Development 121, 333-346 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

Hox genes and the evolution of vertebrate axial morphology

Ann C. Burke, Craig E. Nelson, Bruce A. Morgan* and Cliff Tabin

Department of Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115, USA

*Present address: Cutaneous Biology Research Center, Massachusettes General Hospital-East, 13th Street Building 149, Charlestown, MA 02129, USA

SUMMARY

A common form of evolutionary variation between vertebrate taxa is the different numbers of segments that contribute to various regions of the anterior-posterior axis; cervical vertebrae, thoracic vertebrae, etc. The term 'transposition' is used to describe this phenomenon. Genetic experiments with homeotic genes in mice have demonstrated that *Hox* genes are in part responsible for the specification of segmental identity along the anterior-posterior axis, and it has been proposed that an axial *Hox* code determines the morphology of individual vertebrae (Kessel, M. and Gruss, P. (1990) *Science* 249, 347-379). This paper presents a comparative study of the developmental patterns of homeobox gene expression and developmental morphology between animals that have homologous regulatory genes but different morphologies. The axial expression boundaries of 23 *Hox* genes were examined in the paraxial mesoderm of chick, and 16 in mouse embryos by in situ hybridization and immunolocalization techniques. *Hox* gene anterior expression boundaries were found to be transposed in concert with morphological boundaries. This data contributes a mechanistic level to the assumed homology of these regions in vertebrates. The recognition of mechanistic homology supports the historical homology of basic patterning mechanisms between all organisms that share these genes.

Key words: *Hox* genes, anterior-posterior axis, comparative developmental studies, segment identity, paraxial mesoderm, chick, mouse

INTRODUCTION

Differences along the anterior-posterior (A-P) axis in many organisms are set up by the spatial and temporal expression of a common set of genes early in development, despite extreme variation in adult morphology. The *Hox* genes are a family of regulatory genes expressed along the A-P axis in most metazoans. Because homeobox genes encode transcription regulators active in pattern formation during ontogeny, they are obvious factors to study in the investigation of the mechanistic basis for ontogeny, as well as in the relationship between ontogeny and phylogeny. This paper presents a comparative study of the developmental patterns of homeobox gene expression and morphogenesis along the A-P axis between related animals that have homologous regulatory genes but different axial morphologies.

The participation of different numbers of segments in any given region of the vertebrate body and the different positions of the appendages relative to the A-P axis, have provoked comment and theory from morphologists for centuries. While common generative rules govern mesodermal segments within individual organisms (serial homology), and common ancestry accounts for their presence in all chordates (historical homology), details of this segmental organization differ dramatically between related organisms resulting in a variety of axial formulae, defined here as the number of vertebrae of each morphological type, e.g. cervical, thoracic, lumbar, etc. In 1906, E. S. Goodrich proposed the term 'transposition' to describe evolutionary changes in the number of segments included in any vertebral region. Goodrich surmised that serially and historically homologous regions behave as pliable elements that can slide up or down the A-P axis during evolution. Lankester (1910) expanded the description with a useful musical analogy, comparing an axial structure or region to a tune that can be transposed up or down the scale.

The pattern of variation and constraint in axial formulae among different classes of vertebrates demonstrates that transposition has been an important evolutionary phenomenon in the establishment and radiation of different vertebrate groups (Gadow, 1933; Carroll, 1988). Mammals are a vertebrate class with extreme morphological variation. With very few exceptions however, they are constrained by the fixed number of seven cervical vertebrae whether they be whales or giraffes. Birds are not constrained in this character, and vary from 13 (pigeons and swifts), to 25 (swans) cervical vertebrae. Extremes among the vertebrates include the Cretaceous plesiosaur, *Elasmosaurus* sp. which had as many as 76 cervical vertebrae; snakes, with as many as 350 individual vertebrae of equivocal types; and the modern anurans (frogs) with never more than 9 and as few as 6 total presacral vertebrae. Even when the total number of pre-caudal vertebrae is almost the same, as is the case between chickens and mice, the relative length of specific regions, such as cervical versus thoracic, can vary considerably.

Members of the *Hox* family of homeobox genes are expressed along the A-P axis at specific levels in the central

nervous system and the paraxial mesoderm in the vertebrate embryo. They show sharp anterior boundaries proceeding successively from anterior to posterior in an order 'colinear' with their relative chromosomal position (Duboule and Dollé, 1989; Graham et al. 1989). It is widely assumed that the conserved colinearity of *Hox* genes reflects their primary role in some aspects of embryonic patterning. Mis-expression and inactivation of *Hox* genes in mice and of their homologues in *Drosophila* show that *Hox* genes are among the factors responsible for specific regional identity along the A-P axis. In mice, perturbation of the expression pattern of *Hox* genes by application of retinoic acid can result in the homeotic transformation of vertebrae from one type to another (e.g. cervical to thoracic: Kessel and Gruss, 1990, 1991; Kessel, 1992). Kessel and Gruss proposed that a '*Hox* code' or combination of *Hox* genes expressed in each somite specifies the unique morphology of different vertebrae.

The link between gene expression and vertebral morphology suggests that *Hox* genes may have played an important role in the evolution of specific axial variation. The axial *Hox* code proposed by Kessel and Gruss (1990) could function in a number of ways. In principle, the *Hox* genes could simply mark an A-P co-ordinate system with expression boundaries that do not vary with respect to some fixed point along the somitic ladder in different species and thus would be numerically invariant between species at each axial level. Variation in vertebral morphology would be achieved by alteration in the response of downstream genes to a given *Hox* code. In all species, *Hox* gene expression domains would map consistently to segment number within a body plan regardless of transpositional morphology. In this case the primary events in the evolution of axial formulae would be downstream of the *Hox* genes.

Another alternative is that the *Hox* gene expression domains are transposed along the axis in register with morphology. In this model, a specific combination of *Hox* genes expressed in the same segment would determine the morphological identity of the resultant vertebra. The primary event in evolution of axial formulae would be an alteration in the expression of (upstream) regulators of the *Hox* cluster, or the response of cluster members to those regulators.

The data from mis-expression studies and homozygous deletions of *Hox* genes in mice do not distinguish between the models outlined above. Experimental changes in the *Hox* code could result in homeotic phenotypes whether the code acts directly or indirectly on morphology. Moreover, data garnered from a single organism do not carry evolutionary information needed to resolve this issue. Comparative studies expose phylogenetic variability and provide insight into how genes affect morphology since the molecular genetic mechanisms constrain the ways that evolutionary differences in morphology are achieved. We therefore compared the axial *Hox* code in embryonic chickens and mice, animals with different axial formulae, to determine whether the *Hox* code is transposed in parallel with vertebral anatomy or maintains a constant segmental registration.

MATERIALS AND METHODS

Axial formulae

For the purposes of this study, an axial formula is defined as the combined numbers of vertebrae that contribute to each distinct region of the vertebral column. In tetrapods these regions include the cervical, thoracic, lumbar, sacral, and coccygeal or caudal vertebrae (Fig. 1).

The axial formula of the mouse is typical of most rodents. There are 7 cervical vertebrae, 13 thoracic, 6 lumbar, 4 sacral, and a variable number of caudals (20+).

The chick has 14 cervical, 7 thoracic, 12-13 lumbosacral, and 5 coccygeal vertebrae. The goose has 17 cervical, 9 thoracic, 13-14 lumbosacral and 5 coccygeal vertebrae. The axial formula in birds is complicated by the fusion of multiple vertebrae (including the 7th thoracic in the chicken) into the 'synsacrum', a character unique to birds. The articulation of the pelvis with the vertebral column is extensive in birds, and involves many more vertebrae than the relatively simple sacrum in other tetrapods. The vertebral fusions within the synsacrum have resulted in some disagreement on the identity of individual vertebra (Gadow, 1933). The identity of the individual vertebrae comprising the synsacrum in Fig. 1 have been assigned according to Nickel et al. (1977). These numbers agree with our observations in the chick, and includes 4 lumbar, and 9 sacral vertebrae (those united by parapophyses and diapophyses to the ilium. They are referred to jointly as lumbosacral vertebrae.

Criteria for assignment of axial level in whole-mount embryos

Chicken, goose and mouse embryos, like other amniotes, have five occipital somites. These are incorporated into the occipital region of the skull (de Beer, 1937). Fate mapping experiments on chick embryos suggest that each trunk somite contributes to two adjacent vertebrae, in keeping with the theory of *Neugliederung*, or resegmentation (Remak, 1855). Cells from the caudal portion of one somite, and cells from the anterior part of the next posterior somite together form a complete vertebra (Bagnall et al., 1988; Couly et al., 1993). There is currently some debate on the reality of vertebral resegmentation (see Stern, 1987), as well as on the fate of the anterior somites (C. Ordahl and B. Christ, personal communication). In this study we have defined an axial fate map as shown in Fig. 1A, and described as follows: The anterior portion of the 5th sclerotome is incorporated into the occiput, while the posterior half-sclerotome of that somite contributes to the body of the atlas, the first cervical vertebra. The sixth somite contributes to the atlas and the axis (2nd cervical vertebra), the 7th somite to the axis and the 3rd cervical vertebra, and so on. Thus, for both the chick and the mouse, the first trunk vertebra (pv1, the atlas) forms from somite 5-6, the 10th vertebra from somite 15-16, etc. The axial, somitic level of the anterior limit of expression are therefore always designated by two numbers.

The anterior limits of expression of *Hox* genes are reported here relative to somite number. At the stages used in this study (H and H stage 19-26 in the chick; Hamburger and Hamilton, 1951, and embryonic day 9-13.5 (E9-13.5) in the mouse), precise counting of somites is extremely difficult. The anterior somites begin to disperse quite early, while somites are still forming posteriorly. Since anterior somites can not always be accurately counted, the limbs were used as landmarks for determining somite levels (see below).We consider the somite levels reported here to be accurate, plus-or-minus one somite.

The extent and position of the limb buds relative to the axis changes during development. Both the fore and especially the hind limb buds become concentrated posteriorly as development proceeds. In the chick, the forelimb bud first appears (H and H st. 15-16) at the level of somites 15-20. By stage 24, it extends from somite 18-19 through somite 21-22. These somites map to the last cervical, and first three thoracic vertebrae. The chick hindlimb first appears at the level of somite 25-26 to somite 31-32, and by stage 24 is at the level of somite 29-30 through somite 34-35, or the last lumbar and first 5 sacral vertebrae.

