
E
F
d
v
G
H

**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.

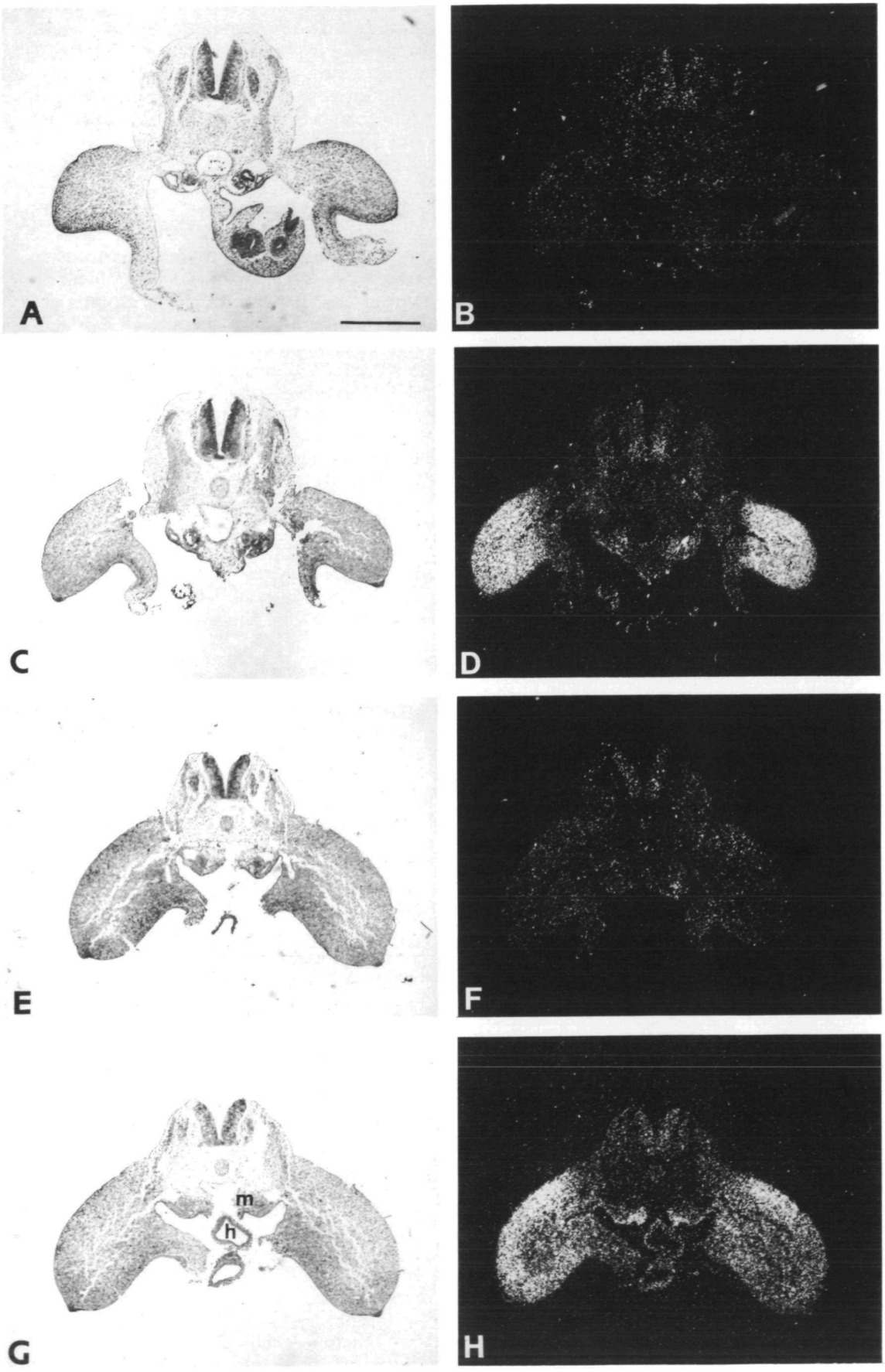
Robert *et al.* 1989; Savard *et al.* 1988; Tabin, 1989; Izpisua-Belmonte *et al.* 1990). In addition, it may be necessary to express a very large number of different homeobox genes in distinct but overlapping gradients to specify uniquely all of the different structures of the limb, despite the similarity 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 late 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.

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 nonsomitic 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 persistant 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 'remnant' of a steeply graded A–P distribution may indicate that even though the pattern


A
B
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m
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G
H

**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.

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.

