Region-specific expression in early chick and mouse embryos of Ghox-lab and Hox 1.6, vertebrate homeobox-containing genes related to Drosophila labial

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Summary

A chick gene homologous to the Drosophila homeobox gene labial has been cloned and sequenced. Regions of additional sequence identity outside of the homeobox reveal a close relationship to the mouse gene Hox 1.6. Northern blot analysis demonstrates that Ghox-lab and Hox 1.6 transcripts are both present at high levels during early stages of chick and mouse development, with a subsequent decline in abundance to very low levels by the time limb mesenchyme begins to differentiate. In situ hybridization analysis of chick embryos shows intense expression of Ghox-lab mRNA by Hamburger and Hamilton stage 4 (avian "mid gastrula") and by stage 6 (pre-somatic neural plate) with expression decreasing shortly thereafter. The pattern of Ghox-lab RNA expression in these early embryos divides the embryo into an anterior and a posterior compartment. At stage 6, considerable signal is observed in the posterior two thirds of the embryo, while none is detected in the anterior third which is fated to become the head. This pattern is purely regional in nature, and does not follow boundaries defined by known tissue types. In situ hybridization of Hox 1.6 probes to mouse embryos of day 7.5 or 8.0 indicate that the Hox 1.6 transcript has a temporal and spatial distribution very similar to that of Ghox-lab in the chick embryo.

Key words: pattern formation, chick embryo, mouse embryo, homeobox gene, gastrulation, primitive streak, in situ hybridization.

Introduction

During early development of the vertebrate embryo, specializations of morphology and cell type appear at defined sites along its anteroposterior axis. How this pattern of cell differentiation takes form is a key problem long pursued by classical embryology. In Drosophila a family of related genes clustered in the Antennapedia complex (ANT-C; Mahaffey and Kaufman, 1989) and the Bithorax complex (BX-C; Duncan, 1987) specify parasegmental identity along the anteroposterior axis. Encoded within these genes are variants of a 60 amino acid motif known as the homeobox (Gehring, 1987; Scott et al. 1989 for reviews). The homeo domain contributes a sequence-specific DNA-binding activity (e.g. Desplan et al. 1988; Otting et al. 1988) which in turn allows the gene product to selectively regulate transcription (Driever and Nüsslein-Volhard, 1989; Krasnow et al. 1989; Struhl et al. 1989; Winslow et al. 1989). Vertebrate genes have been identified that encode close cognates of most Drosophila homeoboxes within the ANT-C and BX-C (McGinnis et al. 1984; reviewed e.g. in Wright et al. 1989). Recently it has become clear that these vertebrate genes are arranged in the genome as four clusters. These clusters have arisen early in the evolution of chordates through duplication of a single ancestral cluster (reviewed in Akam, 1989). Since the time of this duplication there has been strong conservation of cognate homeobox sequences. As in Drosophila, all transcription units are oriented in the same direction within a cluster, and the relative order of cognate genes within the clusters has been retained (see Akam, 1989 and references therein).

Systematic comparisons of expression patterns of vertebrate homeobox genes have been reported for the mouse clusters Hox 1 (e.g. Duboule and Dollé, 1989) and Hox 2 (e.g. Graham et al. 1989). In the case of Hox...
2, for example, a gene located at the 5′ end of the cluster is expressed in a caudal region of the spinal cord. Successively more 3′ genes are found to have boundaries of expression that are located at progressively more anterior positions in the central nervous system (Graham et al. 1989). Such a relationship between gene position and domain of expression and function has been noted previously in Drosophila by Lewis (1978). Taken together, these conserved features of gene sequence, gene organization and gene expression pattern indicate that the molecular basis of pattern specification along the anteroposterior axis already existed in an ancestral organism common to insects and vertebrates.

The Drosophila labial gene (lab) and its only vertebrate homolog characterized to date, Hox 1.6 (Baron et al. 1987; also named ERA-1, LaRosa and Gudas, 1988) occupy the extreme 3′ positions within the ANT-C and Hox 1 clusters, respectively (Baron et al. 1987; Diederich et al. 1989). Consistent with the ordering scheme alluded to above, the labial gene product is expressed and functions in the most anterior domain of any homeotic gene within ANT-C (Diederich et al. 1989). Likewise, Hox 1.6 has been reported to have the most anterior expression boundary of any gene in the Hox 1 locus (Duboule and Dollen, 1989). This paper describes the isolation, sequence analysis and expression pattern of a chick gene, designated Ghox-lab (Gallus homeobox gene homologous to labial). The sequence of the homeo domain of Ghox-lab is closely related to that of mouse Hox 1.6 and of Drosophila labial. Ghox-lab and Hox 1.6 are expressed at high levels during primitive streak and head fold stages, considerably prior to somitogenesis. By contrast, embryos undergoing late somitogenesis and organogenesis express much lower levels of Ghox-lab and Hox 1.6. This early, transient expression raises the possibility that these labial-related genes are among the first homeobox genes activated during the organization of the vertebrate anteroposterior axis.

Material and methods

Cloning Ghox-lab

A probe was prepared from the 545 bp XhoI–BglII fragment (nts 347 to 892) of a Hox 1.6 cDNA (LaRosa and Gudas, 1988). This probe contained most of the homeobox in addition to upstream sequence of the message. The probe was labeled to high specific activity with [32P]dCTP by random hexamer priming (Feinberg and Vogelstein, 1983). Chick genomic DNA prepared from White Leghorn embryos (from SPAFAS, Norwich, CT) was digested with various combinations of EcoRI, BamHI, HindIII, SstI and XhoI, electrophoresed in 1% agarose, and transferred to Genescreen (Du Pont). Hybridization of probes and filters was carried out at 42°C in 50% or 35% formamide, 10% dextran sulfate, 1× Denhardt’s solution without bovine serum albumin, 4× SSC, 10 mM-Tris–HCl pH 7.4, 1% SDS, 100 μg/mL poly A. After adding 32P-labeled DNA probe to the prehybridization mixture at 4×106 cts min−1 mL−1 probe (1×SSC=150 mM-NaCl, 15 mM-sodium citrate). Washes were in 2× SSC at 50 or 65°C.