The mouse forelimb bud appears at E9 extending from somite level

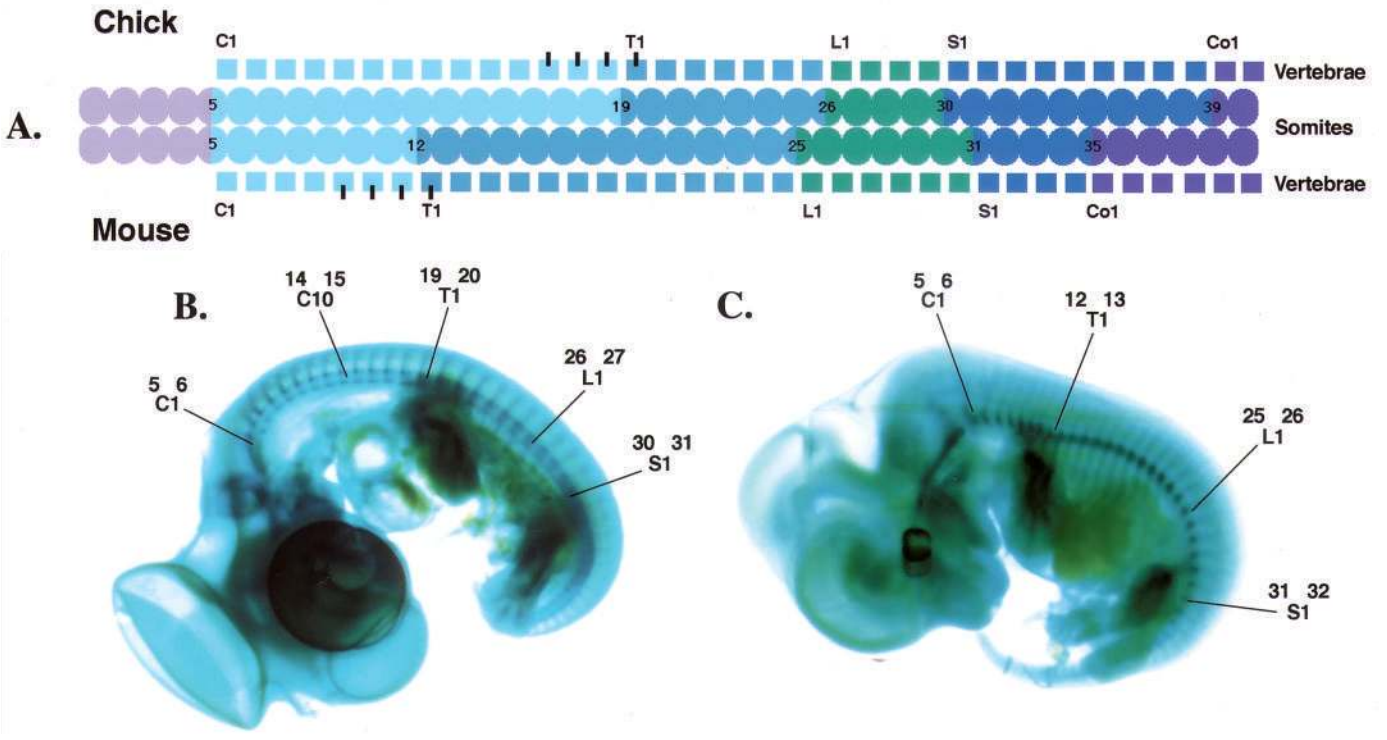

Fig. 1. (A) Schematic representation of the axial formulae of the chick and the mouse. The somites are shown as ovals, the vertebrae by squares. The spinal nerves contributing to the brachial plexus are represented by black bars. (B) A stage 25-26 chick embryo stained with alcian green and cleared in methyl salycilate. Vertebral numbers are indicated by letter and number, flanked by the numbers of the associated somites ($^{5}C1^{6}$ = first cervical, fifth and sixth somites). (C) A day 13 (E13) mouse embryo stained with alcian green and cleared in methyl salycilate. Vertebral and somite numbers are designated as in B.

8-9 to somite 13-14. The hindlimb, appearing at E10, extends from somite level 23-24 to 28-29. At E13, the forelimb is still located at the level of somites 8-9 to 15-16, while the hindlimb has shifted considerably and lies from the level of somite 29-30 to somite 34-35, centered on the first sacral vertebra. At chick stage 26 and mouse E13, the levels of the limbs relative to somites can be confirmed by counting the vertebral bodies in cleared and stained embryos (Fig. 1B,C).

Whole-mount in situ hybridization

Embryos of chick, goose and mouse embryos were fixed overnight in 4% paraformaldehyde, rinsed in PBS, dehydrated in a methanol series and stored in 100% methanol at −20°C. In situ procedures followed that of Riddle et al. (1993).

Preparation of riboprobes

DNA templates were made by linearizing pBluescript plasmids (Stratagene) with *Hox* inserts ranging from 300 bp to 2 kb (Table 1). RNA probes were transcribed with T3 or T7 RNA polymerase (Boehringer) from DNA templates with digoxigenin UTP (Boehringer), precipitated with 4 M LiCl, resuspended in TE, pH 8, and stored at −20°C.

Whole-mount immunohistochemistry

The methods used for immunohistochemistry were provided by C. Wright (personal communication). Embryos of albino *Xenopus* at stages 27-30 were removed from their egg capsules and membranes, fixed in Dent's fix (20% DMSO in methanol) overnight, bleached with 10% H_2O_2 for 24 hours and then stored in 100% methanol at −20°C. The embryos where hydrated into TBST, (10 mM Tris, pH 6.8, 150 mM NaCl, 0.05% Tween-20), and then blocked for 1 hour in TBST with 20% fetal calf serum. Immunolabeling was achieved with the Xlhbox-1 antibody (provided by E. de Robertis) at a concentration of 1:50 in blocking solution at room temperature overnight. Embryos were washed in five 1-hour bathes of TBST followed by incubation with goat anti-rabbit IgG conjugated to HRP (Jackson Labs) at a concentration of 1:1000, overnight at 4°C. The antibody detection reaction was done with 0.05 mg/ml diaminobenzidine, and 0.02% H_2O_2 in TBST at pH 5.5. The reaction was stopped with 100% methanol, embryos were cleared in 2 parts benzyl benzoate: one part benzyl alcohol.

RESULTS

We have examined the relationship between the expression domain of *Hox* genes and morphological boundaries along the main body axis of the mouse and the chick embryo with whole mount in situ hybridization. We report externally visible boundaries in the paraxial mesoderm observed at stages where anatomy can be unambiguously compared between different vertebrates. Axial boundaries are assigned based on somite counts and in relation to external landmarks (see Materials and Methods). After determining the anterior border of expression for 23 *Hox* genes in the chick, we selected 16 *Hox* genes that we expected to be particularly informative for comparison in the mouse. The expression patterns of many *Hox* genes have been published for the mouse (reviewed by Kessel and Gruss, 1990, 1991). However, in order to ensure a consistent manner of comparison with the chick and to resolve ambiguities and conflicts in the published levels (M. Kessel, personal communication), we constructed our own axial *Hox* map for the mouse with 16 genes. Identical in situ procedures were used for chick

Table 1. Chick and mouse genes used in this study

A. Chick

Gene name	Size (kb)	Restriction sites	Description
a-4	0.9	*Pst-Xho*	5′+B+3′
a-9	0.9	linker-*Sma*	5′
a-10	0.5	*Hin*f1-*Hin*f1	5′+B
a-11	1.4	linker-*Nco*1	5′+B+3′
a-13	0.7	*Sac*1-*Bam*H1	5′+B
b-4	1.3	*Eco*R1-*Eco*R1	5′+B+3′
b-8	0.7	*Eco*R1-linker	B+3′
b-9	0.4	linker-linker	5′+B+3′
c-4	0.6	linker-*Kpn*1	5′+B
c-5	0.6	*Sac*1-*Sac*1	B+3′
c-6	1.4	linker-*Not*1	5′+B
c-8	1.2	linker-*Bgl*2	5′+B
c-9	0.45	linker-*Sma*	5′
c-10	1.1	linker-*Sac*1	5′+B
c-11	0.4	linker-*Hinc*2	5′
d-4	0.5	PCR	5′+B
d-8	0.7	Bg12-linker	B+3′
d-9	0.4	linker-*Sma*1	5′
d-10	1.7	linker-linker	5′+B+3′
d-11	0.6	linker-*Nar*1	5′
d-12	0.55	linker-*Eco*R1	5′
d-13	0.5	*Pst*1-*Xho*1	5′+B

B. Mouse

Gene name	Size (kb)	Source
a-4	0.9	R. Krumlauf
a-9	0.7	H. Haack/P. Gruss
b-4	0.3	R. Krumlauf
b-7	0.8	J. Deschamps
b-8	0.9	J. Deschamps
b-9	2.2	R. Krumlauf
c-4	1.0	P. Sharpe
c-5	0.3	P. Sharpe
c-6	0.5	K. Bentley
c-8	0.45	K. Bentley
c-9	0.6	M. Capecchi
d-8	2.0	D. Duboule
d-9	1.1	D. Duboule
d-10	0.45	D. Duboule
d-11	1.1	D. Duboule
d-12	1.1	D. Duboule

(A) Chick *Hox* genes The size and defining restriction sites of the DNA templates used to generate riboprobes are listed, as well as the position of the template relative to the homeobox. For instance, the probe for *Hoxa-4* was made from a template of 900 base pairs between *Pst* and *Xho* sites that includes the homeobox (B) and sequence both 5′ and 3′ to the box (5′+B+3′). Chick *Hox* genes were isolated from stage 24 chick limb cDNA libraries, and described in detail elsewhere (Nelson et al., unpublished data). In brief, identity was determined for the majority of the genes by comparison with published chick cDNA fragments. Identity of *Hoxa-10*, *Hoxa-13*, and *Hoxb-9* was assigned based on sequence identity with published mouse and human clones, and also, in the case of *Hoxa-10* and *Hoxa-13*, on comparison with published chick expression patterns. Identity of the genes in the chick *Hoxc* cluster was based upon comparison with published mouse and human sequence, consistency of expression patterns within the cluster, and pulse-field southern blots that are consistent with physical linkage of the genes encoding the cloned cDNAs. (B) The size of the templates for the mouse *Hox* probes used in this study and the source of the DNA.

and mouse embryos, and identical criteria were used to assign axial boundaries relative to somite number.

Table 2 gives an overview of the *Hox* genes examined for both chicken and mouse in this study. The gene expression domains in the paraxial mesoderm follow the same sequence as the physical order of the genes within each of the four *Hox*

Table 2. Summary of anterior somitic *Hox* expression boundaries

Gene	Mouse level	Chick level	Anatomical region
Hoxa-4	7-8	10-11	Cervical
Hoxb-4	6-7	7-8	Cervical
Hoxc-4	7-8	10-11	Cervical
Hoxd-4	–	7-8	Cervical
Hoxc-5	10-11	17-18	Cervical
Hoxc-6	12-13	19-20	Thoracic
Hoxb-7	15-16	–	Thoracic
Hoxb-8	Amb.	Amb.	Thoracic
Hoxc-8	17-18	23-24	Thoracic
Hoxd-8	18-19	Amb.	Thoracic
Hoxa-9	23-24	25-26	Thoracic
Hoxb-9	Amb.	25-26	Thoracic
Hoxc-9	23-24	25-26	Thoracic
Hoxd-9	29-30	29-30	Lumbar
Hoxa-10	–	29-30	Lumbosacral
Hoxc-10	–	30-31	Lumbosacral
Hoxd-10	31-32	30-31	Lumbosacral
Hoxa-11	–	34-35	Lumbosacral
Hoxc-11	–	37-38	Lumbosacral
Hoxd-11	34-35	39-40	Lumbosacral/caudal
Hoxd-12	35-36	40+	Caudal
Hoxa-13	–	40+	Caudal
Hoxd-13	–	40+	Caudal

–, not done.
Amb, Ambiguous level.
Many of the murine expression levels have been previously reported; see text for details.

clusters, a phenomenon called colinearity (Duboule and Dollé, 1989; Graham et al. 1989). The four *Hox* clusters (*Hox*a, *Hox*b, *Hox*c, and *Hox*d) are thought to represent the products of two sequential duplications of a single ancestral gene cluster during the evolution of the vertebrates (Krumlauf, 1992; Kappen and Ruddle, 1993). *Hox* genes in different clusters that are homologous to the same gene in the ancestral cluster (united by strong sequence similarity) are called paralogues. There are 13 paralogue groups in the four *Hox* clusters, though every cluster does not have a representative member of every paralogue (Fig. 2). Expression patterns will be described below in order of paralogue groups.

