

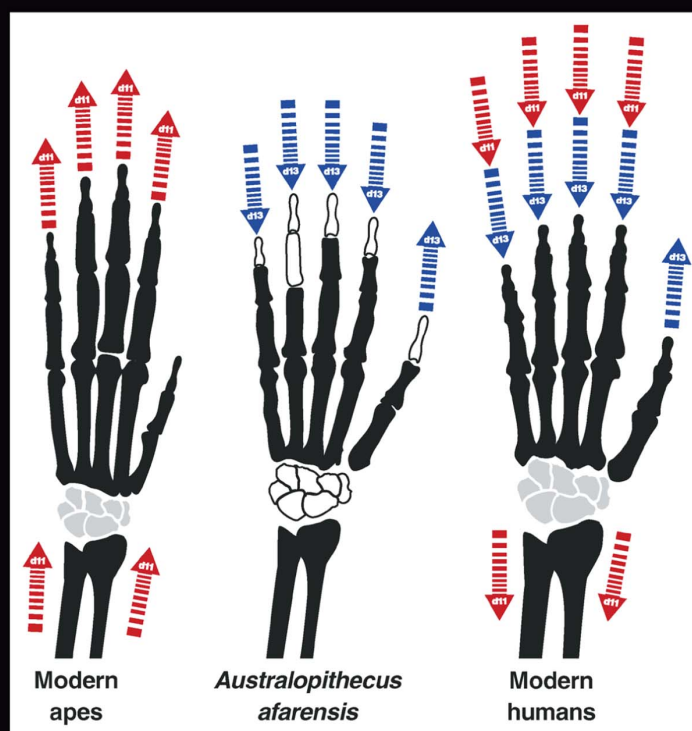
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Patterns of Correlation and Covariation of Anthropoid Distal Forelimb Segments Correspond to Hoxd Expression Territories

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ABSTRACT Anthropoids in general and hominoids in particular exhibit differential adaptations in forearm and digital skeletal proportions to a diverse array of locomotor modes. Hox genes act as selector genes with spatially regulated expression patterns during development. Their expression in the forelimb appears to define modules that specify differential skeletal growth. Here we explore forelimb skeletal proportions in a large sample of anthropoids from a background provided by Hoxd expression patterns in late-stage murine embryonic forelimbs. Interspecific correlation and principal components analyses of primate forelimb data indicate that morphological variation in anthropoids reflects well-defined developmental modules downstream of Hoxd expression. The phalanges of digit one appear to represent a single growth module, whereas the metacarpals and manual phalanges of the posterior digits correspond to a second, independent, expression territory that extends proximally into the distal zeugopod. In particular, hominoids show very high correlations among the posterior digits and the independence of digit one. In addition, the distal radius is generally highly correlated with the posterior digits and not digit one. Relying on established functional differences among Hox paralogs, we present a model that parsimoniously explains hominoid forearm and digital proportions as a consequence of downstream effects of Hox. We, therefore, suggest that Hox-defined developmental modules have served as evolutionary modules during manual evolution in anthropoids. *J. Exp. Zool. (Mol. Dev. Evol.)* 310B:240–258, 2008. © 2007 Wiley-Liss, Inc.

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The generalized primate hand consists of four posterior rays (referred to as medial in anatomical literature) of moderate length and which display substantial opposition to the pollex (i.e., thumb). This basal pattern has been substantially modified in several higher primates (Fig. 1). For example, within hominoids the length of the posterior digits (relative to body size) is greatest in highly suspensory gibbons and siamangs (Hylobatidae). Also relatively long are the posterior digits of orangutans (*Pongo*) and, to a lesser extent, those

of chimpanzees (*Pan*). Only the more terrestrial gorilla (*Gorilla*) displays digital proportions that are similar to the ancestral, cercopithecoid-like

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digital proportions displayed by the early Miocene hominoid genus *Proconsul* (Napier and Davis, '59). In this respect, the long digits of hylobatids, *Pongo* and *Pan* are derived for the Hominoidea. At the other extreme, the posterior digits of modern humans appear to be relatively short, even in comparison with those of Miocene hominoids.

The loss of an opposable first ray has occurred infrequently in anthropoid evolution. However, as their names imply, the New World monkeys (NWMs) *Ateles* and *Brachyteles* and the Old World monkey (OWM) *Colobus* possess hands that combine exceptionally long posterior metacarpals with pollical phalanges that are either exceedingly short or absent altogether (Fig. 1) (Straus, '42; Tague, '97). These genera rely on forelimb suspension during feeding and locomotion.

One of the most significant concepts to have emerged from developmental analyses of the last 20 years is that of "modularity" (Gilbert et al., '96; Wagner and Altenberg, '96; Raff, '96; Gerhart and Kirschner, '97; Winther, 2001; Schlosser, 2004). Modules constitute integrated series of relatively distinct, autonomous components that serve to partition and sequester different parts of the embryo, thus reducing the impact of any changes on the organism as a whole. The limb bud is an ideal example of such an autonomous structure (Raff, '96). This has been demonstrated by both limb transplantation and regeneration studies in urodele amphibians (Bryant et al., '81, '82; Bryant and Muneoka, '86). However, as is evident from the diverse array of limb specializations seen in vertebrates, the limb bud itself must be further subdivided into a number of smaller units also amenable to modular change.

