Spatio-temporal gene expression analysis from 3D in situ hybridization images

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Chapter 5

Gene expression and digit homology in the chicken wing.

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Case study: late zebrafish and cross species development

ABSTRACT

The bird wing is of special interest to students of homology and avian evolution. Fossil and developmental data give conflicting indications of digit homology if a pentadactyl ‘‘archetype’’ is assumed. Morphological signs of a vestigial digit I are seen in bird embryos, but no digit-like structure develops in wild-type embryos. To examine the developmental mechanisms of digit loss, we studied the expression of the high-mobility group box containing *Sox9* gene, and bone morphogenetic protein receptor 1b (*Bmpr-1b*), markers for precondensation and prechondrogenic cells, respectively. We find an elongated domain of *Sox9* expression, but no *Bmpr-1B* expression, anterior to digit II. We interpret this as a digit I domain that reaches precondensation, but not condensation or precartilage stages. It develops late, when the tissue in which it is lodged is being remodelled. We consider these findings in the light of previous Hoxd-11 misexpression studies. Together, they suggest that there is a digit I vestige in the wing that can be rescued and undergo development if posterior patterning cues are enhanced. We observed *Sox9* expression in the elusive ‘‘element X’’ that is sometimes stated to represent a sixth digit. Indeed, incongruity between digit domains and identities in theropods disappears if birds and other archosaurs are considered primitively polydactyl. Our study provides the first gene expression evidence for at least five digital domains in the chick wing. The failure of the first to develop may be plausibly linked to attenuation of posterior signals.

INTRODUCTION

The homology of avian wing digits is of interest to palaeontologists and evolutionary biologists because it bears on the important question of the evolution of birds. Digit homology is also important for experimental developmental biologists who use the phenotypes (‘‘identities’’) of digits as markers of position along the anteroposterior axis of the limb. Furthermore, because the wing is a well-studied experimental model, it provides opportunities to explore the mechanistic basis of developmental homology. Finally, the history of ideas about the avian wing presents us with an unrivaled catalog of archetypes and recapitulation. Our aim in this article is not to overturn current hypotheses of avian phylogeny, but to ask how developmental mechanisms were modified in the transition from the presumed ancestral state. We also wish to ask whether alternative hypotheses of avian digit homology have been overlooked. We begin by reviewing some of the relevant evolutionary and developmental issues. (Note: all references to ‘‘digits’’ in this article are to those of the forelimb, unless otherwise stated.)

Avian phylogeny

Archaeopteryx lithographica (Fig. 1) has always been central to the debate about avian origins because of its historical fame as a ‘‘missing link,’’ and its possession of a mosaic of avian and more inclusive theropod characters (Ostrom 1976; Christiansen and Bonde 2004). Cladistic analyses support the hypothesis that birds belong to the ‘‘Coelurosauria,’’ a clade of theropod dinosaurs (Gauthier 1986; Sereno 1999). Recent discoveries of nonavian maniraptorans with feathers or feather-like coverings (e.g., *Protarchaeopteryx robusta* and *Caudipteryx zoui*; Ji et al. 1998, 2001; Zhou et al. 2003) support the inclusion of birds in Theropoda. The fossil *Rahona ostromi* also shows a mosaic of avian and theropod features, and phylogenetic analysis places it as a sister species to *Archaeopteryx* (Forster et al. 1998). Although other ancestries for birds have been proposed (notably the thecodont and crocodilian hypotheses; Tarsitano and Hecht 1980; Hecht and Tarsitano 1982; Thulborn and Hamley 1982; Hecht 1985; Moinar 1985; Walker 1985), we will adopt here the consensus view that birds are theropods (Fig. 1).

Digit position and digit phenotype

Digits are traditionally assigned Roman numerals I–V, with reference to a pentadactyl archetype (Fig. 2). These designations have two different meanings: as positional references, the terms I–V describe the spatial relations of digits along the anteroposterior axis of the limb. By contrast, the phenotypes or identities I–V apply to different complexes of morphological characters that relate to the skeleton of a digit. The danger of having one numbering system with two meanings is that circular arguments may be developed about digit homologies that are not independent. Furthermore, it has been questioned whether characters such as digit shape and phalangeal formula can in fact be used to ‘‘identify’’ homologous digits in different species (Goodwin and Trainor 1983). In birds, we are faced with uncertainty both about the positional homologies of the wing digits (because only four distinct digits are seen in the wing), and also their phenotypes (because phalanges and other structures may have been lost or remodeled during evolution).