To clone the 15 kb EcoRI fragment that hybridized with the Hox 1.6 probe, 120 μg of chick genomic DNA were digested to completion with EcoRI, loaded in 1 cm slots of an 0.7% agarose Tris acetate/EDTA gel, (Maniatis et al. 1983), and electrophoresed at 5 V cm−1 with adjacent slots of lambda HindIII digest marker DNA. The 9 to 23 kb region of the gel was excised and purified by electroelution followed by banding in a cesium chloride step gradient. The size fractionated genomic EcoRI fragments were ligated to EcoRI cut arms of lambda vector EMBL 4 (Stratagene), in vitro packaged (Stratagene Gigapack Plus) and plated at 2.5×106 plaques per 625 cm2 plate. Plaque replicas on Gene Screen Plus filters were hybridized as above, and a set of intensely positive clones located and plaque purified. A 5.8 kb EcoRI–BamHI fragment was excised from the 15 kb insert and sub-cloned into the plasmid pGEM 3Z (Promega).

DNA sequence analysis

Sequencing was by the dideoxy chain termination method, using Klenow enzyme (Sanger et al. 1977) or Sequenase (US Biochemicals) following the manufacturer’s protocol. Reactions were carried out on either single stranded phage templates or on double stranded plasmids (Tonguzzo et al. 1988). Fragments with strong homology to the Hox 1.6 probe were sequenced first, followed by adjacent fragments. All fragments were connected by sequence overlaps, and likely coding regions sequenced repeatedly on both strands. Synthetic 17-mer oligonucleotides were used to extend or confirm the sequence. The intron largely represents single-strand sequence.

RNA blot analysis

Chick RNA analysis

Embryos were incubated at 37.5°C and staged according to Hamburger and Hamilton (1951). Embryos were washed and dissected in ice-cold PBS (phosphate-buffered saline) and Dounce homogenized in 4 volumes of a 4 mM-guainidium isothiocyanate mixture. Total RNA was then extracted by the guainidinium/hot phenol method outlined in Maniatis et al. (1983). Poly(A)+ RNA was selected from 10 μg total RNA (Maniatis et al. 1983) using 0.2 ml oligo (dT)-cellulose minicolumns. The entire poly(A)+ fraction was precipitated with 10 μg yeast tRNA, resuspended in sample buffer, and separated by electrophoresis carried out as described previously (Wedden et al. 1989). The chick RNA was transferred electrophoretically to a Genescreen membrane in 25 mM-sodium phosphate at pH 6.8 at 3 V cm−1 for 2 h, according to the directions provided with Genescreen (Du Pont). RNA was cross-linked to the filter by UV (Church and Gilbert, 1984). Filters were prehybridized for 1 h at 42°C in the same mix used for genomic Southerns, plus 5 μg mL−1 poly ribo A. After adding 32P-labeled DNA probe to the prehybridization mixture at 4×106 cts min−1 mL−1, the filters were hybridized with agitation at 42°C for 16 h. To remove non-specific signal, filters were washed twice in 2× SSC at 50°C 10 min, then twice in 2× SSC, 1% SDS at 68°C, 30 min.

Mouse RNA analysis

CF-1 females (Harlan Sprague-Dawley) were mated with B6 SJLF/J (Jackson Labs) males and checked the following morning for vaginal plug formation. Noon on the day of the vaginal plug was considered day 0.5 of gestation. The embryos were dissected free of extraembryonic membranes with the exception of the day 7.5 embryos, which included all membranes minus the trophectoderm. Isolation of total cellular mouse embryo RNA was accomplished as described (Kingston, 1989). Mouse RNA was fractionated on 1% agarose/2.2 M-formaldehyde gels, blotted and hybridized as
described (LaRosa and Gudas, 1988). The probe used encompassed nts 1098 to 1953 of the mouse Hox 1.6 gene (LaRosa and Gudas, 1988).