Paralogue groups 1, 2 and 3 were not examined in this study. These genes have expression boundaries in the hindbrain and the occipital somites. The chick and mouse have the same number of occipital somites, and the axial expression of *Hox* genes in this region are in register (R. Krumlauf, personal communication).

Paralogue groups 4 and 5

Representatives of the fourth and fifth *Hox* paralogue groups have anterior boundaries of expression within the cervical region of the chick and the mouse. The cervical region of the chick comprises fourteen vertebrae derived from somites 5-19. The mouse cervical region has only seven vertebrae, derived from somites 5-12 (Fig. 1).

The most anterior, or 3′, paralogue group examined was paralogue four, which has a gene in each cluster (Fig. 2). All of these were examined in the chick. *Hoxa-4*, *Hoxb-4*, and *Hoxc*-4 were examined in the mouse (Fig. 3). The expression boundaries of these genes have similar relationships to each other in each animal, *Hox*b-4 is the most anteriorly expressed

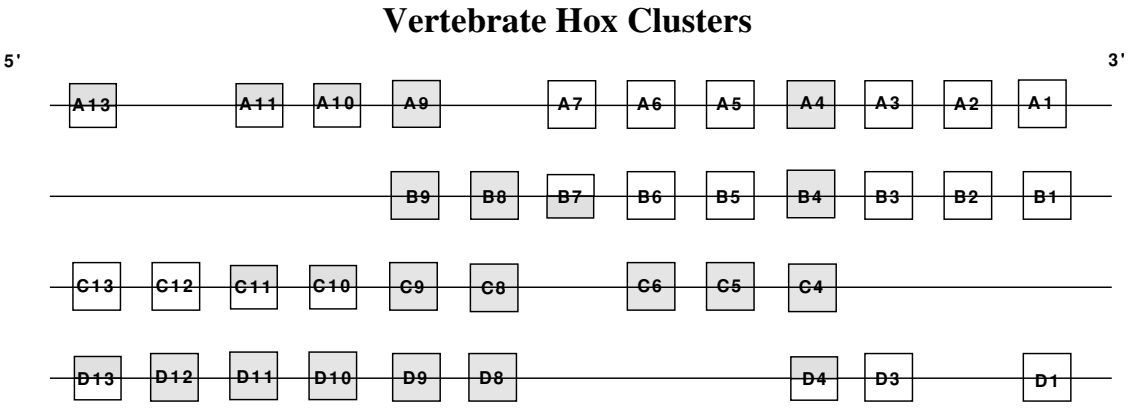

Fig. 2. Schematic representation of the four vertebrate *Hox* clusters. The individual genes are represented by boxes. Filled boxes indicate genes examined in both the chick and the mouse. Half filled boxes indicate genes examined in either the chick (top) or the mouse (bottom).

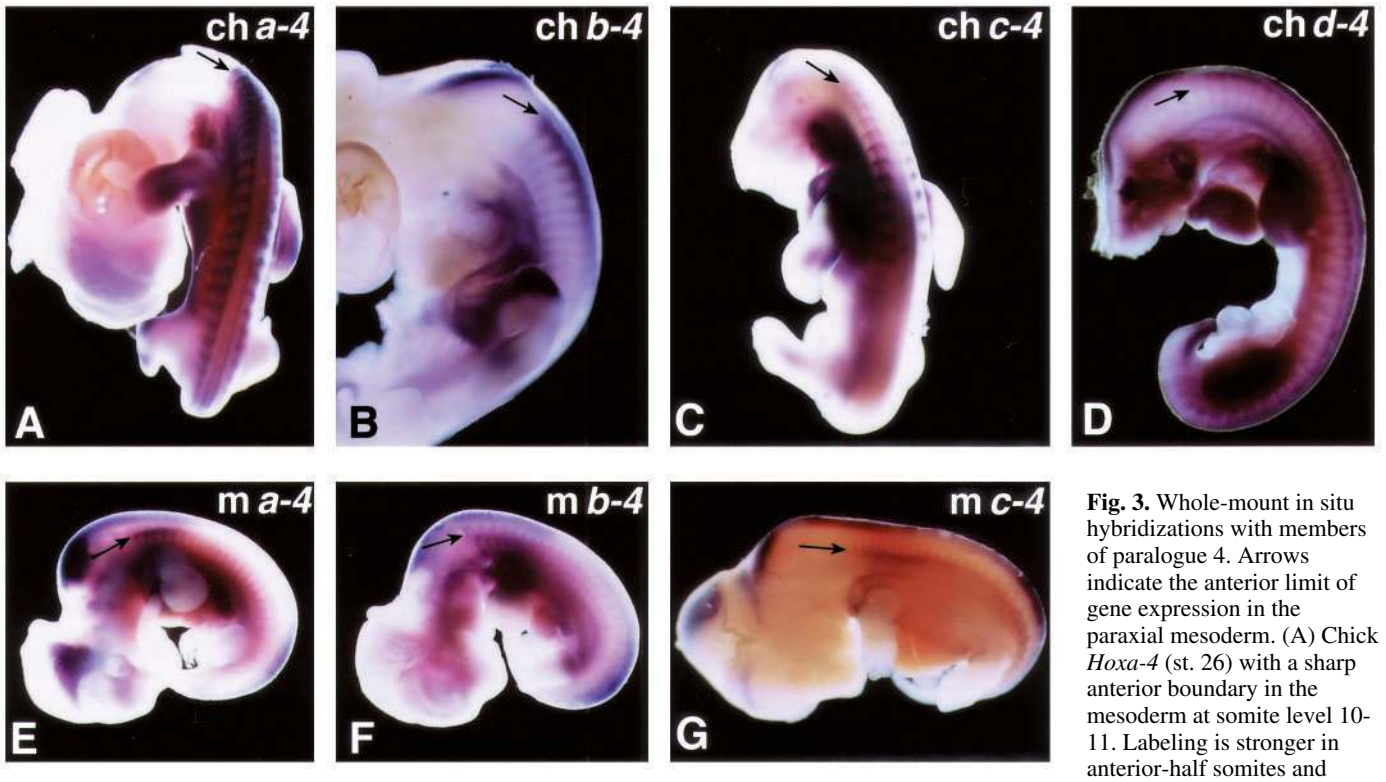

Fig. 3. Whole-mount in situ hybridizations with members of paralogue 4. Arrows indicate the anterior limit of gene expression in the paraxial mesoderm. (A) Chick *Hoxa-4* (st. 26) with a sharp anterior boundary in the mesoderm at somite level 10-11. Labeling is stronger in anterior-half somites and extends posteriorly to the base of the tail. (B) Chick *Hoxb-4* (st. 26), with a sharp anterior boundary at somite level 7-8, several segments anterior to the boundary for *Hoxa-4*, in agreement with data from stage 10 embryos that show the 7th as the first *Hoxb-4* labeled somite (R. Krumlauf, personal communication). Labeling quality is similar to *Hoxa-4*, but shows more uniform intensity dorsally across each somite, with peak expression across seven or eight segments, fading posteriorly. (C) Chick *Hoxc-4* (st. 25), is faint with a paraxial boundary at somite level 10-11. Signal covers fewer than ten segments across the brachial region, and fades below detection by mid trunk. (D) Chick (st. 22) *Hoxd-4* has high overall background, but there is clear paraxial labeling that is not present in the sense control (data not shown). The tissue distribution is similar to *Hoxb-4*, only much weaker at all stages examined. The paraxial expression extends 11 segments anterior to the forelimb bud in stage 22 embryos, which places the anterior expression boundary at somite level 7-8, aligned with the boundary of *Hoxb-4*. This is in agreement with Gaunt and Strachan (1994) who found somite 7 to be the first labeled somite at both stage 11-12 and stage 25-26 chicks. (E) Mouse (E12) *Hoxa-4* has an anterior expression boundary in the paraxial mesoderm at somite 7-8. (F) Mouse (E11.5) *Hoxb-4* is slightly anterior to *Hoxa-4* at somite level 6-7. The somitic label extends clearly for only seven segments for both genes, extending over the axial level of the forelimb, and fading out in the trunk. (G) Mouse (E13) *Hoxc-4* is expressed with a clean paraxial boundary at somite level 7-8, in agreement with data reported by Geada et al. (1992). As in the chick, labeling for *Hoxc-4* is weak, but in the mouse *Hoxc-4* RNA extends further posteriorly along the axis, continuing weakly into the tail. It is not known if these variations in labeling intensity and posterior extent of expression reflects species, stage, or probe differences.

member of group 4 (at somite level 7-8 in the chick and somite level 6-7 in the mouse). Slightly more posterior *Hoxa-4* and *Hoxc-4* share the same boundary in the chick (somite level 10-11), and in the mouse (somite level 7-8). Anatomically these levels are similar in the two species; *Hox*b-4 is associated with anterior cervical vertebrae, and *Hoxa-4* and *Hoxc-4* map to vertebrae towards the middle of the cervical series of both animals.

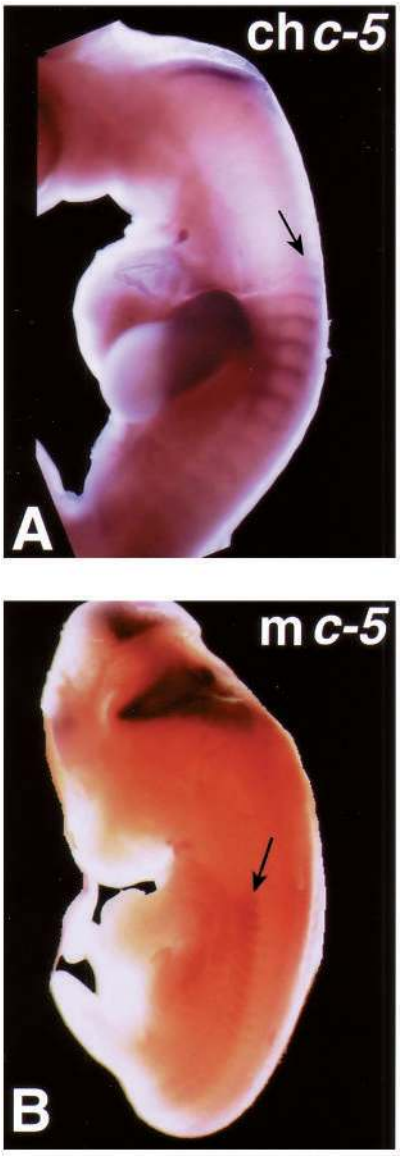

Fig. 4. Whole-mount in situ hybridizations with members of paralogue 5. Arrows indicate the anterior limit of gene expression in the paralaxial mesoderm. The overall labeling quality in both animals is very similar to the quality of *Hoxc-4*. (A) Chick (st. 25) *Hoxc-5* paraxial expression starts seven segments posterior to chick *Hoxc-4* expression, at somite level 17-18, or the thirteenth cervical vertebra. (B) Mouse (E13) *Hoxc-5* expression starts only two segments behind mouse *Hoxc-4* expression, at somite level 10-11, the sixth cervical vertebra. This axial level is consistent with observations of Gaunt et al. (1990).