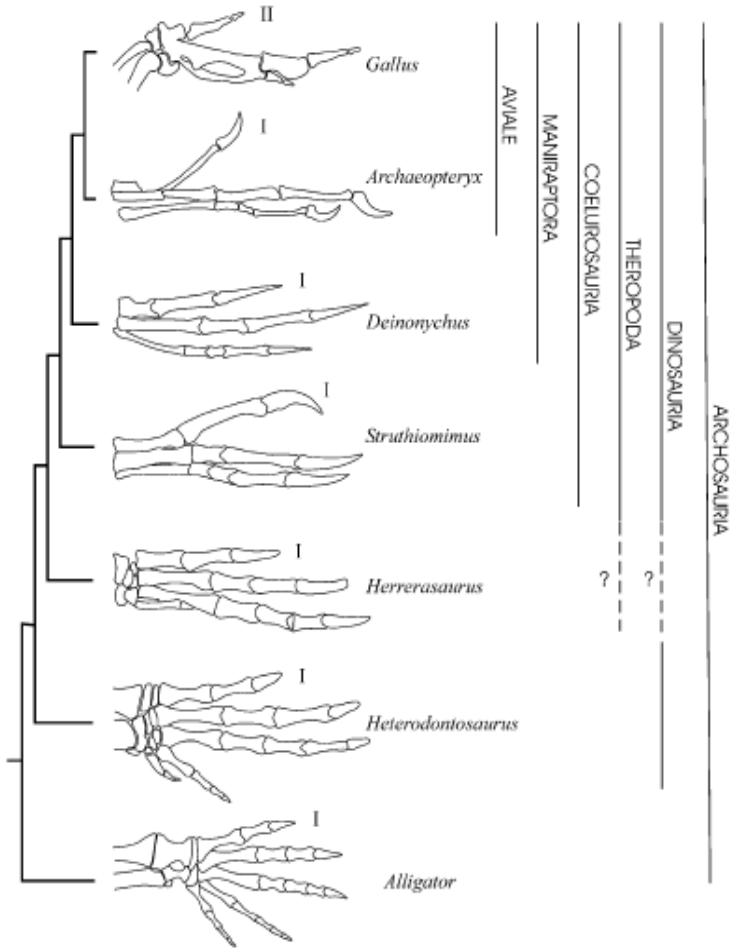


Fig. 1. Schematic illustration of some hand elements in archosaurs (anterior is to the top; some images were inverted to make comparison easier). The phylogeny is modified from Gauthier (1986) and Sereno (1999). The anterior digit is labeled with I or II according to current consensus on digit homologies. Schematic illustrations of the manus are shown and the sources for the drawings are: *Gallus gallus* (Yasuda 2002); *Archaeopteryx lithographica*, ''Maxberg'' specimen (Wellnhofer 1985); *Deinonychus antirrhopus* (Ostrom 1969); *Struthiomimus altus* (Osborn 1916); *Herrerasaurus ischigualastensis* (Sereno 1993); *Heterodontosaurus tucki* (Santa Luca 1980); *Alligator mississippiensis* (Gegenbaur 1864). Names of clades are indicated on the right. The value of the infra-order ''Coelurosauria'' is questioned by Carroll (1987). There is uncertainty, indicated in this figure by the question mark (?), about the taxonomic position of *Herrerasaurus* (Padian et al. 1999; Sereno 1999).

Positional homologies and the problem of vestigial digits

The homologies of cartilaginous and precartilaginous elements at the postaxial margin of the chicken embryo wing are a puzzle, but a plausible vestige of metacarpal V can be identified in alcian blue whole mounts (Montagna 1945; Burke and Feduccia 1997). Hinchliffe and Hecht (1984) identified an intriguing triad of postaxial elements: (a) the vestigial metacarpal V, lying laterally along the proximal part of metacarpal IV, and becoming reduced or disappearing completely; (b) the elongated pisiform, lying at the

lateral border of the wing, near the palmar aspect of the carpus; and (c) an element ''X,'' possibly an avian apomorphy, that lies near the proximal end of metacarpal IV and near the palmar aspect of the carpus, and that may persist to adulthood (see also Montagna 1945). Element X was formerly identified as an extension or process of the pisiform (Montagna 1945; Hinchliffe 1977). Part of element X was described by Montagna (1945) as fusing with his ''centrale IV,'' whereas another part was said to persist in the adult wing as a tuberosity on metacarpal III. Histogenesis at the pre-axial margin is less clear, despite numerous studies (the classical literature is reviewed by Holmgren 1933; Montagna 1945). Labelling for early cartilage matrix with $35SO_4$ (Hinchliffe 1977) fails to find a vestigial digit I. However, a condensation for distal carpal I was claimed by Montagna (1945). Recently, a small avascular zone (Kundrát et al. 2002), cell condensation (Kundrát et al. 2002; Larsson and Wagner 2002), or small cartilage nodule (Feduccia and Nowicki 2002), next to digit II, has been cited as evidence of a digit I vestige in *Gallus gallus* and *Struthio camelus* (the ostrich). There is a tuberosity for attachment of *extensor carpi alualae radialis longus* and *ligamentum elasticum prepatag*iale at the base of metacarpal II (Yasuda 2002). This tuberosity has been interpreted as evidence of a vestigial digit I, although this interpretation has been contested (Montagna 1945). A finding of great significance comes from studies of chicken wings experimentally infected with *Hoxd-11*-encoding retroviral constructs. A supernumerary digit, resembling wildtype digit II, develops in the vestigial digit I position in some cases (Fig. 4F in Morgan et al. 1992). This suggests that a digit I domain exists in that position, and can be rescued if the tissue is experimentally posteriorized. Naming a vestigial anterior digit in the chick as ''digit I'' assumes that ancestral digits have been correctly identified. Basal archosaurs are thought to show a trend toward reduction of digits IV and V (Romer 1956; Wagner and Gauthier1999). Thus the manus of the dinosaur *Herrerasaurus ischigualastensis*, possibly a basal theropod, shows reduced digits IV and V (Sereno 1993). The vestigial digits are positioned toward the palmar surface of the manus (Fig. 1). This and other evidence can be used to reconstruct a scenario in which the reduction of digits IV and V in basal archosaurs is continued in the lineage leading to birds (Fig. 1; see also Gauthier 1986; Wagner and Gauthier 1999). Debates about the taxonomic position of *Herrerasaurus ischigualastensis* (Sereno 1993; Padian et al. 1999; Sereno 1999; Galis et al. 2003; Larsson and Wagner 2003) do not completely overturn these arguments because crocodilians also show reduction of digits IV and V. For example, in the extant *Crocodylus porosus* (estuarine crocodile), digits I–III are prominently clawed, whereas IV and V are much smaller (Kükenthal 1893).