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

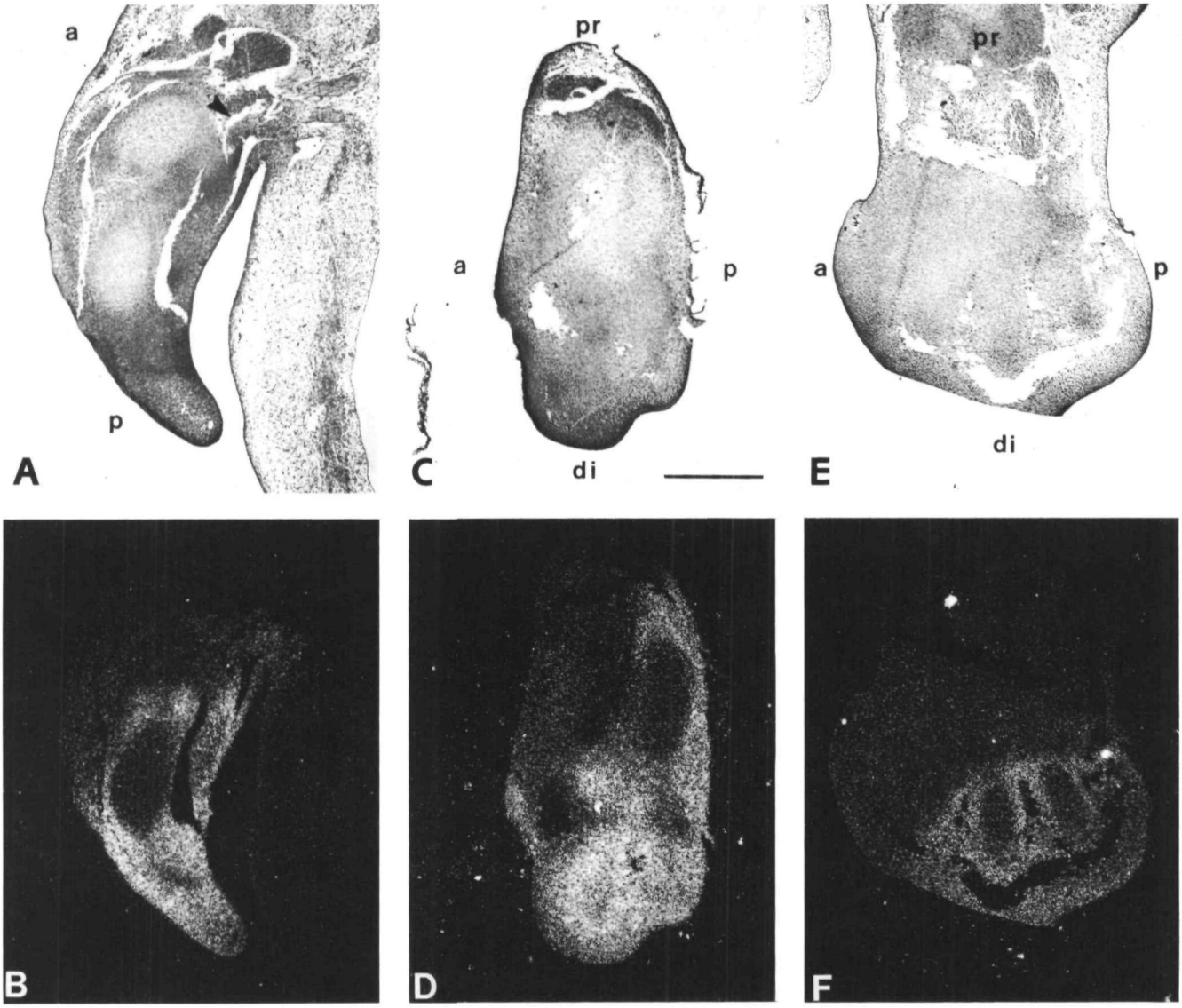


**Fig. 9.** Expression domains of *Ghox 4.7* at a later stage of limb development. Distinctions in limb-type expression of *Ghox 4.7* are evident by stage 28 (A–F). Coronal (A–B) and parasagittal (C–F) sections through stage 28 wing (A–D) and stage 28 leg (E–F) illustrate the intense labeling seen in 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.

*Ghox 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 *Ghox 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 (*XlHBox 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 mesenchymal 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.