Hoxa and *Hoxd* gene expression patterns demarcate boundaries of various overlapping developmental domains in the limbs of birds and mammals (i.e., Nelson et al., '96; Fig. 2). They initially function as selector genes that specify positional identity, localize the zone of polarizing activity and modulate cell adhesion and proliferation during specification of the limb skeleton (Yokouchi et al., '91; Davis et al., '95; Yokouchi et al., '95; Zákány et al., '97; Akam, '98; Pradel and White, '98; de la Cruz et al., '99; Zákány et al., 2004; Carroll et al., 2005). The first "phase" of Hox expression patterns the proximal part of the limb skeleton, including at least a substantial portion of the forearm (zeugopod) (Tarchini and Duboule, 2006). In an independently regulated, second phase of expression, *Hoxd* gene expression patterns the digits (Spitz et al., 2003). Although

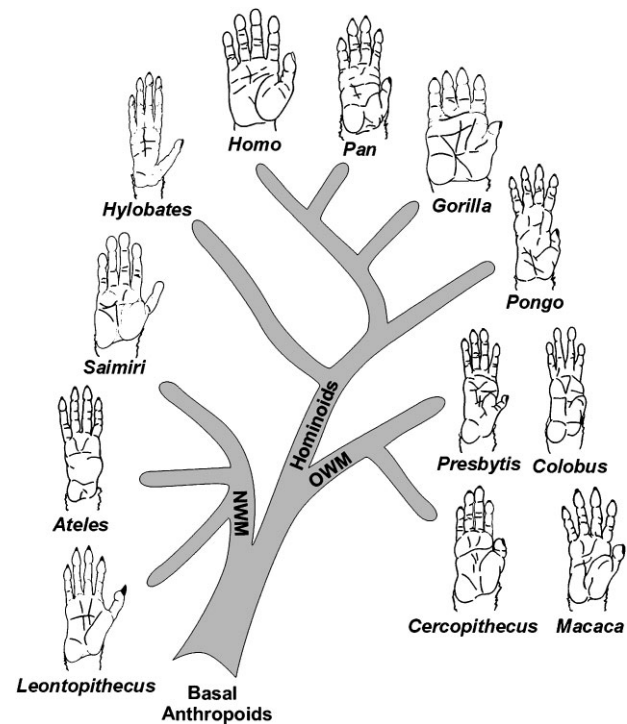


Fig. 1. A phylogeny of anthropoid hand evolution. New World monkeys (NWMs) likely diverged from the ancestral anthropoid stock during the late Eocene/early Oligocene, but fossil evidence of the earliest NWMs is sparse. On the basis of comparisons of extant taxa, ancestral NWM digit proportions appear to have been most similar to those of *Saimiri*. In comparison, the posterior digits of atelines and other suspensory forms are substantially elongated. Digit proportions in Callitrichids (e.g., *Leontopithecus*) are similar to the presumed ancestral condition. However, these "dwarfed" anthropoids have reevolved claws in place of nails, and have therefore been excluded from the present analysis. Fossil hominoids make their initial appearance at approximately 23Ma in the form of *Proconsulids* (not shown). Although extant hominoids (with the exception of humans) all display some suspensory habits and are characterized by long posterior digits, their proportions in early hominoids were more similar to those of ancestral anthropoids. Note that humans and gibbons are both distinguished by large thumbs. The radiation of OWMs is relatively recent. Fossil evidence suggests an initial divergence into Colobine and Cercopithecin forms around 10Ma. The posterior digits in the possibly ancestral OWM *Victoriapithecus* (not shown) are quite short, indicating a possible terrestrial adaptation similar to, and possibly retained by, many extant cercopithecines (e.g., *Theropithecus*, *Papio*, *Macaca*). In comparison, the posterior digits of extant colobines are relatively long.

considerable attention has been focused on the first and second phases of Hox gene expression in the limb, little is currently known about their substantially later expression and its effects on long-term growth during skeletogenesis. Nonetheless, numerous experiments suggest that Hox genes may function at later stages of development

