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Short Contribution

Conservation in the Hox code during morphological evolution

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ABSTRACT The expression domains in paraxial mesoderm of the chicken embryo are described for *Hoxb-3*, *a-4* and *c-6* genes, and these are compared with published expression data for the corresponding genes in the mouse. In both species, it is found that the anterior limits of *Hoxb-3* and *a-4* expression lie in the upper cervical region, and the anterior limits of *Hoxc-6* expression lie in the upper thoracic region. This finding is remarkable because the cervical region, or neck, of the chicken (with fourteen cervical vertebrae) is much longer than that of the mouse (seven cervical vertebrae). The results suggest that the Hox code, at least in the development of homologous axial structures, is conserved between species (*Hoxb-3* and *a-4*, for example, being associated with an anterior cervical phenotype; *Hoxc-6* being associated with an anterior thoracic phenotype). The results also suggest that an evolutionary change in body proportions is accomplished by a shift in the relative positions of Hox expression domains during embryonic development.

KEY WORDS: *chicken, mouse, Hox, evolution*

Modern birds and mammals, although separated by three hundred million years of evolution (Gilbert *et al.*, 1986), differ from each other not in the gross organization of their bodies but in the relative sizes and proportions of their constituent parts. Morphological evolution proceeds by changes in these proportions (Thompson, 1942). During embryonic development, each part of the body develops according to the blend of Hox genes (the Hox code) that it is expressing (e.g. McGinnis and Krumlauf, 1992; Ramirez-Solis *et al.*, 1993). An evolutionary change in body proportions could therefore be established either by a shift in the relative positions of these zones of Hox expression, or alternatively by a change in the cellular interpretation of the Hox code. Evidence is now presented that supports the former of these possibilities. *Hoxb-3* and *Hoxa-4* are found to be expressed in the anterior cervical region of both mouse and chicken, but *Hoxc-6* expression, a marker in both species of anterior thorax, is shifted posteriorly in the chicken, accommodating the increased number of cervical vertebrae, and the longer neck length, found in the bird.

Vertebrae develop from embryonic somites, and it is the cervical vertebrae that define the neck. Between species, the length of the neck varies widely. In mammals, change in the length of the neck is mediated by change in the size of the cervical vertebrae, and their number (with very few exceptions; Yapp, 1965) remains constant at seven. Birds, in contrast (and in common with their forerunners, the dinosaurs), show varied neck length by change in the number of cervical vertebrae. This number varies in birds from nine to twenty-five (Yapp, 1965), with the chicken having fourteen (Sisson and Grossman, 1966).

Chicken *Hoxb-3*, *a-4*, and *c-6* genes (previously *Hox-2.7*, *-1.4* and *-3.3*; Scott, 1992) were identified among cDNA clones isolated from a 10-day chicken embryo library (Fig. 1). These clones were chosen for analysis in the present study because the anterior expression boundaries of corresponding genes in the mouse are known to provide good markers of anterior cervical (*b-3* and *a-4*, Gaunt *et al.*, 1988; Sham *et al.*, 1992) and anterior thoracic (*c-6*, Gaunt *et al.*, 1988; Jegalian and De Robertis, 1992) vertebrae. As detected by whole-mount *in situ* hybridization, the anterior boundary of chicken *Hoxb-3* expression within mesoderm is seen to lie in somite 5, with increasing levels of expression over somites 5 to 7 (Fig. 2A,A'). For chicken *Hoxa-4*, the anterior boundary within mesoderm lies in somite 7, with increasing levels of expression over somites 7 to 9 (Fig. 2B,B'). For chicken *Hoxc-6*, a rise in the abundance of transcripts is seen over somites 20 to 24 (Fig. 2C). For all three genes, expression within neurectoderm extends anterior to the boundaries in mesoderm (Fig. 2). At the stages shown (Fig. 2A,B), expression of the genes in neurectoderm is in a state of forward spreading, and has not yet reached the definitive anterior boundaries (Gaunt and Strachan, 1994). Definitive expression boundaries in somites are, in contrast, established prior to their separation from presomitic mesoderm (Gaunt and Strachan, 1994), and newly formed somites, or adjacent presomitic mesoderm, are already determined with respect to their developmental fate (Kieny *et al.*, 1972).