Phenotype homologies (identity) of avian digits

What are the phenotypic homologies of the three avian wing digits? Considerations of phalangeal formula, and the presence of a semilunate carpal (assumed to represent fused distal carpals I and II) have been used among other characters to assign the phenotypes I–II–III to avian digits (Wagner and Gauthier 1999; Chatterjee 2004). Adult birds commonly have an ossified phalangeal formula of 1–2–1 (our unpublished observation on specimens in the National Museum of Natural History/Naturalis, Leiden, The Netherlands), although it is always possible that tiny distal phalanges have been lost in preparation. A phalangeal formula of 2–3–3 is reported for goose embryos (*Anser sp*.; Schestakowa 1927) and 2–3–1 for adult ostriches (*S. camelus*; Kundrát et al. 2002). A

formula of 2–3–2 is reported for *G. gallus* by some authors (Chamberlain 1943; Yasuda 2002). In summary, modern birds have a lower phalangeal count for at least the third digit than nonavian theropods, where the formula is typically 2–3–4 (Wagner and Gauthier 1999). Two models have recently been proposed to account for these data. Both models assume that birds retain digit positions II–IV of nonavian theropods, and both acknowledge the incongruity that the phenotypes correspond to those of digits I–III (Fig. 2). The Frame Shift model solves the problem by proposing that embryonic digit domains II–IV have undergone a homeotic transformation so as to adopt the more anterior phenotypes of I–III (Wagner and Gauthier 1999). The solution presented by the Pyramid Reduction hypothesis (Kundrát et al. 2002) is that avian wing evolution involved bilateral loss of digits, and that the remaining central digits (II–IV) have simply been remodelled during evolution, with attendant loss of phalanges. They have thereby converged on the I–II–III phenotype (Fig. 2).

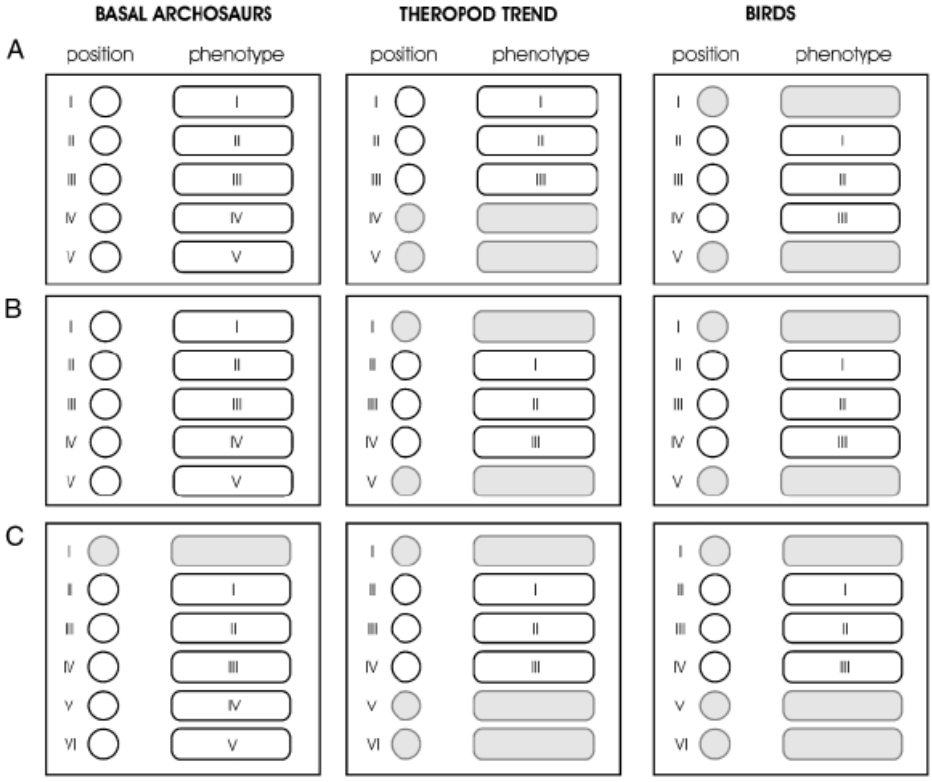


Fig. 2. Schematic representation of some models that attempt to correlate digit position with digit identity in birds. The adult situation is shown, and grey shading indicates that a particular digit is not visible in the adult (but may be present as a vestigial embryonic domain, as is likely the case for positions I and V for birds). (A) Frame Shift model, in which the digital domains take on new phenotypes (Wagner and Gauthier 1999). (B) Bilateral reduction of digits I and V, and convergence of the remaining central digits on a I– III phenotype (Kundrá t et al. 2002). (C) A model that rejects the pentadactyl archetype in favour of hexadactyly. The attraction of this model is that there is continuity of digit position and phenotype across the phylogeny. The disadvantage is that there is no direct evidence in its support.

The primary axis

One further line of evidence for the homology of chicken embryo wing digits comes from the time-sequence in which cartilage elements differentiate in the embryo, and their spatial relations with each other as they develop (Holmgren 1933; Shubin and Alberch 1986). In tetrapod embryos, alcian blue preparations show characteristic arrangements and staining intensities of limb cartilage elements. At certain stages, a chain of precociously differentiated elements may be seen running proximodistally along the limb-the primary axis of Burke and Alberch (1985).

The primary axis is presumably an expression of heterochronies in the underlying patterning mechanisms active at earlier stages. It is unlikely that the axis itself dictates the course of digit development because it only becomes visible after positional values have been encoded in a particular mesenchymal cell population (Wolpert and Hornbruch 1990; Cohn et al. 2002). Extensive surveys (Holmgren 1933; Shubin and Alberch 1986) show that the primary axis in the forelimb passes through the humerus, ulna, ulnare, distal carpal IV (if present), and digit IV. This pattern is seen in all five-fingered tetrapods except urodeles, where the axis passes through digit II. Birds show a primary axis passing through digit IV, so as to make the three bird digits II–III–IV (Burke and Feduccia 1997).

Developmental mechanisms and digit homology.