In situ hybridization of chick embryos
Embryos were collected, rinsed in PBS and then fixed in ice-cold freshly prepared 4% paraformaldehyde/PBS for 1 h. They were then rinsed in 0.9% NaCl and dehydrated through a graded series of ethanol, xylene/xylene 1:1, xylene, xylene/wax 1:1 and finally wax (Paraplast). 7 μm sections were cut, mounted on slides, de waxed, pretreated and prehybridized as described in Wedden et al. (1989). A 293-base [35S]UTP-labeled riboprobe (nts 280 to 573, see Fig. 1; specific activity of 107 cts min⁻¹ μg⁻¹ ) was applied to the tissue sections at a final concentration of 0.02 ng μl⁻¹ hybridization mix, and hybridization was carried out at 50°C for 5 h. Washes to remove non-specifically bound probe were as follows: (1) two washes in FSM (50% formamide, 2×SSC, 20 mM-2-mercaptoethanol) at 65°C for 30 min each, (2) two washes in STE (4×SSC, 20 mM-Tris-HCl pH 7.4, 1 mM-EDTA) at 37°C for 15 min, (3) digestion with 4 μg ml⁻¹ RNAase A (Boehringer) in STE, 30 min 37°C, (4) one wash in STE containing 20 mM-2-mercaptoethanol at 37°C for 10 min, (5) two washes in FSM at 65°C for 45 min each. Sections were briefly rinsed at room temperature in saline, water, and were finally air dried. Exposure to Cronex film and NTB-2 emulsion autoradiography were as described in Zeller (1989). Slides were stained in 5 μg ml⁻¹ Hoechst 33258 dye in water for 2 min, followed by rinsing 2 min in water. The slides were air dried first at room temperature and then 50°C for one hour, followed by mounting in Balsam-Salicylate (5 g Canada balsam, at 42°C; washed at 50°C) Southerns of £coRI, BamHl, HindlH digest revealed a single band of 545 bp homeobox-containing DNA sequence reveals homology with mouse Hox 1.6 and Drosophila labial
In order to isolate a chicken homolog of mouse Hox 1.6 (Baron et al. 1987; LaRosa and Gudas, 1988), Southern blots of chicken genomic DNA were probed with a 545 bp homeobox-containing Xho1–BglII (nts 347 to 892) fragment of a Hox 1.6 DNA clone (LaRosa and Gudas, 1988). Low stringency (hybridization 35% formamide, at 42°C; washed at 50°C) Southern of EcoRI, BamHI, HindIII and SstI digests revealed a single

Stage 1
The embryo is a flat disc composed of epiblast and hypoblast. Cells accumulate at the posterior end of the embryo, where the primitive streak will soon begin to form. The epiblast, which is analogous to the inner cell mass of the mouse, will form the embryo proper while the hypoblast will give rise to extraembryonic structures. (mouse day 6, egg cylinder)

Stage 4, definitive streak stage (18–19 h)
The primitive streak, which is the avian equivalent of the amphibian blastopore, is fully formed. The primitive streak consists of two elongated ridges separated by the primitive groove (see also Fig. 6). At the anterior end of the primitive streak is Hensen’s node, the chick equivalent of the Spemann organizer. Epiblast cells migrate inwards through the streak and become mesoderm and endoderm. This represents the avian equivalent of amphibian gastrulation. (mouse day 7)

Stage 5, head process stage (19–22 h)
Anterior to Hensen’s node the notochord (head process) arises in the form of a rod of condensed mesenchyme. Hensen’s node begins to be displaced posteriorly. (mouse day 7.5)

Stage 6, head fold stage (23–25 h), see Fig. 6
The medullary plate (primordium of CNS) forms anterior to the retreating node. Anterior tissue buckles up from the disc to form a head fold. (mouse day 7.75)

Stage 7, (23–26 h)
The first pair of somites appears anterior to the retreating node. Somites will continue to be laid down in an anterior-to-posterior fashion. (mouse day 8)

Stage 15, (50–55 h)
Head, fore-, mid- and hindbrain as well as the optic cups are clearly defined structures. There are about 25 somites. The limb rudiments are about to emerge. (mouse day 8.75)

Stage 24, (approx. 96 h)
Limb rudiments display elbow and knee. Hand plate and foot plate formed but digits and toes not yet demarcated. (mouse day 11.5)

Stage 32, (approx. 7.5 days)
Limbs have well formed toes and digits, web between autopods is thinning. (mouse day 13)

Stage 46, (approx. 21 days)
Hatching.

Results

DNA sequence reveals homology with mouse Hox 1.6 and Drosophila labial
In order to isolate a chicken homolog of mouse Hox 1.6 (Baron et al. 1987; LaRosa and Gudas, 1988), Southern blots of chicken genomic DNA were probed with a 545 bp homeobox-containing Xho1–BglII (nts 347 to 892) fragment of a Hox 1.6 DNA clone (LaRosa and Gudas, 1988). Low stringency (hybridization 35% formamide, at 42°C; washed at 50°C) Southerns of EcoRI, BamHI, HindIII and SstI digests revealed a single

Synopsis of chick development
To facilitate cross-species comparisons with other vertebrate embryos such as mouse and Xenopus, we provide a brief timetable and description of the stages of chick development relevant to this study. For details of early chick development, see Hamburger and Hamilton (1951) and Bellairs (1986). See Rugh (1977) and Theiler (1989) for details of mouse development. The Hamburger and Hamilton chick stage is followed by the equivalent day embryo in the mouse.
strong band and one to three weaker bands. In the case of EcoRI, for example, the strong band migrating at 15 kb and one fainter band migrating at 12 kb were still visible after stringent hybridization conditions (50% formamide, 42°C, washed at 65°C). Size fractionated EcoRI digested genomic DNA was cloned into lambda EMBL4. Several positive clones were isolated at a frequency of one per 8000. Each contained a 15 kb EcoRI insert and a 5.8 kb EcoRI-BamHI subfragment which hybridized strongly to the probe.