Hoxc-5 is the only member of the 5th paralogue group studied here (Fig. 4). *Hoxc-5* is also expressed in the cervical region, but it is widely separated by somite number between chick and mouse (somite level 17-18: chick, versus somite level 10-11: mouse). The expression boundary marks the same anatomical level, however; the somites contributing to the second to last cervical vertebrae.

Paralogue group 6

*Hox*c-6, the only member of the sixth paralogue group examined, has an anterior boundary of expression at the level of the first thoracic vertebrae in both the chick and the mouse (Fig. 5A,B). In the chick *Hox*c-6 labeling begins at somite level 19-20, two vertebrae behind *Hox*c-5 expression, aligned with the middle of the forelimb bud. In the mouse *Hox*c-6 expression begins at somite level 12-13, two vertebrae behind *Hox*c-5 expression and in axial alignment with the middle of the forelimb bud.

Paralogue groups 7 and 8

All members of the seventh and eighth paralogue groups examined have anterior expression boundaries in the thoracic region of both chick and mouse. *Hox*b-7 expression was only examined in the mouse. The anterior-most somite with positive labeling is somite 15-16, at the position of the forelimb's posterior boundary with the body wall, mapping to the 4th thoracic vertebrae (Fig. 6B).

All three members of the eighth paralogue group, *Hoxb-8*, *Hoxc-8* and *Hoxd-8*, were examined in both the chick and the mouse (Fig. 6). *Hoxb-8* labeling is clearly stronger in the mid-trunk mesoderm than the cervical region of both animals, but no specific somite number could be assigned in either animal (data not shown). *Hoxc-8* axial expression borders in both the chick and the mouse lie in the trunk region, posterior to the forelimb. The anterior limit of *Hoxc-8* expression in the chick corresponds to somite level 23-24, or the fifth thoracic vertebra (Fig. 6A). In the mouse, the anterior-most region to show *Hoxc*-8 expression is somite level 17-18, corresponding to the sixth thoracic vertebra. *Hoxd-8* expression in the paraxial mesoderm of the chick does not resolve into a clear A-P boundary. Signal comes up gradually behind the forelimb and increases in the trunk but no discrete axial level could be assigned (data not shown). In situ hybridizations with *Hoxd-8* in the mouse however, show a positive signal in the paraxial mesoderm that comes on at somite level 18-19, or the seventh thoracic vertebra (Fig. 6D).

Paralogue 9

The complete ninth paralogue set was studied for both the chick and the mouse, and gives a similar picture in each animal (Fig. 7). *Hoxa-9*, *Hoxb-9* and *Hoxc-9* map near the end of the thoracic series in both animals. In the chick, their anterior expression boundaries lie four or five segments anterior to the hind limb and four segments posterior to the fore limb. This corresponds to somite level 25-26, or the last thoracic vertebra (T7). In the mouse, *Hoxa-9*, *Hoxc-9* have anterior expression boundaries four or five segments anterior to the hindlimb, which in the mouse is nine or ten segments behind the forelimb. This maps to somite level 23-24, representing the second to last thoracic vertebra (T12). *Hoxb-9* labeling is stronger in the posterior trunk, but does not form a discrete boundary, and no somite number was assigned.

Unexpectedly, the expression boundary of *Hoxd-9* does not conform to the axial level observed for its paralogues in either animal, but rather are shifted posteriorly towards the lumbosacral transition. In the chick *Hoxd-9* expression is three or four segments behind the expression of *Hoxa-9*, *Hoxb-9* and *Hoxc-9*. It lies one segment anterior to the hindlimb, at somite level 29-30, representing the last lumbar vertebra (L4). In the mouse, the anterior boundary of expression of *Hoxd-9* lies at the same numbered somite, 29-30, which in the mouse corresponds to the penultimate lumbar vertebra (L5).

Paralogue 10

All three of the *Hox* 10 paralogues, *Hoxa-10*, *Hoxc-10*, and *Hoxd-10* were examined in the chick and *Hoxd-10* was examined in the mouse (Fig. 8). All the tenth paralogue genes are expressed close to the lumbosacral transition. *Hoxa-10* has the most anterior expression in the chick, starting one segment anterior to the hindlimb at somite level 29-30. This axial level represents the last lumbar vertebra (L4), and is aligned with the expression of chick *Hoxd-9*. Boundaries for *Hoxc-10* and

Hoxd-10 lie just behind *Hoxa-10* at somite level 30-31, or the first sacral vertebra. In the mouse, *Hoxd-10* labeling shows an anterior boundary at somite level 31-32, aligned with the midline of the hindlimb, and mapping to the first sacral vertebra, as in the chick (Fig. 8D).

Paralogues 11, 12 and 13

The *Hox*a, *Hox*c, and *Hox*d clusters each have a member in the eleventh paralogue group. All three were examined in the chick and *Hoxd-11* was examined in the mouse (Fig. 9). The expression boundaries of paralogue eleven genes in the chick are not as close together as members of other paralogue groups, although they all have expression boundaries within the sacral series, as does *Hox*d-11 in the mouse. *Hoxa-11* in the chick has the most anterior expression boundary, at somite level 33-34, overlapping with the hindlimb bud and corresponding to the fourth sacral vertebra. The anterior boundary of *Hoxc-11* lies at somite level 36-37, at the posterior edge of the hindlimb, mapping to the seventh sacral vertebrae. *Hoxd-11* lies more posterior still, about two segments behind the hindlimb, probably at somite 40. The expression of *Hoxd-11* in the mouse falls at the last sacral vertebra (S4) at somite level 34-35, immediately behind the hind limb.

Hoxd-12 was examined in the chick and the mouse, and *Hoxa-13* and *Hoxd-13* were examined in the chick. The paraxial mesoderm expression patterns of these genes are limited to the tail and a somite number was not assigned (data not shown). The anterior extent of *Hoxd-12* in the chick is a distance of 4-5 segments from the rear border of the hind limb. The domain of *Hoxd-12* expression in the mouse is posterior to all the others, at somite level 35-36, the first caudal vertebra. The expression of both *Hoxa-13* and *Hoxd-13* in the chick is found in the distal end of the tail.

Expression boundary of *Hoxc-6* in the domestic goose and *Xenopus*

Our analysis of *Hox* genes in the chick and the mouse suggest that *Hox* gene expression is transposed in concert with morphology. For example, *Hoxc-6* specifically marks a key morphological boundary, the cervical-thoracic transition in the chick and the mouse even though this transition is transposed by seven vertebrae in these two species. To extend this analysis and test the phylogenetic stability of the correlation between *Hoxc-6* and the cervical-thoracic transition, we examined the expression of *Hoxc-6* in two additional species with very different cervical lengths: the domestic goose and the frog *Xenopus* (Fig. 5).

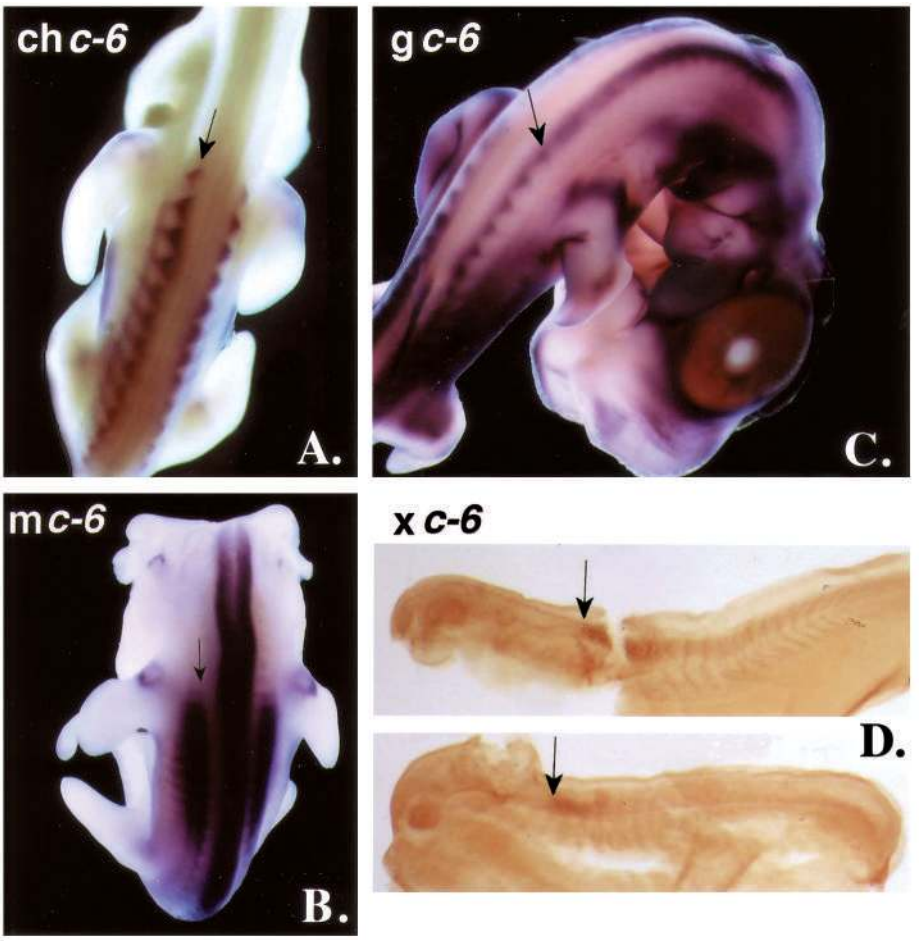

Fig. 5. Whole-mount in situ hybridizations with members of paralogue 6. Arrows indicate the anterior limit of gene expression in the paraxial mesoderm. (A) Chick (st. 25) *Hoxc-6* expression boundary lies at somite level 19-20, aligned with the middle of the forelimb, at the first thoracic vertebra (T1). The paraxial mesoderm is labeled in an 'arcade' pattern along the flank: intersegmental labeling forms arches that meet dorsally in mid-segment, defining each somite. Somite labeling continues posteriorly into the tail. Laterally in the mid-flank there is a band of nearly continuous expression running anterior-posterior along the original boundary between somitic and lateral plate mesoderm. Lateral to this, in the body wall are streaks of inter segmental expression that trace the myosepta. (B) Mouse (E12) *Hoxc-6* expression is aligned with the middle of the forelimb at somite level 12-13, the first thoracic vertebra, consistent with data reported by Jegalian et al. (1992). The mouse does not show an 'arcade' pattern of expression but the label is very strong in a near-continuous region of trunk mesoderm that covers approximately eight segments and extends laterally to the horizontal septum. Ventrally, the label is inter segmental, tracing the myosepta and rib positions in the body wall. (C) Goose at the morphological equivalent of a stage 25-26 chick embryo hybridized with chick the *Hoxc-6* probe. Two different probes were used, both giving positive results with a variable degree of background that is comparable in chick and goose embryos. The labeling pattern is very similar to that of the chick including anterior-proximal limb labeling reported elsewhere (Sharp et al. 1988: Nelson et al. unpublished data), and a partial arcade pattern of somitic labeling. The paraxial mesoderm labeling starts at an axial level that aligns with the middle of the forelimb, at somite level 22-23, the first thoracic vertebra. (D) Whole-mount immunohistochemistry with XlhBox-1 antibody on stage 35 *Xenopus* embryos. Labeling begins at the border between somite 3 and somite 4, forming a continuous border across the neural tube and the mesoderm in agreement with labeling reported by Oliver et al. (1988).