Many recent studies have expanded our knowledge of the molecular mechanisms of limb patterning (reviewed by Sanz- Ezquerro and Tickle 2003). Studies on *sonic hedgehog* (*Shh*) cast doubt on previous ideas about digit identity being a simple readout of a morphogen gradient (Yang et al. 1997; Ahn and Joyner 2004). In fact, patterning appears to be much more complex, with distinct phases. The same genes may have different roles at early stages, when broad domains are established in the limb (Dudley et al. 2002; Richardson et al. 2004) and later stages, when digits differentiate according to particular identities. Members of the bone morphogenetic protein (BMP) family of growth factors are involved both in the formation of prechondrogenic condensations and their later differentiation (Pizette and Niswander 2000). The later actions of *Bmp2* could include the specification of digit identity itself (Yang et al. 1997). The complexity of interactions, and lack of true independence between patterning mechanisms, are among possible objections (Feduccia 1999; Kundrát et al. 2002) to the Frame Shift hypothesis (Wagner and Gauthier 1999); thus, the Frame Shift might require a whole suite of mechanisms to be modified in concert to yield a phenocopy of the nonavian theropod digits. Lack of independence between mechanisms has been shown using loss-of-function studies in mice (Zákány et al. 1997). These suggest that posterior *Hox* genes regulate both the number of initial primordia formed, and the subsequent shapes of the digits. Further studies suggest that there are two critical phases of posterior *Hox* gene expression in the mouse, the first establishing anteroposterior polarity and the second involved in the readout of digit identities; in this model, posteriorly expressed *Shh* acts as a relay between the two phases (Zákány et al. 2004). The time of action of *Shh* has been analyzed in *Shh* homozygous mutant mice, leading to the suggestion that the proximal limb elements are already specified at early stages, and have normal polarity; but successively more distal elements require *Shh* both for their initial specification, and for the establishment of normal phenotype (Chiang et al. 2001). In support of the Frame Shift

hypothesis, digit primordia can undergo anterior transformation when BMP signaling is attenuated by local implantation of Noggin (Dahn and Fallon 2000). These studies also support the idea that digit phenotype is not irreversibly fixed at the early condensation stage. Time of exposure to *Shh* may also be important (Harfe et al. 2004), raising the possibility that a Frame Shift could be based on heterochrony (for more on heterochrony in limb diversification, see Blanco et al. 1998; Blanco and Alberch 1992).
Digit II specification in particular is thought to be dependent on a low threshold of *Shh* exposure (Harfe et al. 2004), and digits can undergo transformations of identity when *Hox* genes are misexpressed (e.g., the posterior transformation seen in birds injected with the RCAS-Hoxd-11 retroviral construct; Morgan et al. 1992). The Pyramid Reduction model (Kundrát et al. 2002), and its attendant loss of phalanges, can be considered in the light of studies on phalanx development. The number of phalanges on each digit may be controlled in part by the duration of limb outgrowth. In the dolphin flipper, there is evidence that prolonged outgrowth on digits II and III selectively leads to hyperphalangy (Richardson and Oelschlaeger 2002). Termination of outgrowth, and therefore formation of the distal phalanx, may be signaled by disappearance of *Fgf8* expression in the apical ectodermal ridge (Merino et al. 1998).

Aims and objectives
We have investigated these issues using whole-mount *in situ* hybridization, and alcian blue staining for hyaline cartilage matrix. Markers of early digit formation included *Sox 9*, and *BMP receptor 1B* (Merino et al. 1998; Pizette and Niswander 2000; Karsenty and Wagner 2002; Chimal-Monroy et al. 2003). The *Sox9* gene belongs to the high-mobility group (HMG) box superfamily of DNA-binding proteins, and is one of the earliest markers of limb mesoderm destined to form cartilage (Chimal-Monroy et al. 2003). It is probably a differentiation factor, and not a patterning molecule, being expressed in cells that have already been patterned with respect to the limb axes, but have not yet started to condense and differentiate (Akiyama et al. 2002). Expression has been shown to be detectable in the stage 22 chick limb (Healy et al. 1999). *Bmpr-1B*, a secreted protein receptor, follows the expression of *Sox9* closely (Healy et al. 1999; Chimal-Monroy et al. 2003). It is expressed in prechondrogenic aggregates, immature chondrocytes, and perichondrium (Pizette and Niswander 2000). *Bmpr-1B* expression has been reported in the chick limb at stage 24 (Merino et al. 1998; Healy et al. 1999). We also examined the expression of *Wnt-14*, a relatively late marker that shows hybridization to interdigital mesenchyme and future joints (Hartmann and Tabin 2001).

MATERIALS AND METHODS

Chicken embryos (*G. gallus*) of stages 26–33 (Hamburger and Hamilton 1951) were used. These stages cover the principal phases of digit formation (Sanz-Ezquerro and Tickle 2003). Limbs were dissected from embryos ranging from 4.5 to 8 days and processed for mRNA *in situ* hybridization. cDNA clones for *Wnt14, Bmpr- 1B*, and *Sox9* were kindly provided, respectively, by C. Tabin, L. Niswander, and J. M. Hurle. Anti-sense and sense RNA probes were synthesized and labeled with digoxigenin (Roche Diagnostics GmbH, Penzberg, Germany). Sense RNA probes were used as negative controls. *In situ* hybridization was performed on the dissected chick limbs according to

standard protocols (Wilkinson 1998). Samples were treated with concentrations of proteinase K ranging from 10 mg/ml for stages 25–26 to 50 mg/ml for stages 31–34 for 10–15min at room temperature. Hybridization was performed at 65–68*1*C in 50% formamide. Color reactions were developed with NBT/BCIP substrate (Roche).
To further characterize patterns of *Sox9* and *Bmpr-1B* expression, stage 30 and 31 wings from the embryos hybridized as above were embedded in Technovit 7100 (Heraeus Kulzer Gmbh, Wehrheim, Germany). Transverse sections of 10 mm were cut on tungsten knives, and counterstained with Neutral Red. Three-dimensional computer reconstructions were made as previously described (Verbeek 2000). The onset of alcian blue staining (whole limbs were stained with 0.3% in acid alcohol), and of gene expression, were recorded for each skeletal element in the chick fore- and hindlimbs. The sequences were then compared.