The 5.8 kb EcoRI-BamHI genomic fragment was further analyzed by restriction mapping. Strongly hybridizing regions and their flanking DNA were sequenced. Since the sequence is not derived from a cDNA clone, certain features of the organization of the Ghox-lab gene and of its mRNA are inferred from homology with Hox 1.6 (Baron et al. 1987; LaRosa and Gudas, 1988). Beginning at the 5' end of the gene (Fig. 1) there are glucocorticoid response element (GRE)-like sequences (nts -162 to -149) and an SP1 binding site (Proudfoot and Brownlee, 1976) located at nucleotide 351. Eukaryotic mRNA is capped at the 5' end, and polyadenylated at the 3' end. The open reading frame which ends at nucleotide 1594 encodes a run of fourteen amino acids, twelve of which are identical to those found in the corresponding region of Hox 1.6 (LaRosa and Gudas, 1988). Nucleotides 1 to 42 encode a motif of 1.6, 213-272, and shows strong homology to the homeobox of vertebrate homeoboxes (Wright et al. 1989). The homeobox (Fig. 2 C) encompasses amino acids 213-272, and shows strong homology to the homeobox of Hox 1.6 (56 of 60 amino acids are identical) and that of Drosophila labial (51 of 60 amino acids are identical). As can be seen in Fig. 2 C, Ghox-lab homeobox has substantially less homology to Antp (39 amino acid identity). Amino acids 273-276 are the same as in Hox 1.6, but the remaining portions of the two proteins' 3' to the homeobox are highly diverged. The entire Ghox-lab protein has a predicted length of 309 amino acids, similar in size to the predicted 336 amino acids of Hox 1.6. By contrast, labial protein of Drosophila contains 629 amino acids (Diederich et al. 1989). The Ghox-lab protein coding region is followed by a 1.1 kb untranslated sequence which ends shortly after the polyadenylation site (Proudfoot and Brownlee, 1976) located at nucleotides 2051-2057 (Fig. 1).

The genomic DNA sequence also provides good evidence for the existence of a splice between nucleo-
The predicted splice of CUCCUUACAG/C sequence which closely fits the sequence for a 5' splice site (Green, 1986). (3) The 3' which is in perfect agreement with the consensusary of this region contains a CAG/GUGAGU motif stretch of non-homologous sequence. (2) The 5' bound- alignment is interrupted by the insertion of a 0.7 kb Hox 1.6 lab cDNA, this evidence is as follows (see Fig. 1): (1) When the tides 604 and 605 (amino acid 202, alanine). The lab, Hox 1.6 evidence is as follows (see Fig. 1): (1) When the homeo domains of Ghox-lab and Hox 1.6 that extends in front of the homeo domain. Note the splice sites of the two proteins is located at the same position (triangles). (C) Sequence comparison Note the splice sites of the two proteins is located at the same position (triangles). (C) Sequence comparison between the homeo proteins of Drosophila Antennapedia, Ghox-lab, Hox 1.6 and Hox 1.6 Antennapedia.

tides 604 and 605 (amino acid 202, alanine). The evidence is as follows (see Fig. 1): (1) When the Ghox-lab sequence is aligned with the Hox 1.6 cDNA, this alignment is interrupted by the insertion of a 0.7 kb stretch of non-homologous sequence. (2) The 5' boundary of this region contains a CAG/GUGAGU motif which is in perfect agreement with the consensus sequence for a 5' splice site (Green, 1986). (3) The 3' boundary of the inserted region contains a CUCCUUACAG/C sequence which closely fits the requirements of a 3' splice site (Green, 1986). (4) A likely branch point sequence is located within the intron, at the first A residue, 37 nucleotides upstream of the 3' splice site. It has the sequence AGCUAAC, which fits the consensus, and is very similar to the branch point, for example, in the Drosophila fuz gene intron (AGCUAAAG; Reed and Maniatis, 1985). (5) The predicted splice of Ghox-lab is located at a position precisely homologous to that of Hox 1.6 (Fig. 2B). In summary, Ghox-lab resembles a typical vertebrate homeobox gene. It contains two exons, the second of which encodes the homeobox, and in addition a long 3' untranslated region.

**Nature of Ghox-lab and Hox 1.6 mRNA species**

Northern blots of chick poly(A)^+ RNA hybridized with a Ghox-lab probe containing nts 280 to 573 (located in the upstream exon) reveal two major bands, corresponding to RNAs of 2.3 and 3.2 kb, that are present in similar amounts (Figs. 3 A and B). The 2.3 kb band is consistent with a transcript which begins at around −50 nt upstream of the first AUG codon, is spliced once at nucleotides 604/605 to remove the 0.7 kb intron, and then terminates near nt 2070 following the polyadenylation signal (Fig. 1). Using Northern blots we have attempted to characterize the molecular nature of the 3.2 kb transcript. These efforts led to the following conclusions. (1) The 3.2 kb transcript is unlikely to be an elongated version of the 2.3 kb mRNA that uses an alternative poly A addition site. A 1.2 kb DNA probe derived from sequences located 90 bp downstream of the poly A site at nts 2051 to 2057 fails to detect either the shorter or the longer RNA (data not shown). (2) The 3.2 kb mRNA is not a simply an unspliced version of the shorter mRNA, since a 260 bp probe centered in the intron does not hybridize (data not shown). (3) Both the 2.3 kb and 3.2 kb mRNAs are detected by a probe to the homeobox-containing exon, and by each of two probes (nts −219 to 279 or nts 280 to 573) spanning the upstream exon. Hence both transcripts cover the homeobox and at least a substantial portion of the DNA upstream of the 604/605 splice site. A remaining possibility is that the 3.2 kb message has a different promoter located further upstream. There is precedent for such an arrangement in other vertebrate homeo-boxes (Oliyer et al. 1988; Boncinielli et al. 1989), and in these cases alternate splicing results in a truncated protein. We are in the process of resolving the issue by sequence analysis of Ghox-lab cDNA clones.

The Hox 1.6 mRNA in mouse embryos appears as a broad band of about 2.4 kb (Fig. 3 C), consistent with the two transcripts of 2.2 and 2.4 kb characterized previously by LaRosa and Gudas (1988). This result differs greatly from that of Baron et al. (1987). Their Northern data suggested the presence of 10 kb and 4.5 kb transcripts in day 9, 10, and 13 mouse embryos and of 1.0 and 1.4 kb transcripts in adult intestine. We cannot explain this result since sequence analysis clearly suggests that these clones encode identical genes.