Fig. 3 shows the relationship between somite and vertebral addresses (Bagnall *et al.*, 1988; Couly *et al.*, 1993), and summarizes the anterior boundaries of *Hoxb-3*, *a-4* and *c-6* expression

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A

Chicken Hoxb-3                          EFHFNRYLCRPRRVEMANLLNLSERQIKIWFQNRRMKYKKDEKSKGMGSSSG
Mouse   Hoxb-3                          ****************************************Q*A**LA****

Chicken Hoxb-3      GPSPTGSPPQPMQSSAGFMNALHTMSSNYDAPSPPSFNKPHQNAYAMSTNYQNPIKGCPSQQKYTNT-AP
Mouse   Hoxb-3      ****A*********T********S*TPS**S****A*G*G******LPS***P*L***GAP***PP*P*S

Chicken Hoxb-3      EYDPHVLQGNGVAYGTPSMQGSPVYVGGN-YVDSLPT-SGPSLYGLNHLPHHQAANMDYSGPPQMPPSQH
Mouse   Hoxb-3      **E*****A**G*****T**********GG*A*P**PPA**********S**PSG*L**N*AAP*G*N**

Chicken Hoxb-3      HGPCEPHPTYTDLSSHHASSQGRIQEAPKLTHLtrm
Mouse   Hoxb-3      ****D*************PP*************trm

B

Chicken Hoxc-6      GVGYGADRRRGRQIYSRYQTLELEKEFHFNRYLTRRRRIEIANALCLTERQIKIWFQNRRMKWKKES
Mouse   Hoxc-6      ********************************************************************
Chicken Hoxb-6      TGSSFGPA*****T*T*************************HS**********************N
Mouse   Hoxa-6      *AV**SHG*****T*T*************************************************N

Chicken Hoxc-6      NLSSTLSGAGGGTAAAADSLA-KEEGYCSQMLGAVtrm
Mouse   Hoxc-6      **T*****G***AT--****GG***KRGETEEEKQKEtrm
Chicken Hoxb-6      K*L*SSQLSAEEEEEKTAEtrm
Mouse   Hoxa-6      K*INSTQAS*EDSE*K*GEtrm
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Fig. 1. Identification of chicken *Hoxb-3* and *c-6* genes. (A) *The chicken Hoxb-3 gene identified by the similarity of its predicted protein sequence with that of mouse Hoxb-3 (Sham et al., 1992), and by its overlap with a published fragment of chicken Hoxb-3 sequence (Scotting et al., 1990).* **(B)** *The chicken Hoxc-6 gene identified by the similarity of its protein sequence with that of mouse Hoxc-6 (Sharpe et al., 1988), and by its differences from chicken Hoxb-6 (Wedden et al., 1989) and mouse Hoxa-6 (Colberg-Poley et al., 1985). The chicken, like mammals, does not apparently possess a Hoxd-6 homeobox gene (A. Kuroiwa, personal communication). Among Hox genes, isoleucine at homeodomain position 7 appears to be uniquely characteristic of Hoxc-6 (Acampora et al., 1989). The homeodomains are boxed, and asterisks indicate identity with the chicken sequence. Both the Hoxb-3 and the Hoxc-6 cDNA clones are incomplete in 5′ regions.*

found in chicken somites and mouse prevertebrae (Gaunt *et al.*, 1988; Sham *et al.*, 1992). As measured against the anatomical landmarks of somite or vertebral address, it is seen that the expression domains for *Hoxb-3* and *a-4* are similar, or identical, in mouse and chicken. In contrast, the expression domain for *Hoxc-6* in chicken is, relative to mouse, apparently shifted posteriorly by a distance of about seven somites or vertebrae (Fig. 3).

To check that these findings and conclusions for the chicken remain true at a later stage of development, *in situ* hybridization was performed on sections of $5^{1/2}$ day embryos using *Hoxb-3* and *Hoxc-6* probes (Fig. 4). Consistent with the predictions of Fig. 3, the anterior boundary of expression is seen for *Hoxb-3* as a rise in the abundance of transcripts over prevertebrae 1 to 2 (pv 1-2), and for *Hoxc-6* as a rise over pv 15-18 (Fig. 4).