Homology of gene expression domains
A major problem in comparing patterns of developmental gene expression is that early expression domains may initially represent primordia of several adult bones, and then segregate into individual bone primordia. Thus there is no direct mapping of domains onto single named organs. We therefore have had to designate each domain in terms of its daughters. To make this designation, we mapped the expression domains in processed whole mounts that were then stained with alcian blue, dehydrated in ethanol, and cleared in methyl salicylate.

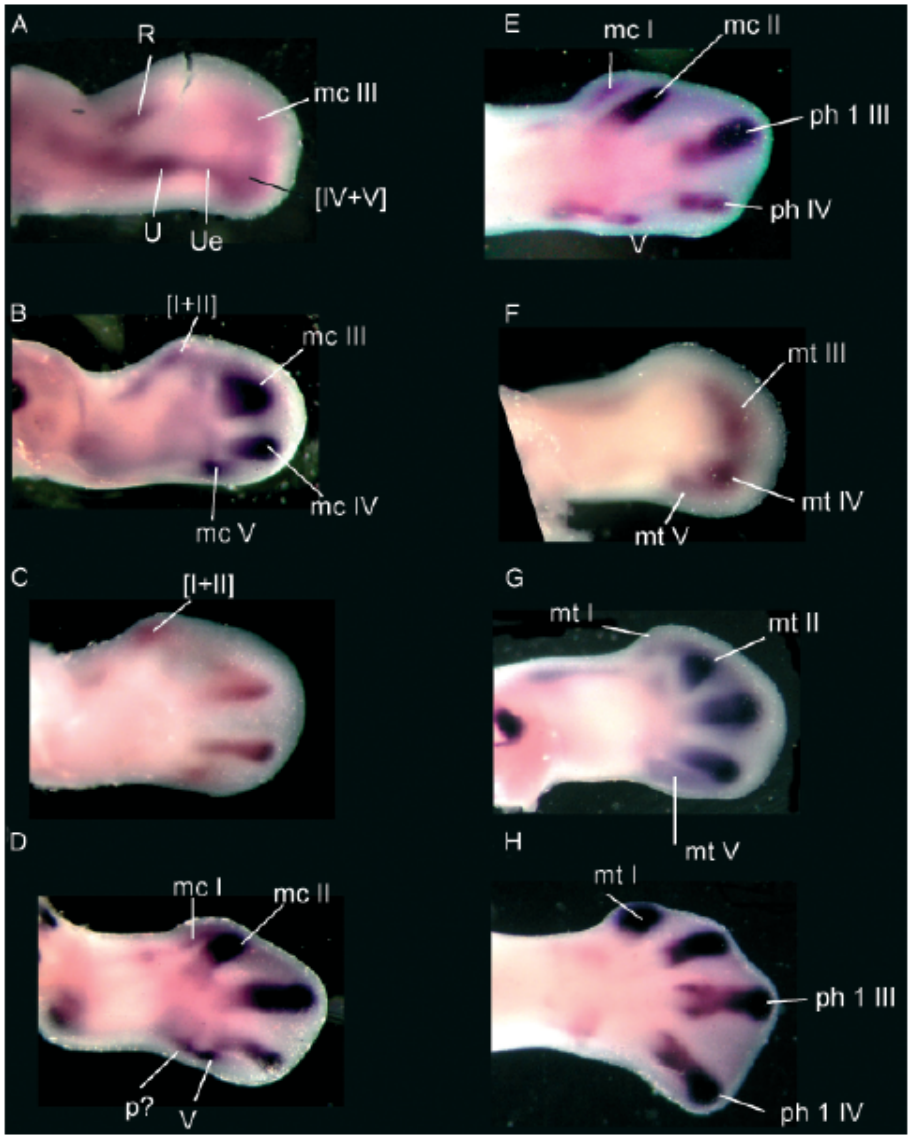
RESULTS

Spatial patterns of gene expression
General points
Sox9 and *Bmpr-1B* were only expressed transiently in the developing cartilage elements, and so they were not enduring markers (as alcian blue is). Thus, we only saw a primary axis in certain early stages (Fig. 3A). Later, however, the proximal elements no longer expressed the molecular markers we used, and so no continuous primary axis was seen along the full proximodistal extent of the limb. No evidence was seen of budding or branching of elements, only the segregation of discrete subdomains from within a common domain (Note: square brackets indicate a common domain for the elements enclosed).

Sox9 expression in the wing
Sox9 was expressed in the elements of the stylopodium, zeugopodium, and digital arch at stages 25 and 26. The digital arch appeared to consist of two separate domains: one for [digit V1IV], and one for the anterior digits (Fig. 3A; although we use the term “digit”, we cannot say without cell marking experiments whether the domain encompasses the entire presumptive digit, or only its metacarpal). At stages 27 and 28, strong expression patterns of *Sox9* could be observed in the three separate digit III, IV, and V domains. Anterior to digit III, another expression pattern was visible, which might be interpreted as a common domain for digit I and II anlagen (Fig. 3B). This domain appeared to divide into two at stage 29 (Fig. 3D). One daughter, which showed strong expression, was for the digit II metacarpal. The other was weaker, and we identify it as a presumptive digit I

metacarpal domain; at no stage was it resolvable into separate bones. It was lodged in the flange of tissue along the anterior margin of the wing that becomes thinned during formation of the prepatagium (Murray and Wilson 1994). A putative pisiform showed hybridization at stage 29 (Fig. 3D). Wings from stage 30 onward showed strong *Sox9* expression patterns for the posterior four digits. The weak *Sox9* domain for digit I was now very distinct, and quite elongated, and lay anterior to digit II, separated from it by a clear interdigital space (Fig. 3E). It bore a striking resemblance to the vestigial digit V domain of *Sox9* expression in the chicken foot (Fig. 3G, see below). Transverse sections through the wing (Fig. 4, B and C) showed that the *Sox9* domain for digit I was close to the ectoderm, on the palmar side of the autopodium, and in alignment with the other four digits, like the avascular zone described previously (Kundrát et al. 2002). This expression pattern was consistent in the eight embryos studied at this stage. At stage 31, hybridization became weaker and at stage 32 was no longer visible. From stage 29, *Sox9* was expressed strongly in the interzones of future joints as previously reported (Hartmann and Tabin 2001; Karsenty and Wagner 2002). At stage 30, the putative pisiform was seen to lie lateral to the ulnare; element ‘‘X’’ was visible between metacarpal V distally, and the pisiform proximally, but lying in a more ventral plane (Fig. 4, E and G).