**Ghox-lab and Hox 1.6 mRNA levels are high during early stages of development and then decline**

Given the sequence homology between Ghox-lab and Hox 1.6, is there also a conservation of function? This should manifest itself, for example, in a similar pattern of expression. To allow a direct comparison between chick and mouse, we have prepared developmental Northern blots of RNA extracted from chick and mouse embryos. Poly(A)^+ mRNA was prepared from whole chick embryos ranging from stage 15 to 32 (for definition of stages see Materials and methods). As can be seen in the Northern blot shown in Fig. 3 A, Ghox-lab expression is high in stage 15 embryos but decreases substantially by stage 22 and is virtually undetectable in older (stage 32) embryos. The proportions of the 2.3 and 3.2 kb RNAs are similar at each of these stages.

RNA was also isolated from mouse embryos over a period of development comparable to that shown in Fig. 3 A for the chick. RNA prepared from day 7.5 mouse embryos (chick stage 5) gave an intense signal when probed with Hox 1.6 cDNA (Fig. 3 C, lane 2). By contrast, RNA prepared from day 7.5 decidual tissue exhibited no signal (Fig. 3 C, lane 1). RNA from the day 8.5 mouse embryo (Fig. 3 C, lane 3), which corresponds roughly to the stage 15 chick embryo (Fig. 3 A, lane 1), exhibited a lower Hox 1.6 mRNA level than that of day 7.5 embryo RNA. As was observed in chick, the message abundance is greatly
reduced by the stage the limb mesenchyme starts to differentiate (Hamburger-Hamilton chick stage 22, and mouse day 10.75). Even less expression is seen in the developmentally most advanced mouse, day 12.5 (last lane in Fig. 3 C), which is comparable to stage 32 in the chick (last lane in Fig. 3 A). These results indicate that \textit{Ghox-lab} and \textit{Hox 1.6} mRNAs exhibit a very similar temporal expression pattern.

\textbf{Spatial localization of \textit{Ghox-lab} mRNA by in situ hybridization in early chick embryos}

The high level of \textit{Ghox-lab} mRNA detected in lane 1 of the Northern blot of Fig. 3 A suggests that \textit{Ghox-lab} is expressed before stage 15. To examine this possibility, definitive streak stage (stage 4) and head fold stage (stage 6) embryos were studied by \textit{in situ} hybridization using \textsuperscript{35}S-labeled riboprobes. Fig. 4 A shows a Hoechst-stained transverse section of a stage 6 embryo. The section passes through the primitive streak slightly posterior to Hensen's node (see scheme in Fig. 6). Fig. 4 B, a darkfield image of silver grains, shows that antisense probe gives high levels of signal after an exposure of 4.5 days. The signal is strongest in the primitive streak but high levels of signal are also seen in the flanking epiblast layer and below in the newly formed mesoderm. More lateral regions of the embryo, corresponding to the \textit{area pellucida}, have considerably less signal. As can be seen in Fig. 4 C, sense riboprobe, hybridized to a neighboring section under the same conditions as used for the antisense experiments, gives essentially no signal. In addition, if \textsuperscript{35}S-antisense probe is hybridized in the presence of a 500-fold excess of non-radioactive antisense riboprobe, the signal is effectively eliminated (data not shown).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{Northern blots of mRNA from chick and mouse embryos showing the abundance of \textit{Ghox-lab} and \textit{Hox 1.6} mRNA. (A) Developmental time course, chick. Poly(A)\textsuperscript{+} RNA was isolated from whole chick embryos at various stages of development and Northern blotting carried out as described in Methods. The poly(A)\textsuperscript{+} RNA selected from 100 \mu g of total RNA was loaded in each lane. Exposures were for 24 h, with one screen. The top of the print marks the gel origin. The lower row in each of A and B represents a reprobing of the filter with chick $\beta$-actin. (B) Spatial distribution, chick. Prior to isolation of RNA, stage 15 chick embryos were dissected into the following sections. (1) Head, anterior to midbrain/hindbrain border. (2) Head/neck, from midbrain/hindbrain border to level of the first somite. (3) Body from the second to the approx. 14th somite. (4) Body from the approx. 15th somite to the posterior end of the embryo. Northern analysis was performed as in A, and the lanes represent RNA from regions 1 to 4. Exposure was for 16 h. (C) Developmental time course, mouse. 11 \mu g of total RNA was gel fractionated, transferred to nitrocellulose, and hybridized with \textsuperscript{32}P-labelled probe ($3\times10^6$ cts min$^{-1}$ ml$^{-1}$). The autoradiograph shown was exposed for 21 h with two Du Pont intensifying screens. The lanes are as follows: de, day 7.5 decidua; 7.5, day 7.5 embryo; 8.5, day 8.5 embryo; 9.5, day 9.5 embryo; 10.5, day 10.5 embryo; 11.5, day 11.5 embryo; 12.5, day 12.5 embryo. The arrow marks the gel origin. An autoradiograph of the blot reprobed with actin is shown in the bottom panel.}
\end{figure}
Ghox-lab expression divides the head fold stage chick embryo into two domains