The riboprobe for chicken *Hoxc-6* was used to examine expression in the domestic goose, following the same general in situ protocol used for the chick. The paraxial mesoderm labeling starts at an axial level that aligns with the middle of the forelimb. In the goose, with 17 cervical vertebrae, this is somite level 22-23, the first thoracic vertebra.

Frogs have highly specialized vertebral morphology, without a clear distinction between cervical and thoracic vertebrae. The axial level of innervation for the forelimb however can be used as a landmark, and derives from segments number 2, 3 and 4. The Xlhbox-1 antibody generated against the *Hoxc-6* protein of *Xenopus* (Oliver et al., 1988; Wright et al., 1989) was used to visualize the anterior expression boundary in the paraxial mesoderm of the chick embryo and in *Xenopus*. Consistent with the in situ data, the anterior boundary in the chick marks the cervical-thoracic transition (data not shown). In stage 35 *Xenopus* larvae (Nieuwkoop and Faber, 1967), the labeling begins at the border between somite 3 and somite 4 (Fig. 5D). This axial level corresponds to spinal nerves that innervate the forelimb in frogs.

DISCUSSION

The evolution of the tetrapod vertebral column into specialized regions has allowed a great deal of functional diversity and adaptation. This diversity arose from an ancestral condition exemplified by the rhipidistian fishes. All fishes exhibit a degree of axial regionalization effected primarily by the extent of the coelom, the position of the median fins, and the morphology of the tail. Tetrapod axial regionalization has apparently evolved from this ancestral condition primarily through adaptations for weight bearing locomotion (Goodrich, 1930; Gadow, 1933; Panchen, 1980).

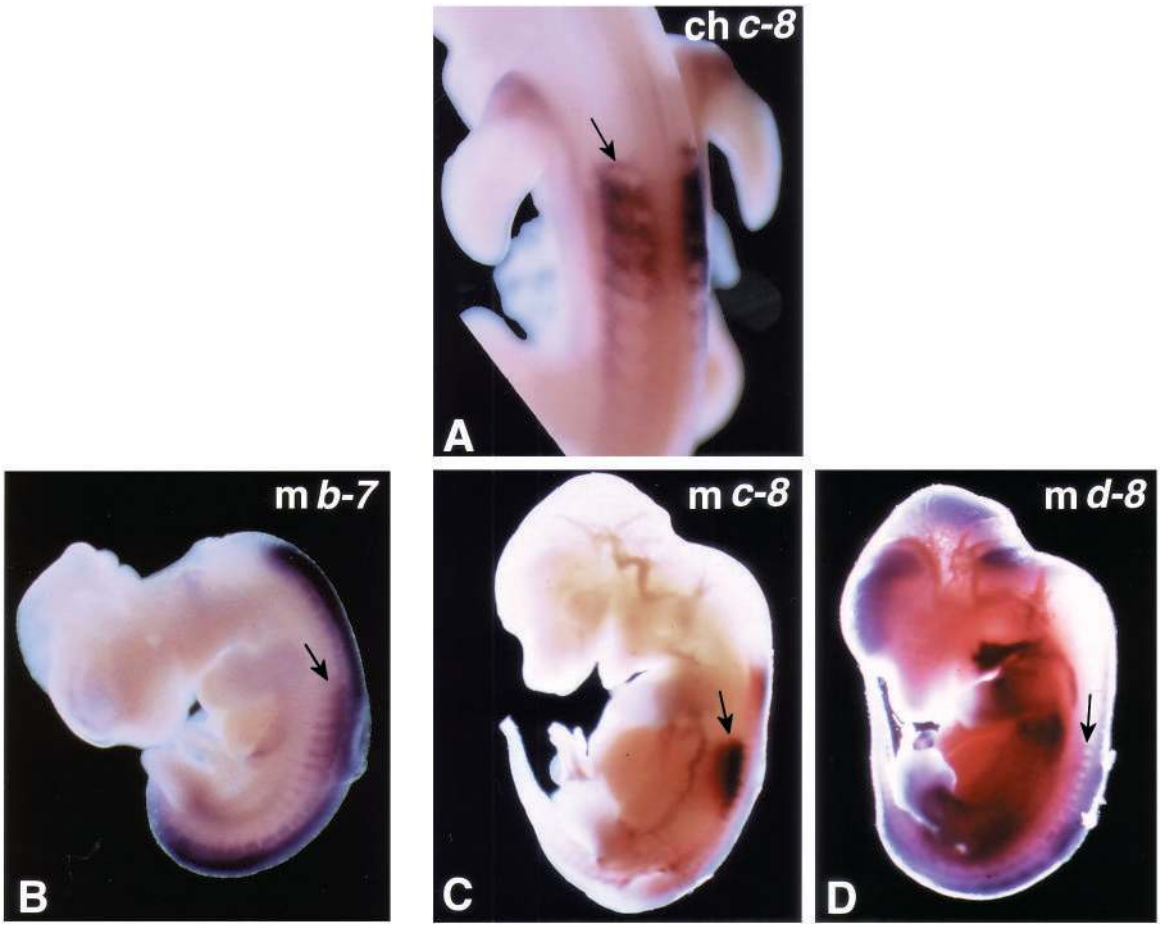

Fig. 6. Whole-mount in situ hybridizations with members of paralogues 7 and 8. Arrows indicate the anterior limit of gene expression in the paraxial mesoderm. (A) Chick (st. 25) *Hoxc-8* with an expression boundary at somite level 23-24, the fifth thoracic vertebra. In this specimen, the expression is very strong in the first three or four labeled segments, and fades posteriorly. Other embryos show uniformly strong labeling from somite level 23-24 into the tail. In stage 19-23 chick embryos, the labeling has a strong posterior-half somite bias, but by stage 25 this is not as obvious, and the labeling takes on the arcade pattern. (B) Mouse (E11.5) *Hoxb-7* shows an expression boundary at somite level 15-16, 4th thoracic vertebra. Kessel and Gruss (1991) give the anterior boundary of *Hoxb-7* as the 4th thoracic vertebra. However, Vogels et al. (1990, 1993) looking at sectioned material place the anterior boundary in the paraxial mesoderm at somite 11, which maps to the 6th and 7th cervical vertebrae. With our probe, signal is gone except in the CNS by E13. (C) Mouse (E13.5) *Hoxc-8*, expression at somite level 17-18, corresponding to the sixth thoracic vertebra (in agreement with Gaunt, 1988). (D) Mouse (E13.5) *Hoxd-8* gives a positive signal in the paraxial mesoderm at somite level 18-19, the seventh thoracic vertebra in contrast to the report of Izpisua-Belmonte et al. (1990) who found a weak signal in the mouse at the tenth thoracic vertebra (somite level 21-22) with increasing intensity posteriorly. Labeling is also strongly positive in the nephric tissue and limb mesoderm.

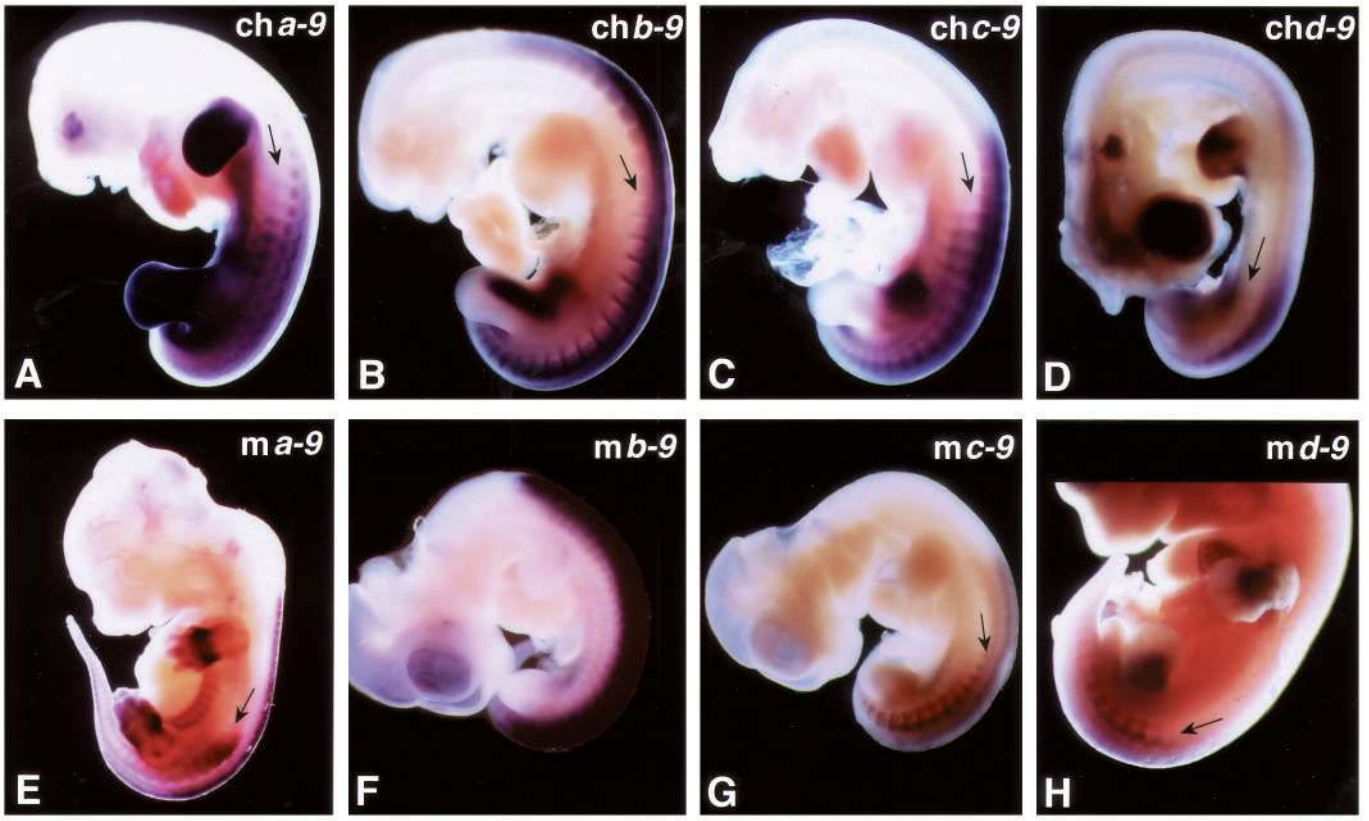

Fig. 7. Whole-mount in situ hybridizations with members of paralogue 9. Arrows indicate the anterior limit of gene expression in the paraxial mesoderm. Chick (st. 24-25) *Hoxa-9* (A) *Hoxb-9* (B) and *Hoxc-9* (C), all show expression at somite level 25-26, or the last thoracic vertebra. Dorsal root ganglia are strongly labeled for *Hoxa-9* and *Hoxb-9* in more anterior segments. Myotomal extensions from the *Hoxc-9*-labeled somites can be clearly seen in the body wall. (D) Chick (st. 23-24) *Hoxd-9* expressed at somite level 29-30, the last lumber vertebra. (E) Mouse *Hoxa-9* (E13.5) and (G) *Hoxc-9* (E11) both show expression at somite level 23-24, or the last thoracic vertebra. The dorsomedial portion of these somites is negative, and label decreases in the base of the tail. Segmental labeling with *Hoxa-9* is also apparent in the leading edge of the body wall closing around the viscera but not visibly continuous with the somites. (F) *Hoxb-9* (E11.5) did not resolve to a distinct somite level, but is stronger in the posterior thoracic region. (H) Mouse (E13) *Hoxd-9* with a boundary at somite level 29-30.