Fig. 3. (A–E) Left chicken wings stage 26–30, after *in situ* hybridization with *Sox9* probe (anterior is to the top, ventral aspect). (F–H) Left chicken hindlimbs stage 26, 27, and 30, respectively, *Sox9* probe. Anterior is to the top, ventral aspect. Roman numerals, digit or metacarpal number; mc, metacarpal; R, radius; U, ulna; Ue, ulnare; ph, phalanx; p?, pisiform?; [mc IV1V], common expression domain for metacarpals IV and V. Some images were inverted to make the orientation consistent.



Sox9 expression in the foot

In the foot (Fig. 3, F–H), expression of *Sox9* showed a pattern similar to that in the wing. A significant difference was that we saw no common domain for digits I and II, as we had seen in the wing. Instead, the foot digit I and II domains appeared to develop as separate domains from the outset. Interestingly, the vestigial foot digit (V) showed a weak, elongated domain of expression of *Sox9* at stage 27 (Fig. 3G). This domain therefore bore a close resemblance to that of the vestigial wing digit I (Fig. 3E). We saw no evidence for more than five digit primordia in the chick foot.

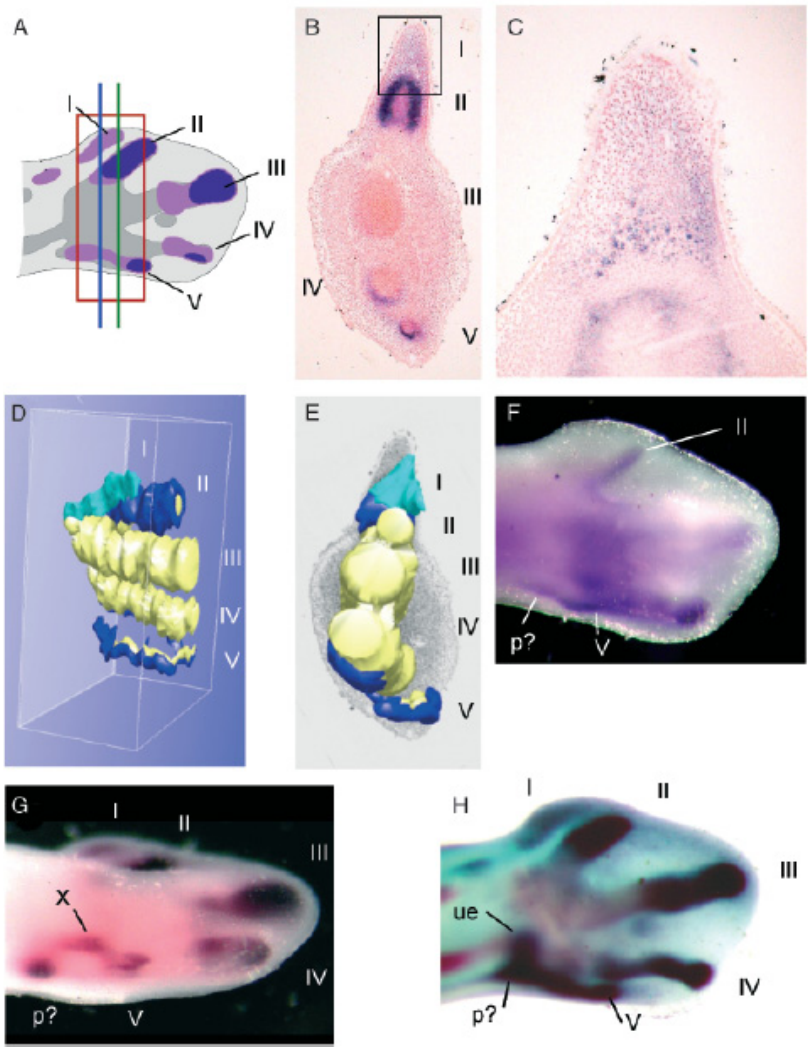


Fig. 4. (A) Schematic interpretation of the *Sox9* gene expression patterns superimposed on cartilage pattern (alcian blue/ in situ double stains).
Roman numerals, digits; vertical green line, plane of section in B; vertical blue line, plane of section in C; dark red box, area reconstructed in D, E.
(B and C) Transverse sections of wings (stage 30, hybridized with *Sox9* probe), neutral red counterstain. Sections from the same specimen, C more proximal than B. (C) Detail from boxed area in B, showing expression of *Sox9* in the noncondensed mesenchyme anterior to digit II. (D) Three-dimensional (3D) reconstruction of the same specimen. Yellow, cartilage; dark blue, gene expression digits II–V; light blue, *Sox9* expression, presumptive digit I. Anterior is to the top. Ventral view. (E) Proximal view of the 3D reconstruction. Anterior to the top. The element at the level ‘‘V’’ may consist of mc V1element X. (F) Left chicken wing, stage 30, *Bmpr-1B* probe. Anterior to the top, ventral view. Distinct prechondrogenic domains are seen in digits II–IV, but not anterior to digit II (labeled II). p?, pisiform. (G) Wing, stage 30, oblique posterior–ventral view, *Sox9* probe. X, element ‘‘X’’; p?, pisiform. (H) Wing, stage 30, overstained in NBT/BCIsubstrate after *Sox9* hybridization, then counterstained with alcian blue and cleared in methyl salicylate. p?, pisiform; ue, ulnare.