To delineate the anterior and posterior boundaries of Ghox-lab expression, sagittal sections of stage 6 were analyzed. The striking result is that both the presumptive brain neuroectoderm and the head fold mesoderm are devoid of Ghox-lab RNA, while transcripts are very abundant in ectoderm, mesoderm and endoderm of the posterior portions of the embryo (Fig. 5 A to D). Note, that the domain of RNA expression extends significantly anterior to Hensen’s node (Fig. 5 A). More importantly, the boundary of expression in the ectodermal layer does not coincide with the posterior border of the medullary plate (see arrows in Fig. 5 B and C and Fig. 6), but falls within it. It follows, therefore, that the anterior limit of expression does not correspond to a perceivable transition in cell type, but instead defines a anterior limit of expression does not correspond to a perceivable transition in cell type, but instead defines a

anterior border similar to that seen in the neuroectoderm of the medullary plate. At first glance, mesodermal cell layers appear to respect essentially the same anterior boundary of expression as ectoderm. However, inspection of a serially reconstructed embryo (data not shown) indicates that, in regions lateral to the midline, Ghox-lab expression does not extend as far anteriorly in mesoderm as it does in the overlying ectoderm. An example of this ‘staggering’ is shown in Fig. 5 C.

The series of sagittal in situ specimens of a stage 6 embryo shown in Fig. 5 B to D reveal that the posterior boundary of Ghox-lab expression shifts forward in successively more lateral regions of the embryo. The posterior boundary of expression corresponds not to the border between area pellucida and area opaca, but is located within the area pellucida. That the posterior and lateral boundaries do not coincide with any well-defined morphological feature is reinforced by serial reconstruction analysis of a frontally sectioned embryo (data not shown). Fig. 6 summarizes the expression pattern seen in stage 6 embryos. The chief feature to emphasize is that Ghox-lab defines a spatial boundary located roughly at the base of the presumptive head, a division apparently retained throughout subsequent development, as will become obvious from data discussed below.

Expression of Ghox-lab in the stage 4 chick embryo

Is Ghox-lab expressed at even earlier stages of development? In order to answer this question, we have carried out in situ hybridization of stage 4 embryos. These embryos are the avian equivalent of the amphibian gastrula and possess a fully extended primitive streak, but have not yet developed a notochord, head fold or neural plate (see Synopsis in Methods). Fig. 5 F shows an in situ hybridization of a stage 4 transverse section which is just posterior to Hensen’s node. After a 4.5 day exposure considerable signal is observed in the primitive streak, the flanking epiblast (upper layer of cells) and the newly formed mesoderm (lower layer). The lateral regions of the embryo consist of area pellucida tissue and show considerably lower levels of signal. It should also be noted that there are significant differences of expression along the anteroposterior axis at this early stage. Examination of another transverse section from the same embryo, just anterior to Hensen’s node (Fig. 5 E), shows very little signal. This section is located in the region corresponding to the presumptive midbrain/hindbrain barrier determined by Spratt (1952) in his fate map of the chick neural plate.

Expression of Hox 1.6 in early mouse embryos

Sequence homology (Fig. 2) and a similar temporal pattern of expression (Fig. 3) indicate that Ghox-lab and Hox 1.6 are closely related genes. This notion is further supported by the fact that their spatial expression patterns are alike. Serial transverse sections of day 7.5 mouse embryos (corresponding to chick stage 5) were hybridized with sense or antisense riboprobes, derived either from the 3’ untranslated region (1136-2216) or the protein coding region (544-736) of Hox 1.6. Grains were detected over the primitive streak following hybridization with antisense probe from the

Fig. 4. In situ hybridization of stage 6 chick embryos sectioned transversely. (A) Hoechst 33258 stained nuclei of a transverse section (see Fig. 6 for location). (B) Darkfield image of the same section, hybridized to an antisense riboprobe of Ghox-lab (nts 280 to 573). (C) Darkfield image of a neighboring section, hybridized and exposed as in B, but with the sense strand of the same probe. Slides were exposed for 4.5 days. Abbreviations: ap, area pellucida, ep, epiblast (ectoderm); pm, primitive mesoderm; ps, primitive streak. The scale bar in A represents 200 µm, and A, B and C are the same magnification.
3' untranslated region (Fig. 7 A), while an adjacent section hybridized to sense riboprobe showed little signal (Fig. 7 B). A similar hybridization pattern was observed with antisense probe from the protein coding region (data not shown). Pretreatment of sections with RNase prior to hybridization abolished all signal (data not shown). The anterior and posterior limits to expression may be seen in Fig. 5 I. The most posterior signal was detected about 5 cell diameters past the amnion in the extraembryonic mesoderm. Another section suggested that the allantois does not synthesize Hox 1.6 mRNA (data not shown). Grains were detected over the primitive streak almost to the tip of the egg cylinder, but since they were never observed at the tip itself, it is presumed that the head process does not synthesize this message. That the spatial boundary of expression seen in Fig. 5 I is maintained as development proceeds becomes clear upon inspection of a day 8 mouse embryo (equivalent to stage 7 in chick). The section depicted in Fig. 7 C demonstrates that silver grains are again observed over the primitive streak and over the somites up to a point near the posterior limit of the foregut pocket.

Later chick embryos express Ghox-lab primarily in neural tube and gut tissues

It is important to determine whether the antero-posterior boundary of Ghox-lab mRNA expression found in early chick embryos persists in more developed embryos. We have studied this question using Northern blots (Fig. 3 B) and in situ hybridization (Fig. 5 G, H). Stage 15 chick embryos are quite well developed, that is, they have a recognizable head with a completely formed optic cup. The trunk has approximately 25 pairs of somites, the limb buds are not yet formed, and the most posterior quarter of the body is presomitic mesoderm.