The findings from *in situ* hybridization at two different stages of chicken development, 56 hours (29 somite) and $5^{1/2}$ days, are therefore consistent in demonstrating that the expression domain for chicken *Hoxc-6* is shifted posteriorly relative to that of mouse *Hoxc-6* by a distance of seven somites or vertebrae (Fig. 3). While it is possible that this shift represents a peculiarity of the chicken *Hoxc-6* gene (and studies upon other Hox genes of the chicken are now needed to confirm the generality of the shift), there is one more obvious and rational interpretation of the findings. Thus, having regard to the greater number of somites required to form cervical vertebrae in chicken than mouse it does hold true for both species that *Hoxc-6* expression increases in intensity over somites and prevertebrae that are destined to form anterior thoracic vertebrae (Fig. 3). The results therefore suggest that the Hox code and its interpretation, at least in the development of homologous structures in birds and mammals, is conserved (*Hoxb-3* and *a-4*, for

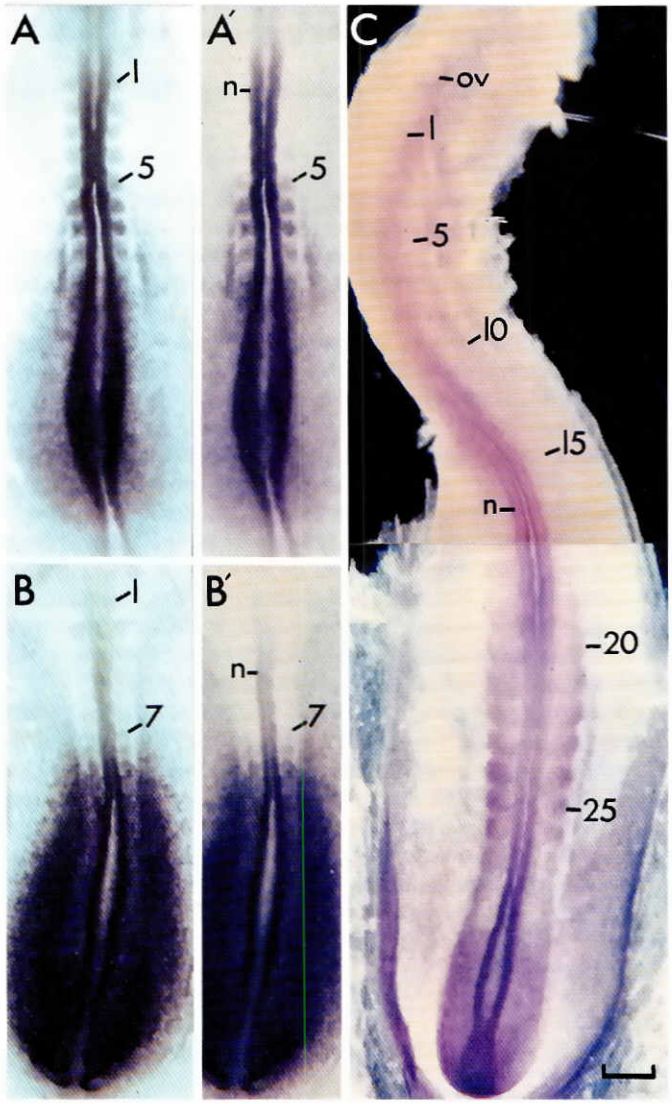

Fig. 2. *Hoxb-3* (A,A′), *a-4* (B,B′) and *c-6* (C) expression detected respectively in 9-somite, 10-somite, and 29-somite chicken embryos. *Numbers denote somite addresses; ov, otic vesicle; n, neurectoderm (neural tube). Embryos are shown viewed by brightfield illumination* **(A,B)**, *by darkfield illumination* **(C)**, *and by darkfield illumination incorporating a groundglass diffuser to enhance contrast between stain and tissue* **(A′,B′)**. *Bar, 0.25 mm.*