Bmpr-1B expression in the wing
The hybridization patterns followed the patterns of *Sox9* expression quite closely and appeared slightly later. At stage 30, no significant expression could be observed in the putative digit I region (Fig. 4F). *Bmpr-1B* expression showed a clear primary axis at stage 26, consisting of hybridization extending from the humerus, and through the ulna to a single common domain for digits IV and V (data not shown).

Bmpr-1B expression in the foot
The notable feature of *Bmpr-1B* expression was strong expression in the vestigial digit V, in contrast to the weak *Sox9* expression in this same digit. *Wnt14* (data not shown) expression was visible initially in the interdigital mesenchyme and along the distal margin of the digital plate. From stage 29 on, *Wnt14* was expressed in the interzones of future joints (Hartmann and Tabin 2001). *Wnt14* expression confirmed that no late digital structures, such as joints, were formed in the digit I Anlage.

Temporal analysis of gene expression during skeletal patterning and development
We made landscape maps showing the relative sequence in which the different cartilage elements were first distinct according to various markers (Fig. 5). As can be seen, there was the expected proximodistal gradient in the appearance of elements, and also some evidence of earlier differentiation along the primary axis. The most notable feature of these maps is that the presumptive *Sox9* digit I domain in the wing (Fig. 5, *Sox9*, wing, arrow) appeared relatively late in the developmental sequence. Thus the temporal homology of the anterior *Sox9* domain was consistent with predictions from the primary axis model, namely that digit I should develop late in the sequence.

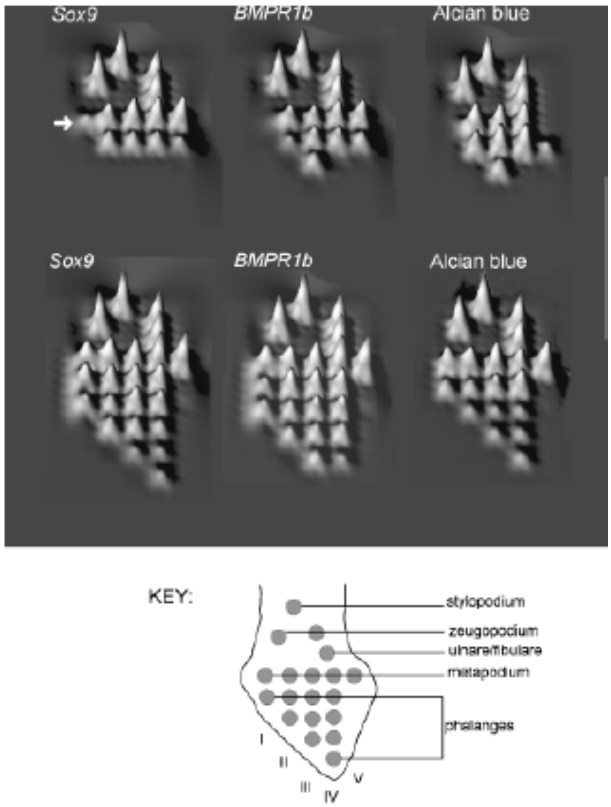


Fig. 5. Developmental timing landscapes showing the relative sequence in which various markers of skeletal formation appear. Schematic palmar views of right limb; proximal is to top, anterior to left (see key for orientation and position of elements). Each element was assigned a rank according to its place in the developmental sequence. The ranks were then inverted so that the earliest elements have a tall peak, and the latest elements have a low peak. Arrow, presumptive wing *Sox9* digit I domain.

DISCUSSION

We have shown evidence of a *Sox9*-expressing domain in the mesenchyme anterior to digit II, and separated from it by a nonexpressing zone. This *Sox9* expression domain was located at the palmar side of the hand, close to the ectoderm of the anterior margin. We suggest that it is reasonable to interpret this domain as being a vestige of digit I, and further argue, on the basis of serial expression patterns, that it splits away from a common digit I–II Anlage around stage 29. This pattern broadly conforms to the ‘‘digit I’’ condensation observed in tissue sections at this stage (Larsson and Wagner 2002). Furthermore, the avascular digit I zone (AZ-1 of Kundrát et al. 2002) resembles in location the digit I *Sox9* domain (compare their Fig. 1C with our Fig. 4B). Finally, the putative *Sox9* digit I domain develops last in the digit sequence, in agreement with the late development of digit I in pentadactyl amniotes (Shubin and Alberch 1986).

Mechanisms of digit reduction and vestige formation

Our study provides evidence that an extensive digit I domain exists in the precondensation limb mesenchyme, but fails to reach condensation and precartilage stages, as shown by lack of *Bmpr-1B* and *Wnt14* expression. This lack of differentiation could explain why no precartilage matrix was found in the putative digit I position by $35SO_4$ labeling (Hinchliffe 1985 book), and supports the idea that the digit I domain shows developmental arrest (Galis et al. 2003).The expression of *Sox9* in this domain is significant, because the gene is thought to be expressed after the initial patterning events have taken place (Akiyama et al. 2002). This supports the idea that in developmental terms, the wing is initially pentadactyl (Kundrát et al. 2002; Larsson and Wagner 2002; Galis et al. 2003). Some block must exist at later stages when digit morphogenesis normally takes place. It is significant that misexpression of *Hoxd-11* leads to formation of a supernumerary digit I in the chick wing digit I (Morgan et al. 1992). Our interpretation of these findings is that the digit I domain fails to develop because it does not receive adequate posterior signals during development; misexpression of *Hoxd-11*, a posterior *Hox* gene, provides those signals and rescues the vestigial digit. We find that other vestigial digits in the chicken show arrest at different points in the sequence of cartilage formation and differentiation (Table 1). Thus wing digit I arrests at the *Sox9*-expressing stage. Foot digit V develops further, expressing *Sox9* weakly, but then showing moderate *Bmpr-1B* expression and some cartilage differentiation. Wing digit V shows strong *Sox9* and *Bmpr-1B* expression, and some cartilage differentiation (summarized in Table 1). We saw no evidence in gene expression patterns for what other workers have described as budding or branching (Shubin and Alberch 1986; Garner and Thomas 2004).What we did observe was the establishment of discrete domains from within a common domain. We do acknowledge, though, that domains remain connected by bridges of less differentiated tissue, and this may create the impression of a branch.