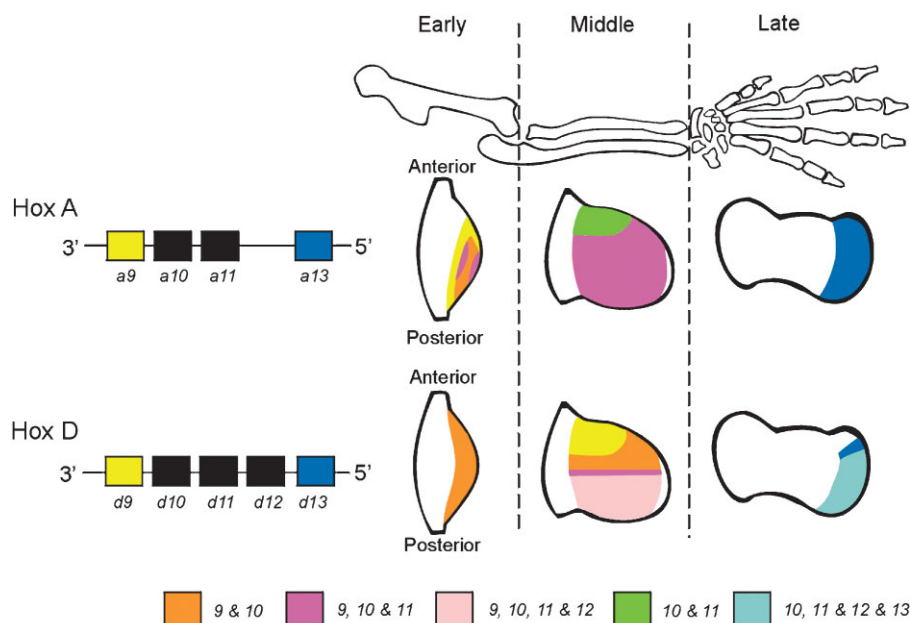


Fig. 2. Diagrammatic expression maps for 5' genes of the Hox A (top) and Hox D (bottom) clusters during early, middle and late stages of forelimb outgrowth. Isolated gene expression domains are coded on the left (genes with no singular expression are colored black). Combinatorial gene-expression domains are coded along the bottom. Because each gene's expression is dynamic (gene domains and the limb bud itself are both changing continuously), such expression maps can only be very approximate. Early and middle expression in the stylopod and zeugopod appear to be guided by a common enhancer (Tarchini and Duboule, 2006), while a separate digit enhancer is known to exist and to regulate later Hox patterning of the autopod (Kmita et al., 2002). These are in situ mRNA hybridization data for the chick forelimb—the mouse forelimb is shown but known to be similar in general Hox expression (Nelson et al., '96; Shubin et al., '97; Wolpert, '98). For color version see Lovejoy et al. (2003).

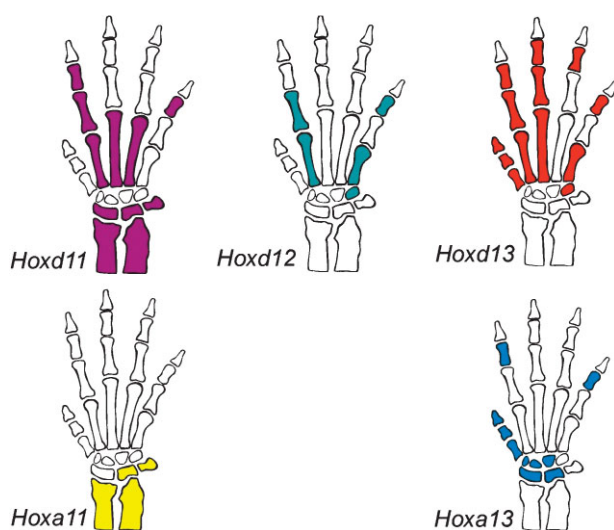


Fig. 3. "Anatomical maps" of bones of the autopod (wrist and hand) and distal zeugopod (radius and ulna) reported to be visibly affected by the deletion of various *Hox* genes. Only bones showing obvious defects are indicated; more systemic effects almost certainly occur which alter growth rate, length, etc. of other elements as well, but the "maps" shown here are likely to delimit expression "territories" of genes whose targets are candidates for growth scaling. To date, *Hoxd10* has not produced a phenotype in the distal zeugopod/autopod (for further discussion see text). Note that only *Hoxd11* is expressed in digits two to five and the distal radius, and that only *Hoxa13* and *Hoxd13* are expressed in the first digit. Because there are two distinct phases of Hox expression, and because our data show none in the short bones of the wrist during later growth (c.f., Fig. 6), the alterations of the carpus illustrated here are presumed to occur as a consequence of earlier Hox expression (data from Small and Potter, '93; Davis and Capecchi, '94; Davis et al., '95; Favier et al., '95; Davis and Capecchi, '96; Fromental-Remain et al., '96).

to modulate longitudinal growth of skeletal elements (Morgan and Tabin, '94; Yokouchi et al., '95; Goff and Tabin, '97; Capecchi, '97; Papenbrock et al., 2000; Zhao and Potter, 2001). These experiments suggest that the transcription factors they encode play a significant role in the

regulation of fetal growth plate activity (Davis and Capecchi, '96; Goff and Tabin, '97). First, complete ablation of *Hoxa11* and *Hoxd11* in the zeugopod results in the loss of normal longitudinal growth in the radius and ulna such that these elements take on the appearance of short bones (Davis et al., '95).

At the other extreme, increasing the normal dosage of *Hoxd11* expression in the distal forelimb of mice results in posterior metacarpals and phalanges that are significantly longer than those of controls (Boulet and Capecchi, 2002). In addition, mice lacking the Hox-binding co-factor *Pbx1* experience reduced growth rate and accelerated endochondral ossification in the affected growth plates (Selleri et al., 2001). Second, individual *Hox* genes appear to specify particular growth patterns. For example, ectopic expression of *Hoxa13* in the zeugopod of mice shortens their radius and ulna, implying that this gene's targets yield a substantially reduced growth rate when in competition with endogenous zeugopod *Hox* genes (Yokouchi et al., '95; Zhao and Potter, 2001). Finally, *Hox* genes have been implicated in the morphological transitions within vertebrate limbs (Shubin et al., '97; Blanco et al., '98; Nemeschkal, '99; Wagner and Chiu, 2001). Specifically, within mammals, the distal/posterior expansion of the autopod in the formation of the bat wing has been shown to correspond with a posterior extension of *Hoxd13* expression (Chen et al., 2005).