We are grateful to Juan-Carlos Izpisua-Belmonte and Denis Duboule for sharing unpublished data and to Robb Krumlauf for helpful discussion. We would also like to thank Greg Dressler, Dave Levens, Lance Liotta, Marie Ranson and Lou Staudt for a critical reading of the manuscript and helpful comments.

## References

Acampora, D., D'Esposito, M., Faiella, A., Pannese, M., Migliaccio, E., Morelli, F., Stornaiuolo, A., Nigro, V., Simeone, A. and Boncinelli, E. (1989). The human *HOX* gene family. *Nucl. Acids Res.* **17**, 10385–10402.

Akam, M. (1987). The molecular basis for metameric pattern in the *Drosophila* embryo. *Development* **101**, 1–22.

Akam, M. (1989). Making stripes inelegantly. *Nature* **341**, 282–283.

Amprino, R. (1984). The development of the vertebrate limb. *Clinical Orthopaedics and Related Research* **188**, 263–284.

Balling, R., Mutter, G., Gruss, P. and Kessel, M. (1989). Craniofacial abnormalities induced by ectopic expression of the homeobox gene *Hox-1.1* in transgenic mice. *Cell* **58**, 337–347.

Blatt, C., Aberdam, D., Schwartz, R. and Sachs, L. (1988). DNA rearrangement of a homeobox gene in myeloid leukaemic cells. *EMBO J.* **7**, 4283–4290.

Breier, G., Dressler, G. R. and Gruss, P. (1988). Primary structure and developmental expression pattern of *Hox 3.1*, a member of the murine *Hox 3* homeobox gene cluster. *EMBO J.* **7**, 1329–1336.

Brickell, P. M. and Tickle, C. (1989). Morphogens in chick limb development. *Bioessays* **11**, 145–149.

Brockes, J. P. (1989). Retinoids, homeobox genes, and limb morphogenesis. *Neuron* **2**, 1285–1294.

Chevallier, A., Kieny, M., Mauger, A. and Sengel, P. (1977). Developmental fate of the somitic mesoderm in the chick embryo. In *Vertebrate Limb and Somite Morphogenesis* (ed. M. Balls, D. A. Ede and J. R. Hinchliffe). pp. 421–432. Cambridge: Cambridge University Press.

Christ, B., Jacob, H. J. and Jacob, M. (1977). Experimental analysis of the origin of the wing musculature in avian embryos. *Anat. Embryol.* **150**, 171–186.

Cleveland, D. W., Lopata, M. A., MacDonald, R. J., Cowan, N. J., Rutter, W. J. and Kirschner, M. W. (1980). Number and evolutionary conservation of $\alpha$- and $\beta$-tubulin and cytoplasmic $\beta$- and $\gamma$-actin genes using specific cloned cDNA probes. *Cell* **20**, 95–105.

Colberg-Poley, A. M., Voss, S. D., Chowdhury, K., Stewart, C. L., Wagner, E. F. and Gruss, P. (1985). Clustered homeo boxes are differentially expressed during murine development. *Cell* **43**, 39–45.

Condie, B. G. and Harland, R. M. (1987). Posterior expression of a homeobox gene in early *Xenopus* embryos. *Development* **101**, 93–105.

Davis, C. A. and Joyner, A L. (1988). Expression patterns of the homeobox-containing genes *En-1* and *En-2* and the proto-oncogene *int-1* diverge during mouse development. *Genes Dev.* **2**, 1736–1744.

Dolle, P. and Duboule, D. (1989). Two gene members of the murine *Hox-5* complex show regional and cell-type specific expression in developing limbs and gonads. *EMBO J.* **8**, 1507–1515.

DOLLE, P., IZPISUA-BELMONTE, J.-C., FALKENSTEIN, H., RENUCCI, A. AND DUBOULE, D. (1989). Coordinate expression of the murine *Hox-5* complex homeobox-containing genes during limb pattern formation. *Nature* **342**, 767–772.

DUBOULE, D., BONCINELLI, E., DE ROBERTIS, E., FEATHERSTONE, M., LONAI, P., OLIVER, G. AND RUDDLE, F. H. (1990). An update of mouse and human *Hox* gene nomenclature. *Genomics* **7**, 458–459.

DUBOULE, D. AND DOLLE, P. (1989). The structural and functional organization of the murine *HOX* gene family resembles that of *Drosophila* homeotic genes. *EMBO J.* **8**, 1497–1505.

EICHELE, G. (1989). Retinoids and vertebrate limb pattern formation. *Trends Genet.* **5**, 246–250.