Chick embryos were dissected along the antero-posterior axis into four segments. RNA was isolated from each segment and then subjected to Northern blot analysis (for details see legend of Fig. 3 B). Poly(A)* RNA from the first segment, comprising tissue anterior to the hindbrain, showed no signal, while the remaining tissue segments exhibited approximately equal levels of expression (Fig. 3 B). These results are in agreement with in situ hybridization analyses. Transverse sections passing through telencephalon, mesencephalon and metencephalon are devoid of signal when probed with Ghox-lab antisense riboprobe (data not shown). We conclude that Ghox-lab is not expressed anterior to the myelencephalon.

Stage 15 embryos that were transversely sectioned either towards the anterior end of the spinal cord (Fig. 5 G) or in the lumbar region (Fig. 5 H), expressed the highest levels of Ghox-lab RNA in the spinal cord and in tissues of the gut. In Fig. 5 G silver grains are distributed over both the endodermal layer of the gut as well as over the surrounding splanchnic mesoderm which separates endoderm and coelom. In the more posterior section (Fig. 5 H), where the gut has not yet fused and the endodermal layer is still thin, the most intense signal overlaps the splanchnic mesoderm. While in early embryos Ghox-lab expression is apparently uniform throughout the entire posterior mesoderm (e.g. Fig. 5 B and C), in the expression in older embryos is quite different. Transcript levels are very low in anterior mesenchyme (Fig. 5 G). Grain density gradually increases in more posterior mesenchyme (Fig. 5 H) and reaches a considerable level in the most posterior region that contains the developmentally most primitive mesoderm, i.e. unsegmented mesenchyme. Hence the spatial distribution of Ghox-lab mRNA follows the anterior-to-posterior gradient of development characteristic of all vertebrates. A final point we wish to make is that at stage 15 the notochord has very little signal (Fig. 5 G and H).

The in situ hybridization results also allow us to make rough quantitative comparisons of expression. As described above, Northern blots show a striking general decline in Ghox-lab mRNA expression from stage 15 to stage 25. Examination of in situ hybridization data indicates that this decline must begin well before stage 15. In Fig. 5 G the maximum signal seen in a 16-day emulsion exposure of the stage 15 embryo has a lower grain density than that seen in a 4.5 day exposure of an identically hybridized stage 6 neural plate embryo (Fig. 5 B). We conservatively estimate that from stage 6 to stage 15 there is at least a 5-fold decline in the maximal level of expression, and an even greater
Homeobox gene expression in early embryos

Fig. 6. Summary diagram of Ghox-lab expression in the stage 6 chick embryo. The drawing shows a dorsal aspect of a Hamburger–Hamilton stage 6 embryo. The region of Ghox-lab expression is indicated by shading and was determined by a serial reconstruction of sagittal sections from the embryo shown in Fig. 5 A–D and, in addition, by serial reconstructions of frontal sections from another stage 6 embryo. A pair of arrows indicates the plane of each section shown in Figs 4 and 5 A to D. Features indicated by lettering are: ao, area opaca (extraembryonic); ap, area pellucida; hf, head fold; hn, Hensen’s node; mp, medullary plate (a raised surface); ps, primitive streak.

decline in the overall level of expression. Stage 4 chicks (Fig. 5 F) have a level of hybridization that is at least as high as that of the stage 6 embryos, suggesting that expression may actually begin even prior to that stage.

A comparison of Ghox-lab expression in stage 15 chick embryos and Hox 1.6 expression in day 9 mouse embryos (see Duboule and Dollé, 1989) reveals that they are similar. For example, cells of the spinal cord of chick and mouse express the gene, and the signal extends well into the hindbrain as far as the otic vesicle. Both genes are expressed in the gut. This similarity in the expression pattern later in development lends additional support to the view that their functions are closely related.

Discussion

Sequence of Ghox-lab and its kinship to Drosophila labial and mouse Hox 1.6

There is increasing evidence from the study of mouse, Xenopus and human homeobox-containing genes that points to the existence of an ancestral ANT-C and BX-C related cluster common to both insects and vertebrates (see Introduction). Early in the evolution of vertebrates this ancestral gene cluster underwent duplication in order to give rise to four clusters. The result of this process is that vertebrate genomes could contain as many as four copies of each ancestral gene. Thus the Drosophila labial homeobox gene, which is located at the extreme 3' end of the ANT-C, could have up to four cognates in vertebrates. Each of these copies presumably has diverged somewhat in sequence and function, but has conserved the distinctively labial character of the homeobox.

LaRosa and Gudas (1988) have pointed out that the homeobox of mouse Hox 1.6 encodes a protein domain which is 85% identical to the homeo domain of Drosophila labial. This sequence homology is consistent with the fact that Hox 1.6 is the most 3' gene known to date in the Hox 1 cluster (Baron et al. 1987). Here we describe the isolation of a chicken gene which we name 'Ghox-lab'. We found that there is indeed considerable similarity between Ghox-lab and the Hox 1.6 gene used for its isolation. The amino terminus, the homeo domain and the 25 amino acids preceding it are almost identical. For example, cells of the spinal cord of chick and mouse express the gene, and the signal extends well into the hindbrain as far as the otic vesicle.
Fig. 7. (C) In situ hybridization of Hox 1.6 probes to mouse embryo sections. Antisense RNA probes (A and C) or sense probes (B) were hybridized to cryo sections of gestational day 7.5 (A and B) and day 8 (C) mouse embryos. Panel A and B show sections perpendicular to the primitive streak. Panel D shows a camera lucida drawing of the sagittal section of the 1–2 somite embryo in panel C. Abbreviations used are: a, amnion; f, foregut; h, heart anlage; hf, head fold; ps, primitive streak; s, somite region. The sections were exposed for 4 days. A, B and C are darkfield micrographs. Scale bar: 200 μm.

negligible amounts by the limb bud stage. Most other murine (reviewed e.g. Holland and Hogan, 1988; Wright et al. 1989) and chick (Ghox 2.1 and 2.2, Wedden et al. 1989; Ghox 2.3, Pang and Eichele, unpublished) homeobox genes continue to be expressed at high levels well beyond the limb bud stage.