Hox proteins affect skeletal development and growth by regulating the expression of downstream targets that more directly control cellular processes such as mitotic rate, apoptosis, cell-cell adhesion and construction of an extracellular matrix (Pradel and White, '98; Lovejoy et al., 2003). Although the exact targets of Hox activity in the developing limb skeleton are currently unknown, they are believed to compete for regulation of the same growth gene targets (Goff and Tabin, '97). In such a way they appear capable of "finely sculpting the limb skeleton" (Davis and Capecchi, '96; p 1175). Often multiple growth plates reside within the same Hox territory (see below). This suggests that Hox territories demarcate a series of growth modules and could therefore modulate the growth rates of all skeletal elements lying within that domain. It follows from this hypothesis that changes in the levels of expression or downstream action of Hox genes within these domains will alter growth in systematic ways, and that this should be detectable in the adult proportions of the elements residing within each such module. The specific goals of this study are therefore twofold: (1) to evaluate *Hoxd* expression in the zeugopod and autopod during the late stages of murine limb development, when subsequent growth plate behavior is likely to be specified; and (2) to evaluate adult anthropoid distal forelimb and digit length proportions to determine if it is consistent

with the observed Hox expression patterns and their previously established functions.

MATERIALS AND METHODS

Analytical design

Hox expression is largely restricted to members of the *Hoxa* and *Hoxd* complexes in the distal forelimb of birds and mammals (Nelson et al., '96; Tarchini and Duboule, 2006; Tarchini et al., 2006). However, as misexpression phenotypes indicate (Fig. 3), it is generally the AbdB-related (Hox paralogy groups nine to 13) members of these complexes that play a significant role in limb patterning. The patterns of expression of the downstream members of these complexes are as follows: all five digits express *Hoxa13* and *Hoxd13*; *Hoxd10* and *Hoxd11* are expressed in the posterior digits and the distal zeugopod (radius and ulna), but not in the proximal zeugopod or the phalanges of digit one. *Hoxd12* is expressed in digits two to five. In the zeugopod only *Hoxa11* and *Hoxd11* appear to have a significant role in skeletal patterning and growth (Davis et al., '95; Nelson et al., '96; Boulet and Capecchi, 2002). Exactly where the first metacarpal falls with respect to these Hox expression domains is presently unclear. Although it is certainly possible that it lies within either the same domain as the remainder of digit one or that of the four posterior digits, it is equally possible that this morphologically distinct metacarpal, whose growth plate is unique in being proximal rather than distal in mammals (Reno et al., 2007), expresses a unique combination of Hox genes altogether.

The Hox expression patterns described above suggest the existence of several potential distal forelimb growth modules (Fig. 4). For example, the combined expression of *Hoxa13* and *Hoxd13* could define an "autopod" module that patterns all five digits. Alternatively, the autopod could be divided into independent "posterior digit" and "digit one" modules on the basis of the additional expression of *Hoxd11* and *Hoxd12* in the posterior digits (note that *Hoxd10* has been shown to have little effect on digit morphology). An independent distal zeugopod/posterior digit module is also conceivable and would be defined by *Hoxd11* expression. Here we evaluate the following hypotheses:

- (1) All five digits belong to a single growth module that is independent of the distal zeugopod. This module is defined by the expression of *Hoxa13* and *Hoxd13* (blue+yellow+green in Fig.4).

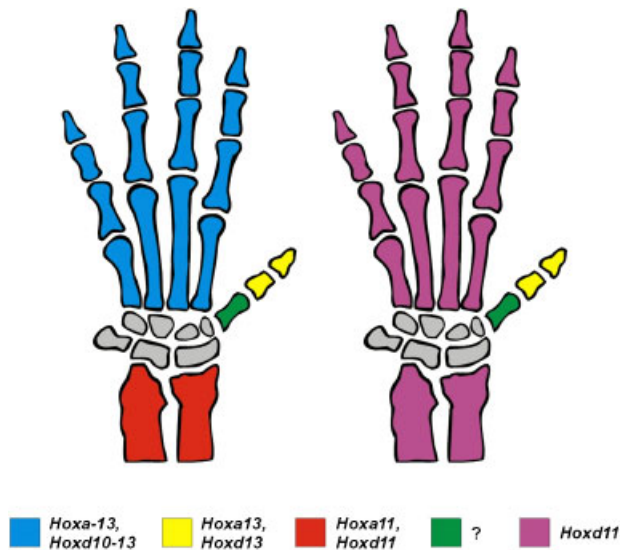


Fig. 4. Proposed *Hox* delimited growth modules within the distal forelimb of anthropoid primates, derived by combining murine *Hox* expression patterns with morphological analysis as described in the text.

- (2) The posterior digits comprise a single *Hox*-defined growth module that is independent of those impacting digit one and the distal radius and ulna. The posterior digit module would be defined by expression of *Hoxd12* (blue in Fig. 4).
- (3) The posterior digits, distal radius and distal ulna comprise a single *Hoxd11*-defined growth module that is independent of the digit one module (purple in Fig. 4).