Table 1. Summary of gene expression and development in normal and vestigial chicken digits

	Sox9	*bmpR-1b*	Alcian blue	Final form
Formed digits	+++	+++	+++	Fully developed
Vestigial digits				
Wing digit I	+	+/–	–	Absent
Wing digit V	+++	++	++ (small)	Greatly reduced metacarpal
Foot digit V	++	+++	++ (small)	Greatly reduced metatarsal

Note that the three vestigial digits in the chicken limbs are arrested at different points in their development and differentiation.

From approximately stage 28 onward, the interdigital region in the wing becomes greatly thinned, as does a flange of tissue along its anterior border, which becomes the prepatagium (see Figs. 3 and 4 in Murray and Wilson 1994). The *Sox9* domain for digit I is embedded in the flange, where it lies in close proximity to the ventral ectoderm (Fig. 4).

Frame shift and bilateral (''pyramid'') reduction hypotheses
Our findings cannot distinguish between the Frame Shift and Pyramid Reduction hypotheses because both of those models accept a vestigial anterior digit in the chick. We are impressed, however, by the rescue of the digit I domain in the chick by *Hoxd-11* misexpression (Morgan et al. 1992). This is consistent with the idea that a shift in anteroposterior positional signalling has occurred in the evolution of birds, such that digit I no longer receives an adequate threshold of posteriorizing signals. One could of course argue that the ''rescued'' digit I in those experiments was in fact a reduplicated digit II produced by localized mimicking of polarizing activity. However, because expression of the *Hoxd-11*-RCAS construct was ubiquitous in the limb bud, and not localized to the anterior border, we think this objection is unlikely. More recent studies reveal that *Hoxd-11* is expressed from the posterior margin of the autopodium to the cartilage condensation of digit II; it is not expressed in the anterior region of the autopodium (Nelson et al. 1996; Goff and Tabin, 1997). It has been shown that *Hoxd-11* ectopically affects condensation and segmentation of digit I (Goff and Tabin, 1997).

Alternative models
Alternatives to the Frame Shift and bilateral reduction models can be considered:
-The anterior vestige in the chicken embryo wing is not a digit but some other structure or primordium;
-Birds are not a clade within the theropods;
-Digit identities are not meaningful units of homology; rather, they are emergent patterns generated non-specifically by interactions between developmental mechanisms (Goodwin and Trainor 1983); and
-The pentadactyl ''archetype'' is false and the archosaur limb may in fact be primitively polydactylous.

We will discuss the polydactyly model in some detail, not because we consider it the most parsimonious explanation, but because it has scarcely been discussed in the context of avian evolution for many decades. It also has the unique virtue of providing continuity between digit position and digit identity across archosaur phylogeny. If the vestigial digit I domain of chicks is primitive for archosaurs, then the ''vestigial digits IV and V'' of *Herrerasaurus* and other archosaurs are in fact digits V and VI (Fig. 2). This would mean that birds could also have a vestigial digit VI as Schestakowa (1927) suggested. Element X or the pisiform are potential candidates for such a vestige. Bardeleben (1889) considered the pisiform of mammals to be a vestige of a sixth digit. This opinion was also held by Holmgren (1952), who viewed the tetrapod limb as primitively seven fingered, on the basis of his extensive developmental studies. Studies in other taxa predict that digital loss should be bilateral, affecting digit I as well as posterior digits (Alberch and Gale 1983). This has always made the asymmetric reduction in archosaurs (affecting digits IV and V) seem anomalous. However, if archosaurs are polydactyl, and have a vestigial digit I domain in their embryos, then there is no anomaly (Fig. 2). Polydactyly is not robustly supported at this time. Most evidence for digit I in birds, and for extra digits generally, is of the ''nodules and shadows'' type, where morphological vestiges in adults, or histological traces in embryos, are interpreted as recapitulated digits. Other difficulties with a polydactyly theory are: embryos from non-avian theropods are not available for

study; no adult archosaur has six distinct digits; there is no evidence for a vestigial digit I in archosaurs outside birds; and we saw no evidence of more than five digital domains of *Sox9* expression in the chick foot in this study.

Examples of supposed extra digital elements are seen in Batrachomorpha, and include the claimed ‘‘postminimus’’ in the pes of some salamanders (e.g., *Hynobius lichenatus*; Hasumi and Iwsawa 2004), and polydactyly in humans (Biesecker 2002). Late Devonian tetrapods were certainly polydactylous (Coates and Clack 1990) and the Early Carboniferous tetrapod *Pederpes finneyae* is speculated to have had a hexadactylous manus (Clack 2002). However, *Casineria kiddi*, possibly an early amniote, has a pentadactyl manus (Paton et al. 1999).

In summary, we have found molecular evidence of a digit I domain in the chicken wing that is specified by early patterning mechanisms, but fails to undergo terminal differentiation. In the light of previous studies where *Hoxd-11* was misexpressed, we suggest that the digit I domain can be rescued by increasing the strength of posterior patterning signals. Conflicts between fossil and developmental data can be eliminated by a Frame Shift, by bilateral reduction, or by assuming that archosaurs are primitively polydactyl. On the basis of current data, no one model of digit homology is more parsimonious than others.

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