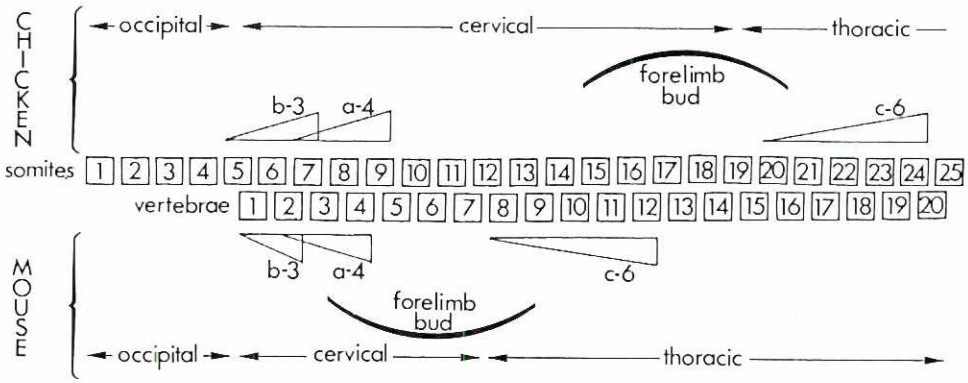

Fig. 3. The relationship between somites, vertebrae, and *Hoxb-3, a-4* and *c-6* expression in chicken and mouse. *Each vertebra forms from the posterior part of one somite and the anterior part of the next (Bagnall et al., 1988). For the first vertebra, this is posterior somite 5 and anterior somite 6 (Couly et al., 1993). Wedges show the increasing abundance of transcripts, detected in somites (chicken) and prevertebrae (mouse, Gaunt et al., 1988; Sham et al., 1992), at the anterior boundaries of the Hox expression domains. The somites (myotome component) and adjacent lateral plate mesoderm that contribute to mammalian (Patten, 1958) and chick (Chevallier et al., 1977) forelimbs are also indicated.*

example, being associated with an anterior cervical phenotype; *Hoxc-6* being associated with an anterior thoracic phenotype). The results further suggest that an evolutionary change in body proportions, such as a lengthening of the neck in birds, may apparently be effected simply by a shift in the relative positions of Hox expression domains established early in embryogenesis.

While the above interpretation can readily be applied to neck length in birds, where there is species-variation in the number of cervical vertebrae, it is less clear at present that it can similarly be applied to evolutionary changes in mammalian neck length (where the number of cervical vertebrae remains constant at seven). An evolutionary lengthening of the mammalian neck also, presumably, requires a shifting apart of the *Hoxb-3/a-4* and *Hoxc-6* expression domains, but it is possible that this shift occurs later in embryogenesis, by a process of enhanced growth within cervical somites and vertebrae. Two distinct mechanisms might therefore exist for the shifting apart of Hox expression domains during morphological evolution: a shift during first establishment of the domains, and a later shift due to enhanced tissue growth. The possibility is not excluded that both types of shift may in fact be due to enhanced tissue growth (distinction between the shifts would then lie in whether enhanced growth occurs in pre- or post-somitic mesoderm), but in what follows it is assumed that this is not the case, and that *Hoxc-6* boundaries in chicken and mouse do indeed form at initially different positions.

The molecular processes that initially set boundaries of Hox expression are, with some possible exceptions in the hindbrain (Sham *et al.*, 1993), not understood. Formation of somites along the anterior-to-posterior axis takes place sequentially in time, and it therefore seems probable that the definitive boundaries of Hox gene expression, formed within the presomitic mesoderm, are also established sequentially in time along the body. What might be the mechanism by which the initial expression domain of a Hox gene is shifted in its position, changing, for example, the length of the