ERSELIUS, J. R., GOULDING, M. D. AND GRUSS, P. (1990). Structure and expression pattern of the murine *Hox-3.2* gene. *Development* **110**, 629–642.

FIBI, M., ZINK, B., KESSEL, M., COLBERG-POLEY, A. M., LABEIT, S., LEHRACH, H. AND GRUSS, P. (1988). Coding sequence and expression of the homeobox gene *Hox 1.3*. *Development* **102**, 349–359.

FRITZ, A. AND DE ROBERTIS, E. M. (1988). *Xenopus* homeobox-containing cDNAs expressed in early development. *Nucl. Acids Res.* **16**, 1453–1469.

GALLIOT, B., DOLLE, P., VIGNERON, M., FEATHERSTONE, M. S., BARON, A. AND DUBOULE, D. (1989). The mouse *Hox-1.4* gene: primary structure, evidence for promoter activity and expression during development. *Development* **107**, 343–359.

GAUNT, S. J., COLETTA, P. L., PRAVTCHEVA, D. AND SHARPE, P. T. (1990). Mouse *Hox-3.4*: homeobox sequence and embryonic expression patterns compared with other members of the *Hox* gene network. *Development* **109**, 329–340.

GEHRING, W. J. (1987). Homeoboxes in the study of development. *Science* **236**, 1245–1252.

GILMAN, M. (1987). Ribonuclease protection assay. In *Current Protocols in Molecular Biology* (F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith and K. Struhl, eds). pp. 4.7.1–4.7.8. New York: John Wiley and Sons.

GRAHAM, A., PAPALOPULU, N. AND KRUMLAUF, R. (1989). The murine and *Drosophila* homeobox gene complexes have common features of organization and expression. *Cell* **57**, 367–378.

HAMBURGER, V. AND HAMILTON, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88**, 49–92.

HAN, K., LEVINE, M. S. AND MANLEY, J. L. (1989). Synergistic activation and repression of transcription by *Drosophila* homeobox proteins. *Cell* **56**, 573–583.

HAYASHI, S. AND SCOTT, M. P. (1990). What determines the specificity of action of *Drosophila* homeodomain proteins? *Cell* **63**, 883–894.

HILL, R. E., JONES, P. F., REES, A. R., SIME, C. M., JUSTICE, M. J., COPELAND, N. G., JENKINS, N. A., GRAHAM, E. AND DAVIDSON, D. R. (1989). A new family of mouse homeobox-containing genes: molecular structure, chromosomal location, and developmental expression of *Hox 7.1*. *Genes Dev.* **3**, 26–37.

IZPISUA-BELMONTE, J.-C., DOLLE, P., RENUCCI, A., ZAPPAVIGNA, V., FALKENSTEIN, H. AND DUBOULE, D. (1990). Primary structure and embryonic expression pattern of the mouse *Hox-4.3* homeobox gene. *Development* **110**, 733–745.

KAPPEN, C., SCHUGHART, K. AND RUDDLE, F. H. (1989). Two steps in the evolution of *Antennapedia* class vertebrate homeobox genes. *Proc. natn. Acad. Sci. U.S.A.* **86**, 5459–5463.

KESSEL, M., BALLING, R. AND GRUSS, P. (1990). Variations of cervical vertebrae after expression of a *Hox-1.1* transgene in mice. *Cell* **61**, 301–308.

KESSEL, M. AND GRUSS, P. (1990). Murine developmental control genes. *Science* **249**, 374–379.

KESSEL, M., SCHULZE, F., FIBI, M. AND GRUSS, P. (1987). Primary structure and nuclear localization of a murine homeodomain protein. *Proc. natn. Acad. Sci. U.S.A.* **84**, 5306–5310.

KOZAK, M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* **44**, 283–292.

KRUMLAUF, R., HOLLAND, P. W. H., MCVEY, J. H. AND HOGAN, B. L. M. (1987). Developmental and spatial patterns of expression of the mouse homeobox gene, *Hox-2.1*. *Development* **99**, 603–617.

MACCABE, J. A., ERRICK, J. AND SAUNDERS, J. W. JR (1974). Ectodermal control of the dorso-ventral axis of the leg bud of the chick embryo. *Devl Biol.* **39**, 69–82.

MAHON, K. A., WESTPHAL, H. AND GRUSS, P. (1988). Expression of homeobox gene *Hox 1.1* during mouse embryogenesis. *Development* **104 Supplement**, 187–195.