The only other known Ant-like vertebrate homeobox-containing gene for which there is evidence of significant expression in the pre-somatic neural plate stage is Hox 1.5 (Gaunt, 1987; reviewed in Holland and Hogan, 1988). Hox 1.5 could not be detected in the ‘early’ day 7.5 mouse embryo, but was found to begin expression by ‘late’ day 7.5. The signal appears to increase further by day 8 (Gaunt, 1988). We have found that Hox 1.6 is already expressed at high levels in day 7.5 mouse embryos (Figs. 5 I, 7 A), and it may be expressed at even earlier stages, which have yet to be examined.

In the chick, we find Ghox-lab mRNA expressed at high levels at Hamburger-Hamilton stage 6 (‘early neurula’), and even earlier, at stage 4 (‘mid gastrula’). Stage 4, the definitive streak stage, corresponds to day 7.25 in the mouse, and by this chronology Ghox-lab expression begins at a more primitive phase of development than Hox 1.5. Another special feature of the Ghox-lab and Hox 1.6 genes is that they are turned off relatively early in development. In contrast, Hox 1.5 and other Hox cluster genes continue to be expressed in embryos with well developed limbs, clearly showing that they act later within a temporal hierarchy. The fact
that Hox 1.6 occupies an extreme 3' position in the Hox-1 cluster (Duboule and Dollé, 1989), and that labial homologs Ghox-lab and Hox 1.6 exhibit early embryonic expression, lends support to the view that there is a 3' to 5' sequence of gene activation within Hox clusters. We wish to further propose that the transient nature of expression seen in these vertebrate labial homologs sets them apart from other homeobox genes, and that their primary function is confined to early development. Perhaps the greater degree of sequence conservation observed in other Hox cluster genes reflects the fact that they play additional roles later in development, and therefore have additional structural constraints.

Spatial expression pattern of Ghox-lab and Hox 1.6

The study of Duboule and Dollé (1989) described the spatial distribution of Hox 1.6 in mouse embryos from day 8.25 to day 9.5, but data was not available for earlier stages. As discussed in the previous section, our report demonstrates that Hox 1.6 is expressed even earlier. Fig. 5 I clearly shows that Hox 1.6 is expressed in a 7.5 day primitive streak stage embryo and that the transcripts are found in the entire primitive streak. The anterior border of expression extends approximately to the posterior border of the head process. 8 day embryos with one or two somites have signal extending from the most posterior extreme of the body, reaching anteriorly as far as the foregut pocket (Fig. 7 C and D). This pattern of expression is reminiscent of that described by Duboule and Dollé (1989) for 'day 8' (8-10 somite) embryos.

Expression of Ghox-lab divides the definitive streak (stage 4) and head fold (stage 6) chick embryo into two distinct regions. Serial reconstructions of stage 6 embryos clearly demonstrate that cells in the posterior third of the embryo do not express Ghox-lab, whereas cells in the posterior region are Ghox-lab positive. In stage 4 embryos, the anterior boundary of Ghox-lab expression is located in Hensen's node. Studies are in progress to determine the precise relationship between this boundary and the fate map, but at present it seems that the territory of Ghox-lab expression at stages 4 and 6 reaches into presumptive hindbrain tissue. Specifically, the stage 4 section shown in Fig. 5 E, which is negative for Ghox-lab expression, passes through a zone that the fate map (Spratt, 1952) predicts will become the boundary region between mid- and hindbrain. As described in Results, midbrain and forebrain, once developed, lack Ghox-lab transcripts. The section shown in Fig. 5 F is situated just posterior to Hensen's node and shows high levels of expression. In Spratt's fate map the presumptive neural plate in this territory is fated to become spinal cord and, as shown in Fig. 5 G and H, spinal cord does indeed express Ghox-lab. These two examples indicate that (1) the descendants of cells that are Ghox-lab positive in the early embryo express the gene well into the stage of organogenesis; (2) the descendants of Ghox-lab negative cells remain negative throughout development. How is Ghox-lab transcription maintained in the positive cells? One possible mechanism is autoregulation, a feature exhibited by a number of transcriptional regulators, including the Drosophila Deformed protein (Kuziora and McGinnis, 1988).

An important and open question is how Ghox-lab and Hox 1.6 are activated. An extensive body of work in the amphibian embryo shows that inductive signals are among the earliest cues in development (Smith, 1989). For example, Rosa (1989) found that a mesoderm-inducing factor activates the transcription of Mix.1 which contains a divergent homeobox motif. Perhaps Ghox-lab and Hox 1.6 are also induced by a diffusible factor secreted in the early embryo. Among possible candidates for such a factor is retinoic acid. Reasons for suggesting this are: (1) retinoic acid has been shown to induce the expression of Hox 1.6 in teratocarcinoma cells (LaRosa and Gudas, 1988), and (2) recent studies by Durston et al. (1989) have revealed that retinoic acid causes a transformation of the antero-posterior axis of Xenopus. The Hox clusters in general and, as this study shows, the vertebrate homologs of labial in particular, appear to be involved in the regional specification of the anteroposterior pattern. Perhaps retinoic acid acts by regulating the expression of Hox cluster genes.

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