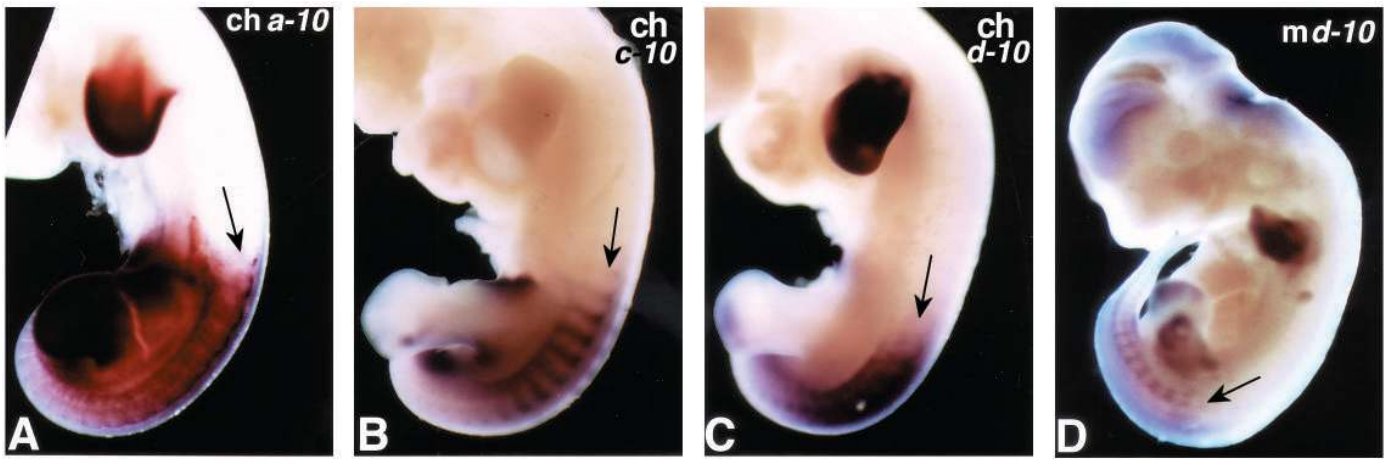

Fig. 8. Whole-mount in situ hybridizations with members of paralogue 10. Arrows indicate the anterior limit of gene expression in the paraxial mesoderm. (A) Chick (st. 24) *Hoxa-10*, is expressed at somite level 29-30, the last lumbar vertebra. Labeled somites show a posterior bias and strongly positive dorsal 'caps'. (B) *Hoxc-10* (st. 26) and (C) *Hoxd-10* (st. 25) share a boundary behind *Hoxa-10* at somite level 30-31, or the first sacral vertebra. Somites labeled with *Hoxc-10* have the arcade character, like the other chick *Hox*c genes, and the labeling with *Hoxd-10* resembles that of *Hoxa-10* without the 'caps'. (D) Mouse (E12.5) *Hoxd-10* shows an anterior boundary at somite level 31-32, mapping to the first sacral vertebra. There is an intersegmental bias resulting in a striped effect.

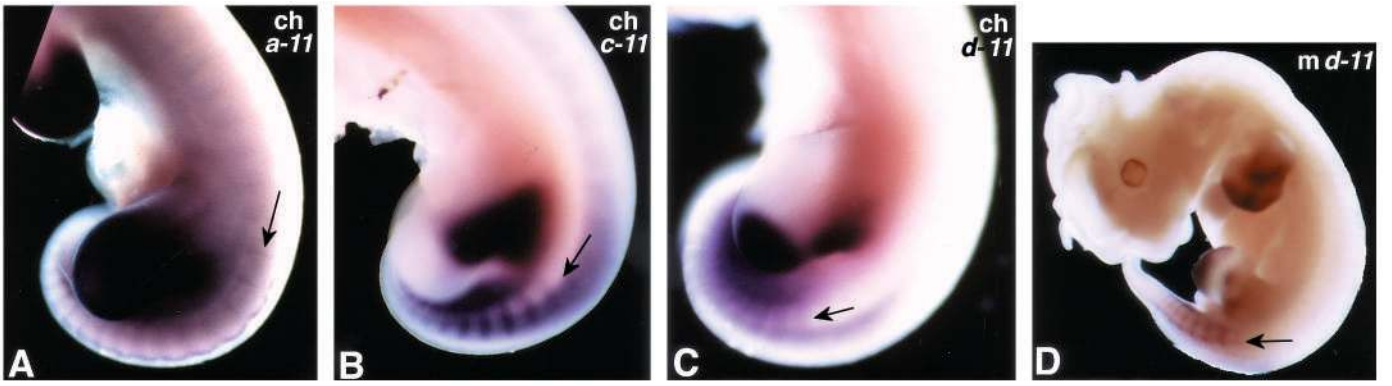

Fig. 9. Whole-mount in situ hybridizations with members of paralogue 11. (A) Chick (st. 24) *Hoxa-11* at somite level 33-34. (B) Chick (st. 24) *Hoxc-11* at somite level 36-37. (C) Chick (st. 24) *Hoxd-11* at somite 40. (D) Mouse (E12.5) *Hoxd-11* at somite level 34-35. Arrows indicate the anterior limit of gene expression in the paraxial mesoderm.

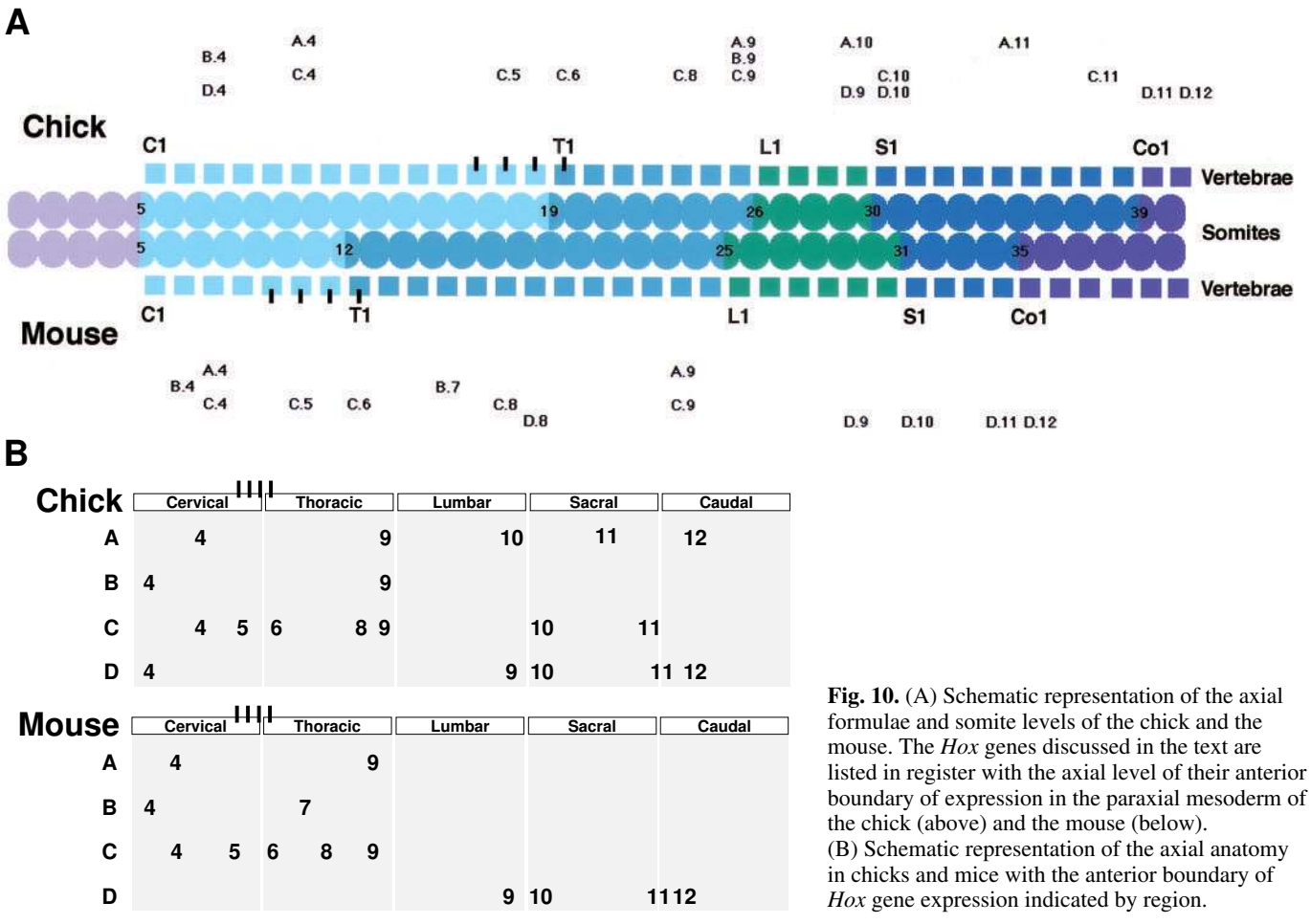

Fig. 10. (A) Schematic representation of the axial formulae and somite levels of the chick and the mouse. The *Hox* genes discussed in the text are listed in register with the axial level of their anterior boundary of expression in the paraxial mesoderm of the chick (above) and the mouse (below). (B) Schematic representation of the axial anatomy in chicks and mice with the anterior boundary of *Hox* gene expression indicated by region.

Vertebral diversity in tetrapods ranges from the secondarily achieved uniformity of snakes and other limbless tetrapods, to the highly specialized morphology of the turtle's shell, the giraffes' long neck and the prehensile tail of certain monkeys. The early developmental pattern from unsegmented mesoderm, through somites, into vertebrae, has not altered substantially between fishes and tetrapods. The subsequent specializations and subtleties of structure are, however, significant characters in the distinctions between vertebrate groups.

We have evaluated the correlation between morphological regions of the vertebral column and the anterior-most expression of *Hox* genes as determined by whole-mount in situ hybridization (Fig. 10). It should be noted that this technique lacks the sensitivity and resolution to draw specific mechanistic conclusions about the roles of *Hox* genes at a given axial level. This is exemplified by the fact that in mice carrying homozygous deletions of certain *Hox* genes, phenotypic effects are observed anterior to the detectable limits of *Hox* gene

expression (e.g. *Hoxa-4*, Kostic and Capecchi, 1994; *Hoxb-4*, Ramirez-Solis et al., 1993). As pointed out by Kostic and Capecchi (1994), one explanation for this is that these genes are expressed at a significantly lower level anterior to the boundary of expression observed in the whole-mount in situ hybridizations. The limits of expression we define here are not necessarily the absolute anterior limits of expression in all cases. However, in every *Hox* gene examined in cross-species comparison, the expression boundaries are consistently associated with morphology, not with absolute somite number, in animals with different axial formulae. The relative shifts in *Hox* gene expression that we observe in different areas along the axis reflect the relative expansion and contraction of morphological regions. This implies a role for the *Hox* genes in the evolution of axial variation.