MAVILIO, F., SIMEONE, A., GIAMPAOLO, A., FAIELLA, A., ZAPPAVIGNA, V., ACAMPORA, D., POIANA, G., RUSSO, G., PESCHLE, C. AND BONCINELLI, E. (1986). Differential and stage-related expression in embryonic tissues of a new human homeobox gene. *Nature* **324**, 664–668.

MEIJLINK, F., DE LAAF, R., VERRIJZER, P., DESTREE, O., KROEZEN, V., HILKENS, J. AND DESCHAMPS, J. (1987). A mouse homeobox containing gene on chromosome 11: sequence and tissue-specific expression. *Nucl. Acids Res.* **15**, 6773–6785.

NAKAMURA, T., MAHON, K. A., MISKIN, R., DEY, A., KUWABARA, T. AND WESTPHAL, H. (1989). Differentiation and oncogenesis: phenotypically distinct lens tumors in transgenic mice. *The New Biologist* **1**, 193–204.

OLIVER, G., DE ROBERTIS, E. M., WOLPERT, L. AND TICKLE, C. (1990). Expression of a homeobox gene in the chick wing bud following application of retinoic acid and grafts of polarizing region tissue. *EMBO J.* **9**, 3093–3099.

OLIVER, G., SIDELL, N., FISKE, W., HEINZMANN, C., MOHANDAS, T., SPARKES, R. S. AND DE ROBERTIS, E. M. (1989). Complementary homeoprotein gradients in developing limb buds. *Genes Dev.* **3**, 641–650.

OLIVER, G., WRIGHT, C. V. E., HARDWICKE, J. AND DE ROBERTIS, E. M. (1988). A gradient of homeodomain protein in developing forelimbs of *Xenopus* and mouse embryos. *Cell* **55**, 1017–1024.

RAGSDALE, C. W., JR. AND BROCKES, J. P. (1990). Retinoic acid receptors and vertebrate limb morphogenesis. In *Structure and Function of Nuclear Hormone Receptors* (ed. M. G. Parker), pp. 269–295. London: Academic Press.

REGULSKI, M., HARDING, K., KOSTRIKEN, R., KARCH, F., LEVINE, M. AND MCGINNIS, W. (1985). Homeobox genes of the *Antennapedia* and *Bithorax* complexes of *Drosophila*. *Cell* **43**, 71–80.

ROBERT, B., SASSOON, D., JACQ, B., GEHRING, W. AND BUCKINGHAM, M. (1989). *Hox-7*, a mouse homeobox gene with a novel pattern of expression during embryogenesis. *EMBO J.* **8**, 91–100.

RUBIN, L. AND SAUNDERS, J. W. JR (1972). Ectodermal-mesodermal interactions in the growth of limb buds in the chick embryo: constancy and temporal limits of the ectodermal induction. *Devl Biol.* **28**, 94–112.

RUBIN, M. R., KING, W., TOTH, L. E., SAWCZUK, I. S., LEVINE, M. S., D'EUSTACHIO, P. D. AND NGUYEN-HUU, M. C. (1987). Murine *Hox-1.7* homeo-box gene: cloning, chromosomal location, and expression. *Molec. cell. Biol.* **7**, 3836–3841.

RUDNICK, D. (1945). Limb-forming potencies of the chick blastoderm: Including notes on associated trunk structures. *Trans. Conn. Acad. Arts Sci.* **36**, 353–377.

SAMBROOK, J., FRITSCH, E. F. AND MANIATIS, T. (1989). *Molecular Cloning: A Laboratory Manual*, second edition, Volumes 1–3. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

SANGER, F., NICKLEN, S. AND COULSON, A. R. (1977). DNA sequencing with chain termination inhibitors. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463–5467.

SAUNDERS, J. W. (1948). The proximo-distal sequence of origin of the parts of the chick wing and the role of the ectoderm. *J. exp. Zool.* **108**, 363–403.

SAUNDERS, J. W. AND GASSELING, M. T. (1968). Ectodermal-mesenchymal interactions in the origin of wing symmetry. In *Epithelial-Mesenchymal Interactions* (ed. R. Fleischmajer and R. E. Billingham), pp. 78–97. Baltimore: Williams and Wilkins.

SAVARD, P., GATES, P. B. AND BROCKES, J. P. (1988). Position dependent expression of a homeobox gene transcript in relation to amphibian limb regeneration. *EMBO J.* **7**, 4275–4282.