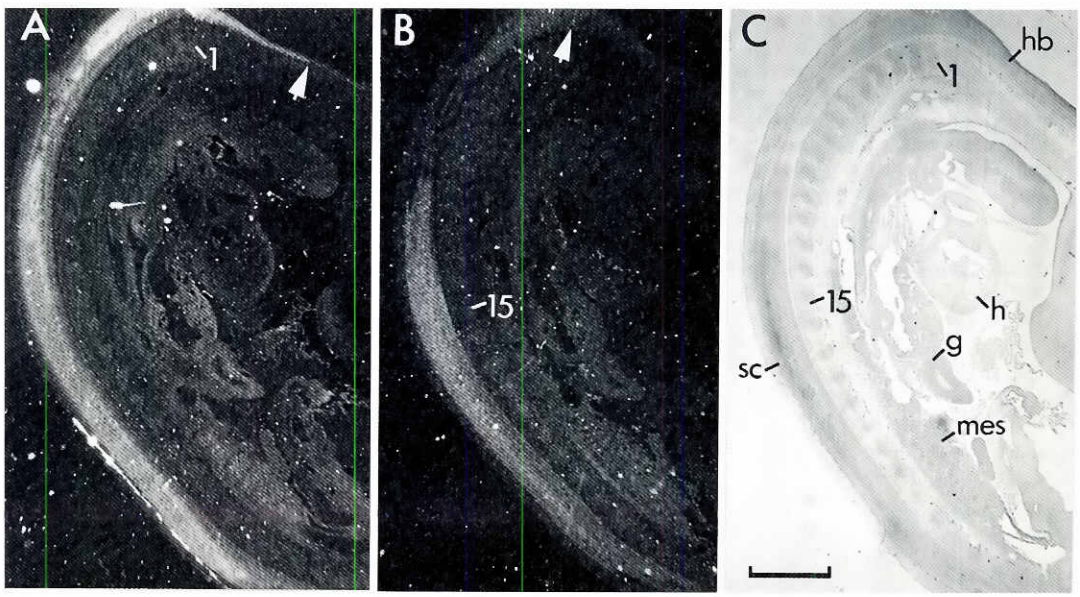

Fig. 4. Hoxb-3 (A) and c-6 (B) expression detected on nearby parasagittal sections from a $5^{1/2}$ day chicken embryo. (A and B) *Dark-field;* **(C)** *bright-field illumination. Numbers denote prevertebral addresses. Arrows indicate the anterior boundaries of expression in the nervous system. hb, hindbrain; sc, spinal cord; mes, mesonephric kidney; g, gut; h, heart. Bar, 1 mm.*

neck during evolution in birds? Two alternative possibilities are now suggested. First, in the context of proposals first made by Duboule and coworkers (e.g., Izpisúa-Belmonte *et al.*, 1991), a posterior shift might primarily be due to a delay in the expression of the Hox gene, so that its anterior boundary becomes established at the level of a later-forming (more posterior) somite. As a second possibility, however, the position of a Hox boundary might primarily be set by signalling molecules diffusing within presomitic mesoderm from the vicinity of, for example, the Hensen's node region. In terms of this second hypothesis, an evolutionary shift in Hox expression could be mediated by a change in either the production, transmission, or reception of such a signal.

Experimental Procedures

cDNA clones were isolated by low stringency screening of a 10-day chicken embryo library (Clontech) with a mouse *Hoxa-3* homeobox probe (McGinnis *et al.*, 1984). Inserts were subcloned into Bluescript KS⁻ (Stratagene), and were sequenced in a series of primer walks using Sequenase Version 2.0 (United States Biochemical). Nucleotide sequences of the clones identified as chicken *Hoxb-3* and *c-6* are deposited in the EMBL Data Library (accession numbers X80113 and X80114 respectively). A third clone was identified as chicken *Hoxa-4* by its identity with the published sequence (Sasaki *et al.*, 1990).

Whole-mount *in situ* hybridization was carried out as described by Conlon and Rossant (1992) using digoxygenin-labeled riboprobes (prepared as described by the manufacturers of the labeling kit, Boehringer Mannheim). *In situ* hybridization to sections, using ^{35}S-labeled riboprobes, was as performed by Gaunt *et al.* (1988). The nucleotide sequences used as probes were the same in both procedures. *Hoxa-4* probe corresponded to residues 1399 to 1925 in the published sequence (Sasaki *et al.*, 1990). Taking the first residue of the homeobox as base 1, *Hoxb-3* probe corresponded to bases 350 to 976 (a HincII fragment), and *Hoxc-6* probe to bases -21 to +414.

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