Hox gene expression conforms to anatomical regions along the axis

The *Hox* expression boundaries that have been examined in the occipital region of the chick conform exactly to those found in the mouse (e.g. Guthrie et al., 1992; Krumlauf, personal communication). There is no transposition of segments in this region between the two species since the segmental pattern of the hindbrain rhombomeres and the number of occipital somites do not differ between these two species. Thus, in the region of the amniote axial skeleton that does not show morphological transposition, *Hox* gene expression boundaries remain constant between species.

In contrast, in regions of the axial skeleton that are transposed between the chick and the mouse, the borders of *Hox* gene expression are shifted in concert with the morphological transposition. For example, *Hoxc-5* is expressed in the cervical region, but at an axial level separated by 7 somites between chick and mouse (somite level 17-18: chick, versus somite level 10-11: mouse). This transposition is in concert with the level of the forelimb and the brachial plexus, suggesting that genes of the fifth paralogue are causally linked to the level of the forelimb.

The expression boundaries of *Hoxc-6* in chick and mice also indicate a causal connection to the position of the limb and the anatomical boundary between the neck and the thorax (C-T transition). This correlation was expanded by examining the expression of *Hox*c-6 in the domestic goose. The domestic goose has 17 cervical vertebrae, and thus the forelimb is transposed caudally by 3 segments relative to the chick. In the goose the anterior limit of expression falls at somite level 22-23, the first thoracic vertebra, and thus is also transposed by three segments relative to the chick, in concert with the position of the forelimb.

The forelimbs of tetrapods can be assigned an axial level based on the number of the spinal nerves that innervate the muscles of the limb. The ventral rami of several different spinal nerves converge together and are concentrated at the base of the limb. The series of nerves that form the brachial plexus bridges the transition between cervical and thoracic vertebrae, and provides a landmark to locate the cervical-thoracic transition in animals with highly derived vertebral morphology (e.g. frogs). In amniotes (which include birds and mammals), the brachial plexus usually comprises four or five spinal nerves. The anterior three or four of these are cervical, and the last one is the first thoracic.

The expression border of *Hoxc-6* is located at the somites that map to the first thoracic vertebra (that carries the last spinal nerve to the brachical plexus) in the chick, the goose and the mouse (Fig. 11). The significance of this association is extended by the pattern of *Hoxc-6* expression found in *Xenopus* and in the zebrafish. In stage 30 *Xenopus* larvae, labeling with the Xlhbox-1 antibody against the *Hoxc-6* protein begins at the border between somite 3 and somite 4 (Fig. 5C). The forelimb bud in metamorphosing *Xenopus* tadpoles arises just behind the gills, and is innervated from spinal nerves 2, 3 and 4, which bridge the cervical-thoracic transition of adult frogs.

In many modern bony fishes, the pectoral fin is located immediately behind the jaw, and no distinction is made between cervical and thoracic vertebrae. (The extreme anterior positioning of the pelvic fin and its migration from its level of innervation, was the major feature behind Goodrich's 1906 description of transposition). Transplantation experiments in amphibians and chicks demonstrate that the segmentation and outgrowth of the spinal nerves is dependent on the mesodermal somites, which in turn are influenced by lateral mesoderm (Detwiler, 1934; Keynes et al., 1987). The number of spinal nerve roots in the limb plexus reflects the number of myotomes that contribute to the limb musculature. Molven et al. (1990)

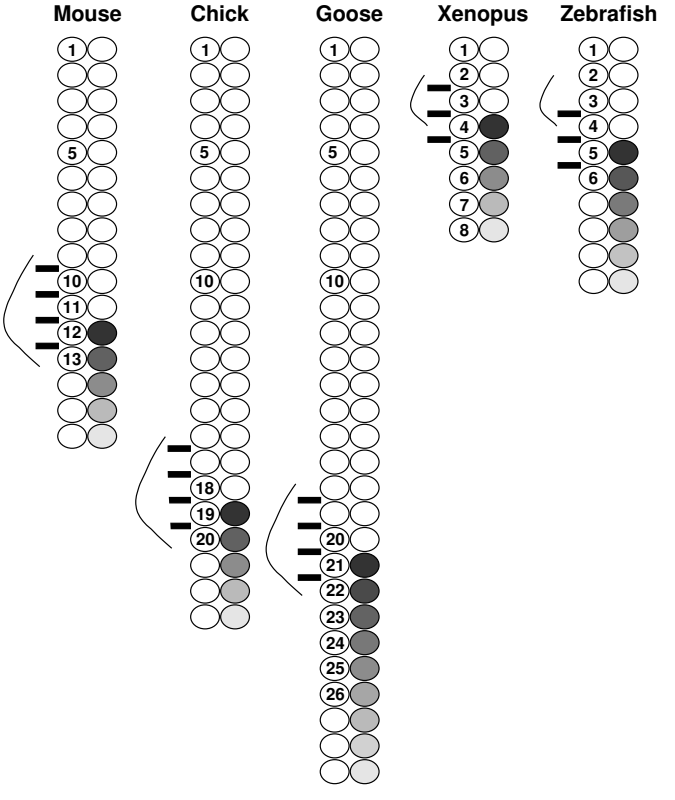

Fig. 11. Schematic representation of the somite levels bridging the cervical-thoracic transition in mouse, chick, goose, *Xenopus*, zebrafish. Black bars represent the spinal nerves of the brachial plexus, and the level of the limb or fin bud is indicated with a curved line. The shaded somite levels indicate the level of *Hoxc-6* expression as determined by whole-mount in situ hybridization, or immunohistochemistry. The level for the zebrafish is taken from Molven et al. (1990).

used the Xlhbox-1 antibody to localize the *Hoxc-6* protein in embryos of the zebrafish, *Brachydanio rerio*. They report the expression boundary in the paraxial mesoderm to fall between somites 4 and 5. In the embryo the finbud appears adjacent to somites two and three, and has anterior-proximal expression of *Hoxc-6*, as seen in the limbs of chicks and mice. The innervation for the pectoral fin (the piscine brachial plexus) comes from spinal nerves 3, 4 and 5 (Myers, 1985). Thus, while the expression of *Hoxc-6* in the paraxial mesoderm does not overlap with the placement of the finbud, it does align with the posterior-most segment that innervates the pectoral appendage in the zebrafish, as it does in an amphibian and three different amniotes (Fig. 11). In the tetrapods this is the first thoracic vertebra.

Additional evidence for the culpability of paralogue group 6 members in the cervical-thoracic transition comes from homozygous deletions of *Hoxa-6* in the mouse (Kostic and Capecchi, 1994). These mice have a rib on the last cervical vertebra, a partial posterior transformation of the seventh cervical vertebra towards a thoracic morphology.

The other *Hox* genes studied are similarly transposed along with morphological regions. Briefly, all members of the seventh and eighth paralogue groups examined here have anterior expression boundaries within the thoracic region in both chick and mouse. The expression boundaries of *Hoxc-8* compared between the two species do not correspond to any obvious morphological landmarks. However, the transposition of this gene expression boundary is clear. If *Hox*c-8 were expressed in the chick at the same absolute somite number at which it is expressed in the mouse, it would be in the chick cervical region rather than the thoracic region.

The expression boundaries of *Hoxa-9*, *Hoxb-9* and *Hoxc-9* are found in close association with the morphological transition from thoracic to lumbar vertebrae in both chicks and mice. In the lumbar region the expression boundaries differ in absolute location by only one or two somites between the two species. This is because the long neck of the chicken is followed by a short trunk, while the relatively short mammalian neck is followed by a long trunk in the mouse. Paralogue group nine also shows a significant disparity in the axial expression of one of its members, *Hoxd-9*, relative to its three cognates. *Hoxd-9* is expressed just anterior to the lumbosacral border in both the chick and the mouse, again an anatomical level that differs in absolute number by only one somite.

Members of the tenth paralogue group examined are grouped at the lumbosacral transition, and members of paralogue eleven are expressed at levels within the sacrum. In contrast to members of the other paralogue groups, whose expression borders are relatively close together, the expression boundaries of *Hox-11* genes are spread over seven somites in the chick. We found *Hoxd-11* expression at the end of the sacral series in the mouse, similar to the anatomical level in the chick. Published reports for *Hoxa-11* in the mouse are four somites anterior to this at the lumbosacral boundary (Small and Potter, 1993), indicating that expression of the *Hox-11* genes in the mouse extend over the full sacral series. The distribution of *Hox-11* gene expression in the chick mimics the extensive expansion of the sacral region in birds. The expression boundaries of the *Hox-12* genes examined were found at the beginning of the caudal series of both species, and members of paralogue 13 were found further posterior in the tail.

Hox genes and the evolution of axial diversity in vertebrates

The full set of *Hox* genes appears to have been present in the common ancestor of tetrapods and modern fishes (F. Vander-Hoeven and D. Duboule, personal communication). This ancestral animal lacked the distinct axial regions present in tetrapods, but it is virtually certain that its *Hox* genes were expressed in an A-P, colinear progression. Primitive *Hox* expression patterns presumably served to define units that have subsequently been elaborated into specialized regions, facilitating the evolution of axial structures along separate, discrete morphological paths in fishes and tetrapods.

The anatomical distribution of *Hox* gene expression boundaries is remarkably consistent between the chick and mouse. The association of individual genes with specific anatomical boundaries is quite clear. Particularly striking is the association of *Hoxc-5* and *Hoxc-6* with the cervical-thoracic transition; the association of *Hoxa-9*, *Hox*b-9 and *Hoxc-9* with the end of the thoracic series; the posterior displacement of the cognate *Hoxd-9* to the end of the lumbar series; followed by the *Hox*-10 members in the initial segments of the sacrum. In the case of *Hoxc-6*, we have extended the comparison to include an amphibian and a fish. The cervical-thoracic transition, and the innervation of the pectoral appendage are further linked by the expression of *Hoxc-6*, even in animals where a morphological transition is not perceived (e.g. fishes).

The axial expression of *Hoxd-9* is anomalous in that this expression level is significantly out of register with the other members of the ninth paralogue group. The significance of the shift in the expression boundary of *Hoxd-9* may have traces in the history of vertebrate anatomy. Based on fossil evidence, the advent of a pelvis attached to the vertebral column (sacrum) was a major transition between tetrapods and their fish-like ancestors. Also, neither fossil or recent amphibians subdivide the trunk into thoracic and lumbar regions, so a distinction between thoracic and lumbar vertebrae is not a universal character of tetrapods, and birds and mammals are derived in this regard (Goodrich, 1930; Gadow, 1933). *Hoxa-9*, *Hoxb-9* and *Hoxc-9* are associated with the transition from thoracic to lumbar vertebrae, and *Hoxd-9* is apparently anchored to the presacral-sacral transition in chicks and mice. It is not unreasonable to suppose that all the genes in the ninth paralogue group shared the same anterior expression boundary at one time. The shift of *Hoxd-9* expression away from its paralogues, resulting in a novel gene expression boundary, may have been instrumental in the evolutionary transition from fish to tetrapod. Alternatively (or subsequently), expression shifts within the *Hox-9* paralogue may have been influential in the evolution of the thoracic-lumbar transition from a more uniform amphibian-type trunk. This hypothesis suggests that the generation of novel gene expression relationships by transposition of expression boundaries could result in the generation of new morphological regions, as well as positional changes in pre-existing regions.

Hox genes as characters or units of biological homology

In the 19th century, the German school of *Naturphilosophie*

developed the concept of *Bauplan* (body plan), to describe the invariant aspects of morphology that unite organisms into natural groups (see Russell, 1916 for review). Invariant aspects of morphology indicate homology, and homology identifies the units that can be compared meaningfully between any two organisms. Originally a purely morphological concept, the idea of *Bauplan* has been expanded today to include molecular data. The regulated A-P expression of Homeobox genes has been used as the primitive character uniting widely divergent groups of animals into a common *Bauplan,* called the *Zootype* (Slack et al., 1993).