If, as we suggest here, *Hox* genes at least partially specify the growth properties of skeletal elements within these domains, then it follows that (1) the *relative lengths* of the elements that lie within a single, *Hox*-defined module should correlate with one another across taxa, and (2) there should be no correlations between elements of different modules that are not caused by other common factors such as body size and overall limb length.

To test these *a priori* hypotheses, we examined the relationships among adult length data for distal forelimb elements in a sample of anthropoid primates of varying locomotor specializations (Table 1). In a previous study, we found evidence of slight founding effects and/or nondirectional genetic fluctuations among anthropoid taxa (Reno et al., 2000). Therefore, in this study the relationships among forelimb lengths were evaluated within three evolutionarily distinct anthropoid radiations: (1) NWMs, (2) OWMs, and (3) hominoids.

Modularity in animals (e.g., Wagner, '89; Nemeschkal, '99) and primates in particular

TABLE 1. Osteological sample

Species	Males	Females
Platyrrhini (NWM)		
<i>Alouatta palliata</i> (AQ)	6	2
<i>Alouatta seniculus</i> (AQ)	4	0
<i>Ateles fusciceps</i> (S)	4	3
<i>Cebus capucinus</i> (AQ)	3	1
<i>Saimiri sciureus</i> (AQ)	1	2
Catarrhini		
Cercopithecidae (OWM)		
Colobinae		
<i>Presbytis frontata</i> (AQ)	1	2
<i>Trachypithecus cristatus</i> (AQ)	3	3
<i>Colobus guereza</i> (AQ)	9	3
Cercopithecinae		
<i>Theropithecus gelada</i> (TQ)	3	2
<i>Macaca mulatta</i> (AQ)	2	4
<i>Macaca fascicularis</i> (AQ)	4	0
<i>Erythrocebus patas</i> (TQ)	3	2
<i>Cercopithecus aethiops</i> (AQ)	4	4
<i>Cercopithecus mitis</i> (AQ)	3	0
Hominoidea		
<i>Homo sapiens</i> (B)	15	15
<i>Pan troglodytes</i> (S)	15	14
<i>Gorilla gorilla</i> (S)	15	13
<i>Pongo pygmaeus</i> (S)	6	13
<i>Hylobates lar</i> (S)	9	4

NWM, New World monkey; AQ, arboreal quadruped; TQ, terrestrial quadruped; S, suspensory; B, biped.

(Cheverud et al., '89; Hallgrímsson et al., 2002; Marroig et al., 2004; Young and Hallgrímsson, 2005) has previously been explored. Although each of these studies differs with regard to its particular aim, most identified functional and/or developmental modules by using patterns of intraspecific correlation to assess morphological integration.

However, if similar phenotypes in two or more skeletal elements are guided by their membership in one or more modules that fact can only be identified via their covariance. Within species, environmental perturbation (e.g., cartilage modeling or growth plate responses to loading) and additional "memberships" in other shared modules are likely to either obscure and/or even impose additional covariation unrelated to the gene territories under consideration [e.g., systemic factors such as the growth hormone/insulin-like growth factor 1 axis (Robson et al., 2002), whole limb modules that may be defined by *Tbx4/Tbx5* (King et al., 2007), or individual variation in genes that may lie downstream of *Hox*]. Only after sufficient directional selection has isolated and collectively enhanced the morphological

phenotypes of any single module will any antecedent gene territory derived linkages then be observable via enhanced covariation. Here we seek to identify modules that have been targets of natural selection, in this case the known territories of specific Hox genes whose effect on skeletal growth appears to have been modulated by locomotor adaptations within the metacarpus and antebrachium. We therefore have focused on modules defined only by morphological correlations among the homologous elements of separate species.

A variety of different statistical procedures can be used to test specific hypotheses of character covariation and modularity (Magwene, 2001; Klingenberg, 2005). Here we use interspecific bivariate correlation ($P < 0.05$; one-tailed) of size corrected skeletal lengths to test for the correlation of anthropoid distal limb segments across taxa, and principle components analysis (PCA) to further assess the intercorrelations of variables and to evaluate their relative independence within each of the proposed Hox modules. Additional details of these procedures are provided below.

Anthropoid distal forelimb metrics & statistical procedures

A summary of the specimens included in this study is provided in Table 1. Skeletal metrics collected from all specimens include the lengths of the proximal (PROX) and distal (DIST) radius. These variables are meant to assess independent growth of the proximal and distal radial growth plates in adult skeletons and as such were measured relative to the nutrient foramen marking the approximate anlagen midpoint at the time of periosteal bud invasion (Payton, '32, '34; Bisgard and Bisgard, '35). As the long bones of the mammalian hand have single growth plates, the maximum lengths of the five metacarpals (MC1–5) were also measured. In OWM and hominoid specimens the lengths of the proximal phalanges of the first (PP1) and third (PP3) digits were collected, and the intermediate phalanx of the third digit (IP3) was measured in hominoids only. The phalanges were either not present, or could not be unambiguously identified in most NWM specimens.