SCHUGHART, K., UTSET, M. F., AWGULEWITSCH, A. AND RUDDLE, F. H. (1988). Structure and expression of *Hox 2.2*, a murine

homeobox-containing gene *Proc. natn. Acad. Sci. U S.A.* **85**, 5582–5586.

SCOTT, M. P., TAMKUN, J. W. AND HARTZELL, G. W. (1989). The structure and function of the Homeodomain. *BBA Reviews on Cancer* **9**, 25–48.

SCOTTING, P. J., HEWITT, M. AND KEYNES, R. J. (1990). Isolation and analysis of chick homeobox cDNA clones. *Nucl. Acids Res.* **18**, 3999.

SHARPE, P. T., MILLER, J. R., EVANS, E. P., BURTENSHAW, M. D. AND GAUNT, S. J. (1988). Isolation and expression of a new mouse homeobox gene. *Development* **102**, 397–407.

SHORT, J. M., FERNANDEZ, J. M., SORGE, J. A. AND HUSE, W. D. (1988). *Lambda ZAP*: a bacteriophage *lambda* expression vector with *in vivo* excision properties. *Nucl. Acids Res.* **16**, 7583–7600.

SMITH, S. M., PANG, K., SUNDIN, O., WEDDEN, S. E., THALLER, C. AND EICHELE, G. (1989). Molecular approaches to vertebrate limb morphogenesis. *Development* **10 Supplement**, 121–131.

STEPHENS, T. D., BEIER, R. L. W., BRINGHURST, D. C., HIATT, S. R., PRESTRIDGE, M., PUGMIRE, D. E. AND WILLIS, H. J. (1989). Limbness in the early chick embryo lateral plate. *Devl Biol.* **133**, 1–7.

SUMMERBELL, D. (1974). A quantitative analysis of the effect of excision of the AER from the chick limb bud. *J. Embryol. exp. Morph.* **32**, 651–660.

SUMMERBELL, D. (1983). The effects of local application of retinoic acid to the anterior margin of the developing chick limb. *J. Embryol exp. Morph.* **78**, 269–289.

SUMMERBELL, D., LEWIS, J. AND WOLPERT, L. (1973). Positional information in chick limb morphogenesis. *Nature* **244**, 492–496.

TABIN, C. J. (1989). Isolation of potential vertebrate limb-identity genes. *Development* **105**, 813–820.

THALLER, C. AND EICHELE, G. (1987). Identification and spatial distribution of retinoids in the developing chick limb bud. *Nature* **327**, 625–628.

THALLER, C. AND EICHELE, G. (1988). Characterization of retinoid metabolism in the developing chick limb bud. *Development* **103**, 473–483.

TICKLE, C., ALBERTS, B. M., WOLPERT, L. AND LEE, J. (1982). Local application of retinoic acid to the limb bud mimics the action of the polarizing region. *Nature* **296**, 564–565.

TICKLE, C., LEE, J. AND EICHELE, G. (1985). A quantitative analysis of the effect of all-trans-retinoic acid on the pattern of chick wing development. *Devl Biol.* **109**, 82–95.

TICKLE, C., SUMMERBELL, D. AND WOLPERT, L. (1975). Positional signalling and specification of digits in chick limb morphogenesis. *Nature* **254**, 199–202.

WEDDEN, S. E., PANG, K. AND EICHELE, G. (1989). Expression pattern of homeobox-containing genes during chick embryogenesis. *Development* **105**, 639–650.

WOLGEMUTH, D. J., BEHRINGER, R. R., MOSTOLLER, M. P., BRINSTER, R. L. AND PALMITER, R. D. (1989). Transgenic mice overexpressing the mouse homeobox-containing gene *Hox-1.4* exhibit abnormal gut development. *Nature* **337**, 464–467.

WRIGHT, C. V. E., CHO, K. W. Y., OLIVER, G. AND DE ROBERTIS, E. M. (1989). Vertebrate homeodomain proteins: families of region-specific transcription factors. *Trends Biochem. Sci.* **14**, 52–56.

WRIGHT, C. V. E., CHO, K. W. Y., HARDEWICKE, J., COLLINS, R. AND DE ROBERTIS, E. M. (1989). Interference with function of a homeobox gene in *Xenopus* embryos produces malformations of the anterior spinal cord. *Cell* **59**, 81–93..

ZWILLING, E. (1964). Development of fragmented and of dissociated limb bud mesoderm. *Devl Biol.* **9**, 20–37.

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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.