The usefulness of the *Bauplan* concept is explicitly manifest in any phylogenetic study, and is employed implicitly by all biologists searching for universal patterns or mechanisms. The data discussed here explores a degree of molecular homology for axial regions among amniotes that strengthens the pre-existing assessment of homology based on morphology. *Hox* gene expression constitutes an additional set of characters with which to homologize segments between organisms, and provides an opportunity to re-examine aspects of morphology that have long been problematic for evolutionary biologists. For instance, the loss of limbs has occurred independantly in snakes and other tetrapods, and the morphological boundaries between axial regions have been largely lost in limbless animals. Data on *Hox* gene expression would clarify regional homologies, and expose the underlying history of these morphological adaptations.

The full significance of a molecular level of homology becomes apparent when one tries to reconstruct the history and direction of morphological evolution. The analysis of molecular characters can lead to the recognition of morphologically cryptic homologies, and can also expose variations of the downstream, interpretive systems of regulatory genes that result in developmental differences. Segmental patterns in phyla such as arthropods, annelids and vertebrates, have long been considered independently derived (not historically homologous) in each lineage because of fundamental developmental differences (cf. Minelli and Peruffo, 1991). However, using molecular characters as a basis for homology, particular segments of arthropods and chordates are homologous in a mechanistic sense, because they share developmental information in the form of homologous homeobox gene expression. Moreover, they may also be homologous in an historical sense if we accept that the common ancestor of chordates and arthropods had distinct anterior-posterior regions definable on the basis of the expression of these genes. A similar relationship has been reported in the development of visual organs, long considered to be independently derived in arthropods and chordates. The *eyeless* gene in *Drosophila* and its homologue *Pax-6* in vertebrates, both function in eye development, indicating an ancient historical homology between these developmentally different structures (Zuker 1994; Quiring et al., 1994).

The analysis presented here supports the view that *Hox* genes are instrumental in the establishment of distinct morphological structures along the axis in vertebrates, thereby determining the level of transitions between different vertebral regions long known to be historically homologous based on morphology. The transposition identified by Goodrich wherein the absolute levels of morphological transitions are shifted between vertebrate species, are likely to have been achieved by evolutionarily changes in expression domains of *Hox* genes along the anterior-posterior axis.

We are grateful to the following people for mouse *Hox* probe constructs: R. Krumlauf (*Hoxa-4*, *Hoxb-4*, *Hoxb-9*); H. Haake and P. Gruss (*Hoxa-9)*; M. Capecchi (*Hoxc-9)*; J. Deschamps (*Hoxb-7 Hoxb-8*); D.Duboule (*Hoxd-8*, *Hoxd-9*, *Hoxd-10, Hoxd-11*, *Hoxd-12*); P. Sharp (*Hoxc-4* and *Hoxc-5*); K. Bentley (*Hoxc-6, Hoxc-8*). E. DeRobertis provided the Xlhbox-1 antibody. E. DiMambro assisted in the characterization of chick *Hox* clones. D. Roberts provided goose embryos. J. Khorana assembled the figures. We thank R. Krumlauf, E. Gilland, and M. Scott for critical reading of the manuscript, and an anonymous reviewer for helpful comments. A. C. B. is supported by a National Institutes of Health, NRSA postdoctoral fellowship. This work was supported by a grant from the National Institutes of Health to C. T.

REFERENCES

Bagnall, K. M., Higgins, S. J. and Sanders, E. J. (1988). The contribution made by a single somite to the vertebral column: experimental evidence in support of resegmentation using the chick-quail chimaera model. *Development* **103**, 69-85.

Carroll, R. (1988). *Vertebrate Paleontology and Evolution.* New York: W. H. Freeman and Company.

Couly, F. G., Coltey, P. M. and Le Douarin, N. M. (1993). The triple origin of the skull in higher vertebrates: a study in quail-chick chimeras. *Development* **117**, 409-429.

de Beer, G. R. (1937). *The Development of the Vertebrate Skull.* Oxford: Oxford University Press.

Detwiler, S. R. (1934). An experimental study of spinal nerve segmentation in amblystoma with reference to the pleurisegmental contribution to the brachial plexus. *J. Exp. Zool.* **67**, 395-441.

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

Gadow, H. F. (1933). *The Evolution of the Vertebral Column.* Cambridge, UK: Cambridge University Press.

Gaunt, S. J. (1988). Mouse homeobox gene transcripts occupy different but overlapping domains in embryonic germ layers and organs: a comparison of *Hox-3.1* and *Hox-1.5*. *Development* **103**, 135-144.

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**, 329-39.

Gaunt, S. J. and Strachan, L. (1994). Forward spreading in the establishment of a vertebrate *Hox* expression boundary: the expression domian separates into anterior and posterior zones and the spread occurs across implanted glass barriers. *Dev. Dynam.* **199**, 229-240.

Geada, A. M., Gaunt, S. J., Azzawi, M., Shimeld, S. M., Pearce, J. and Sharpe, P. T. (1992). Sequence and embryonic expression of the murine *Hox-3.5* gene. *Development* **116**, 497-506.

Goodrich, E. S. (1906). Notes on the development, structure and origin of the median and paired fins of fish. *Quart. Jour. Microsc. Sci.* **50**, 333-376.

Goodrich, E. S. (1913). Metameric segmentation and homology. *Quart. Jour. Microsc. Sci.* **59**, 227.

Goodrich, E. S. (1930). *Studies on the Structure and Development of Vertebrates.* London: Macmillan.

Graham, A., Papalopulu, N. and Krumlauf, R. (1989). The murine and Drosophila homeobox gene complexes have common features of organisation and expression. *Cell* **57**, 367-378.

Guthrie, S., Muchamore, I., Kuroiwa, A., Marshall, H., Krumlauf, R. and Lumsden, A. (1992). Neuroectodermal autonomy of *Hox-2.9* expression revealed by rhombomere transpositions. *Nature* **356**, 157-159.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Exp. Zool*. **88**, 49-92.

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**, 733-45.

Jegalian, B. G., Miller, R. W., Wright, C. V., Blum, M. and De Robertis, E. M. (1992). A *Hox 3.3*-lacZ transgene expressed in developing limbs. *Mech. Dev.* **39**, 171-80.

Kappen, C. and Ruddle, F. H. (1993). Evolution of a regulatory gene family: HOM/*HOX* genes. *Curr. Biol.* **3**, 931-938.
Kessel, M. and Gruss, P. (1990). Murine developmental control genes. *Science* **249**, 347-379.
Kessel, M. and Gruss, P. (1991). Homeotic transformations of murine vertebrae and concomitant alteration of *Hox* codes induced by retinoic acid. *Cell* **76**, 89-104.
Kessel, M. (1992). Respecification of vertebral identities by retinoic acid. *Development* **115**, 487-501.
Keynes, R. J., Stirling, R. V., Stern, C. D. and Summerbell, D. (1987). The specificity of motor innervation of the chick wing does not depend upon the segmental origin of muscles. *Development* **99**, 565-575.
Kostic, D. and Capecchi, M. R. (1994). Targeted disruption of the murine *Hox*a-4 and *Hox*a-6 genes result in homeotic transformations of the vertebral column. *Mech. Dev.* **46**, 231-247.
Krumlauf, R. (1992). Evolution of the vertebrate *Hox* homeobox genes. *BioEssays* **14**, 245-252.
Lankester, E. R. (1910). Metamerism. In *Encyclopedia Britannica*
Minelli, A. and Peruffo, B. (1991). Developmental pathways, homology and homonomy in metameric animals. *J. Evol. Biol.* **3**, 429-445.
Molven, A., Wright, C. V. E., Bremiller, R., DeRobertis, E. M. and Kimmel, C. B. (1990). Expression of a homeobox gene product in normal and mutant zebrafish embryos: evolution of the tetrapod body plan. *Development* **109**, 279-288.
Myers, P. Z. (1985). Spinal motor neurons of the larval zebrafish. *J. . Comp. Neurol.* **236**, 555-561.
Nickel, R., Schummer, A., Seiferle, E., Siller, W. G. and Wight, P. A. L. (1977). *Anatomy of the Domestic Birds.* New York, Heidelberg, Berlin: Springer-Verlag.
Nieuwkoop, P. D. and Faber, F. (1967). Normal Stages of *Xenopus laevis*. Amsterdam: North-Holland Publishing Co.
Oliver, G., Wright, C. V. E., Hardwicke, J. and De Robertis, E. M. (1988). Different antero-posterior expression of two proteins encoded by a homeobox gene in Xenopus and mouse embryos. *EMBO J.* **7**, 3199-3209.
Panchen, A. L. (1980). *The Terrestrial Environment and the Origin of Land Vertebrates.* London: Academic Press.
Quiring, R., Walldorf, U., Kloter, U. and Gehring, W. J. (1994). Homology of the eyeless gene of Drosophila to the small eye gene in mice and aniridia in humans. *Science* **265**, 785-789.
Ramirez-Solis, R., Zheng, H., Whiting, J., Krumlauf, R. and Bradley, A. (1993). *Hoxb-4* (*Hox-2.6*) mutant mice show homeotic transformation of a cervical vertebra and defects in the closure of the sternal rudiments. *Cell* **73**, 279-294.
Remak, R. (1855). Untersuchungen über die Entwicklung der Wirbeltiere. Berlin: Reimer.
Riddle, R. D., Johnson, R. L., Laufer, E. and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **75**, 1401-1416.
Russell, E. S. (1916). *Form and Function.* London: John Murray.
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.
Slack, J. M. W., Holland, P. W. H. and Graham, C. F. (1993). The zootype and the phylotypic stage. *Nature* **361**, 490-492.
Small, K. M. and Potter, S. S. (1993). Homeotic transformations and limb defects in *Hox* A11 mutant mice. *Genes Dev.* **7**, 2318-28.
Stern, C. D. and Keynes, R. J. (1987). Interactions between somite cells: the formation and maintenance of segment boundaries in the chick embryo. *Development* **99**, 261-272.
Vogels, R., de, G. W. and Deschamps, J. (1990). Expression of the murine homeobox-containing gene *Hox-2. 3* suggests multiple time-dependent and tissue-specific roles during development. *Development* **110,** 1159-68.
Vogels, R., Charité, J., de Graff, W. and Deschamps, J. (1993). Proximal cis-acting elements cooperate to set *Hoxb-7* (*Hox-2.3*) expression boundaries in transgenic mice. *Development* **118**, 71-82.
Wright, C. V. E., Cho, K. W. Y., Oliver, G. and DeRobertis, E. M. (1989). Vertebrate homeodomain proteins: families of region-specific transcription factors. *Trends Biol. Sci.* **14**, 52-56.
Zuker, C. S. (1994). On the evolution of eyes: Would you like it simple or compound? *Science* **265**, 742-743.

(Accepted 21 October 1994)

Note added in proof

S. J. Gaunt has recently compared the expression of *Hoxb-3*, *Hoxa-4* and *Hoxc-6* in murine and chick somites and reached similar conclusions to those reported here.

Gaunt, S. J. (1994). Conservation in the *Hox* code during morphological evolution. *Int. J. Dev. Biol.* **38**, 549-552.