In addition to these primary variables of interest, the anteroposterior diameter of the humeral head, the maximum breadth of the distal humeral articular surface (capitulum and trochlea), the maximum diameter of the radial head, and the maximum breadth of the distal radial articulation

were collected from each specimen. The geometric mean of these articular dimensions was calculated for each specimen and served as an estimate of the individual's overall body size (GMEAN). In addition, because there is some evidence that the highly suspensory hylobatids have elongated the entire upper limb as opposed to just its distal segment (Reno et al., 2000) it was necessary to control for the effects of total limb elongation (through a mechanism distinct from that being investigated here) in this group as well. For this reason the total length of the humerus (HUMERUS) was also collected. This measure serves as an indication of limb elongation independent of body size. Interspecific bivariate correlation analysis was applied to the distal forelimb lengths within each higher taxonomic group (i.e., NWMs, OWMs, hominoids). In all three groups tests of correlation were performed using the means of distal limb segment lengths adjusted for body size by GMEAN. In the hominoids, tests of correlation were additionally performed with means adjusted for humeral length (HUMERUS).

Data reduction was further explored using PCA. Variables included in the PCA were the individual measures of the lengths of the distal radius, MC1 and MC3, and the proximal phalanges of digits one and three. These variables present a balanced representation of the variation contributed by each of the hypothesized modules defined by Hox expression. Owing to the absence of phalangeal length data for NWMs, PCA was applied only to the OWM and hominoid samples. As in the bivariate correlation analyses, PCA was performed on variables normalized for body size (GMEAN) in both taxa, and repeated with variables normalized for upper limb length (HUMERUS) in hominoids only.

To further test the correspondence between forearm segment covariation and our proposed developmental Hox modules, we used Mantel's test (Cheverud et al., '89; Nemeschkal, '99) to compare intermatrix correlations between real morphological and hypothetical data matrices. For each taxonomic group, we created a hypothetical Hox module matrix in which elements predicted to reside within the same module were coded as one (1), whereas those in separate hypothetical modules were coded as zero (0). Mantel's test assesses the significance of the correspondence of the two matrices by comparing the intermatrix correlation against a sample of 10,000 correlations generated from random permutations of the two matrices (XLStat).

Hox gene expression in the late stage distal forelimb

Mouse embryos were dissected from the uterine horn of a pregnant female at E13.25, E15.25 and E17.25 and fixed in 4% paraformaldehyde. Embryos were dehydrated in a graded methanol series and stored in 100% methanol at -20°C . Whole-mount in situ hybridization with riboprobes for mouse *Hoxd11*, *Hoxd12* and *Hoxd13* was performed as described in the literature (Nieto et al., '96). Because mutant forms of the *Hoxd10* gene have been found to have no discernable effect on digit morphology (Zákány et al., '97), expression of this gene was not monitored. To facilitate determining the distribution of Hox expression within the forming skeleton, limb buds from each stage were also cleared and stained with alcian blue.

RESULTS

Intercorrelation of intramodular forelimb lengths

To test for the presence of morphological growth modules in the primate forelimb, we first examined patterns of correlation and covariation within the distal forelimbs of anthropoid primates. This was designed to determine whether proximal and distal growth plates are regulated in a coordinated or an independent manner. Species means for body size-corrected (GMEAN) radial and metacarpal lengths are presented in Figure 5. For ease of comparison, species are arranged within each higher taxonomic group (i.e., NWMs, colobines, cercopithecines, hominoids) by increasing length of the MC1. As is evident from this figure, the patterns of interspecific variation in the relative lengths of the four posterior metacarpals parallel one another quite closely in all higher anthropoid taxa. In comparison, the pattern of interspecific variation in the relative length of the first metacarpal is distinct relative to that of the posterior digits. Also evident in Figure 5 is the striking variation in distal radial length present within higher taxonomic categories. Significantly, variation in the relative lengths of the four posterior metacarpals within these groups closely parallels that displayed by the distal radius (particularly in hominoids) and is definitively unlike that displayed by the first metacarpal or the proximal radius.

Results of GMEAN-normalized correlation analysis (Table 2) confirm that the lengths of the four posterior metacarpals are strongly correlated with

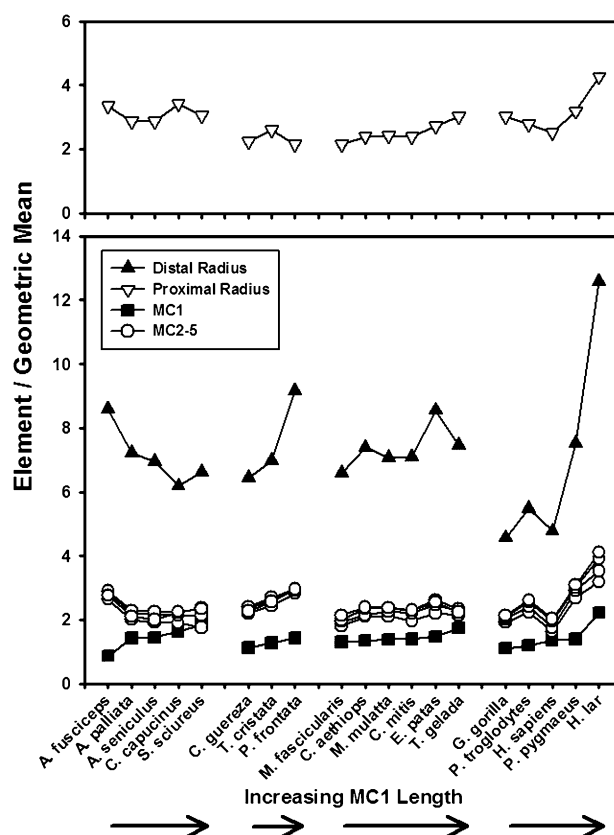


Fig. 5. Size-normalized (GMEAN) metacarpal and distal radius length in anthropoid species arranged according to ascending metacarpal one (MC1) size. Note the common behavior of the posterior four metacarpals and their marked independence of MC1. The distal radius follows the behavior of the posterior rays much more closely, especially within hominoids. Note that human opposability involves dramatic reduction of its posterior four digits.

one another within each higher taxon. The only exception to this finding is NWMs in which the correlations between lengths of MC2 and MC5 failed to reach significance. Also confirmed by these statistics is the distinctive pattern of variation in the relative length of MC1. In OWMs no correlation was found between the length of the first metacarpal and those of the four posterior rays. Interestingly, in NWMs, a significant *negative* correlation obtained between MC1 and MC4 and MC5, and in the combined hominoid sample the length of MC1 correlated positively with all posterior metacarpals except MC5. However, when gibbons were removed from this analysis the length of the MC1 failed to correlate with that of any posterior metacarpal. Similar results were obtained when hominoid distal limb segment lengths were normalized by humeral length (HUMERUS).

TABLE 2. Results of tests of correlation of size-corrected species means

	PROX	DIST	MC2	MC3	MC4	MC5	PP3	IP3	MC1
New World monkeys ($n = 5$; GMEAN)									
DIST	-0.186								
MC2	0.483	0.663							
MC3	0.173	0.893*	0.922**						
MC4	0.154	0.941*	0.843**	0.967**					
MC5	0.084	0.959*	0.761	0.925**	0.990**				
MC1	0.012	-0.924*	-0.570	-0.794	-0.917*	-0.963**			
Old World monkeys ($n = 9$; GMEAN)									
DIST	0.106								
MC2	-0.150	0.819**							
MC3	0.023	0.745*	0.953**						
MC4	-0.022	0.693*	0.927**	0.981**					
MC5	-0.079	0.680*	0.926**	0.961**	0.981**				
PP3	-0.687*	0.210	0.664*	0.621*	0.668*	0.700*			
MC1	0.732*	0.463	0.029	0.035	0.017	0.023	-0.600*		
PP1	-0.005	0.291	0.158	-0.065	-0.126	-0.109	-0.093		0.372
Hominoids ($n = 5$; GMEAN)									
DIST	0.958								
MC2	0.928**	0.978**							
MC3	0.920**	0.970**	0.999**						
MC4	0.903*	0.938**	0.987**	0.992**					
MC5	0.882	0.910*	0.972**	0.979**	0.997**				
PP3	0.922*	0.984**	0.998**	0.996**	0.977**	0.958**			
IP3	0.930*	0.982**	0.998**	0.996**	0.978**	0.958**	0.998**		
MC1	0.896*	0.962**	0.891*	0.874*	0.811*	0.766	0.912*	0.903*	
PP1	0.734	0.858*	0.767	0.745	0.658	0.603	0.802*	0.784	0.959**
Great apes and humans, hylobatids removed ($n = 4$)									
DIST	0.686								
MC2	0.683	0.966*							
MC3	0.688	0.959*	1.000**						
MC4	0.763	0.940*	0.990**	0.992**					
MC5	0.792	0.934*	0.983**	0.986**	0.999**				
PP3	0.613	0.972*	0.994**	0.992**	0.969**	0.957*			
IP3	0.631	0.939*	0.995**	0.996**	0.983**	0.973*	0.991**		
MC1	0.047	0.639	0.459	0.437	0.348	0.325	0.538	0.421	
PP1	-0.470	0.199	0.036	0.013	-0.102	-0.137	0.140	0.025	0.859
Hominoids ($n = 5$; HUMERUS)									
DIST	0.020								
MC2	0.424	0.731							
MC3	0.472	0.657	0.995**						
MC4	0.602	0.569	0.966**	0.984**					
MC5	0.657	0.493	0.933**	0.960**	0.994**				
PP3	0.277	0.805	0.981**	0.963**	0.917**	0.875*			
IP3	0.331	0.833*	0.979**	0.954**	0.899*	0.847*	0.971**		
MC1	-0.703	0.655	0.255	0.174	0.003	-0.099	0.385	0.399	
PP1	-0.907*	-0.108	-0.291	-0.317	-0.458	-0.517	-0.202	-0.214	0.613
Great apes and humans, hylobatids removed ($n = 4$)									
DIST	0.289								
MC2	0.482	0.924*							
MC3	0.503	0.915*	1.000**						
MC4	0.611	0.899	0.987**	0.990**					
MC5	0.653	0.889	0.975*	0.979**	0.998**				
PP3	0.350	0.975*	0.982**	0.977**	0.953*	0.938*			
IP3	0.454	0.910*	0.998**	0.998**	0.978*	0.963*	0.978**		
MC1	-0.768	0.309	0.183	0.162	0.025	-0.033	0.305	0.219	
PP1	-0.914*	-0.258	-0.311	-0.326	-0.458	-0.513	-0.299	-0.262	0.835

Bold values indicate elements that reside in the same Hox module and are thus coded 1 in the corresponding hypothetical matrix for Mantel's test; other values are coded 0.

DIST, distal radius; MC, metacarpal; PP, proximal phalanges; IP, intermediate phalanx.

* $P < 0.05$; ** $P < 0.01$.

In OWMs the length of the PP3 was found to correlate significantly and positively with the lengths of all four posterior metacarpals. In addition, a weak, but nevertheless statistically significant negative correlation was observed between PP3 and the length of MC1. Also in this group, the length of the proximal thumb phalanx failed to correlate significantly with any other distal forelimb element. In the hominoid sample, the lengths of the PP3 and IP3 were found to correlate with all four posterior metacarpals and this correlation persisted even after removal of the hylobatid data. In comparison, no significant correlations were observed between the length of the hominoid proximal thumb phalanx and any other distal forelimb length either when the hylobatids were eliminated from the analysis, or when limb segment lengths were corrected by humeral length.

With respect to the radial dimensions, the length of the proximal radius failed to correlate positively with any distal forelimb dimensions in either NWMs or hominoids (once again, hylobatids removed). In OWMs the length of the proximal radius correlated negatively with the length of the PP3, and positively with the length of MC1. In NWMs the length of the distal radius correlated positively with the lengths of MC3–5. In OWMs the length of the distal radius correlated positively with the lengths of all four posterior metacarpals. In hominoids the length of the distal radius (corrected by GMEAN) correlated positively with the lengths of all metacarpals and phalanges. However, when hylobatids were removed DIST only correlated with the metacarpals and phalanges of the posterior digits. When corrected by HUMERUS, the distal radius was positively correlated with most elements of the posterior digits (MC4 and 5 just failed to reach significance) with hylobatids excluded. In the NWMs, MC1 length demonstrates a significant negative correlation with the length of the distal radius.

Independence of growth modules in the limb skeleton

Results of the principal components analyses performed for OWMs and hominoids are presented in Table 3. Using GMEAN-normalized variables, PCAs extracted three factors from the balanced set of five distal forelimb variables in OWMs and only two factors from these variables in hominoids. In the OWMs, the lengths of the distal radius and MC3 both correlated well with

TABLE 3. Principal component loadings of anthropoid forelimb elements residing within proposed Hox modules

	Old World monkeys—corrected by GMEAN			
Variable	PC1 42%	PC2 29%	PC3 19%	
DIST	0.832	0.109	−0.333	
MC3	0.899	−0.269	0.114	
PP3	0.464	−0.782	0.347	
MC1	0.534	0.759	−0.094	
PP1	0.321	0.413	0.836	
	Hominoids—corrected by GMEAN			
	All genera		Hylobatids removed	
Variable	PC1 83%	PC2 13%	PC1 65%	PC2 30%
DIST	0.972	−0.126	0.941	−0.229
MC3	0.924	−0.326	0.912	−0.359
PP3	0.915	−0.333	0.951	−0.257
MC1	0.947	0.263	0.699	0.664
PP1	0.771	0.621	0.356	0.906
	Hominoids—corrected by HUMERUS			
	All genera		Hylobatids removed	
Variable	PC1 55%	PC2 36%	PC1 57%	PC2 36%
DIST	0.903	−0.034	0.947	−0.037
MC3	0.906	−0.248	0.963	−0.085
PP3	0.960	−0.133	0.978	0.002
MC1	0.459	0.835	0.242	0.952
PP1	−0.001	0.947	−0.114	0.952

DIST, distal forearm; MC, metacarpal; PP, proximal phalanges; PC, principal component.

PC1 (42% of variance). The lengths of the MC1 and PP3 loaded most heavily, but in opposite directions, on PC2 (29% of variance) and the length of the proximal thumb phalanx loaded most heavily on the PC3 (19% of variance). In the hominoid sample corrected by GMEAN (Table 3) the lengths of the distal radius, MC3, PP3, and MC1 all loaded heavily on component one (83% of variance). The length of the proximal thumb phalanx loaded most heavily on component two (13% of variance). Variable loadings obtained from hominoid analyses in which either the hylobatids were removed or the included variables were normalized for humeral length (regardless of inclusion of hylobatids) differed from those above, but were remarkably similar to one another. In the latter three analyses, the distal radius, MC3 and PP3 loaded heavily on PC1 (55–65% of the variance), whereas the lengths of the MC1 and PP1 loaded heavily on PC2 (30–36% of variance).

Late stage expression of Hoxd genes delineates growth modules during limb skeletogenesis

We next tested the hypothesis that the growth modules identified above are related to compartmentalization of growth zones by differential *Hoxd* gene expression. Given the similarity of long bone proportions in the mouse with those of basal anthropoids, we used the mouse limb as a proxy system. The results of our murine in situ hybridizations during late stage skeletogenesis are shown in Figure 6. Observations made on whole-mount specimens were further confirmed on histological sections (data not shown). *Hoxd11* expression was present in the distal (but not proximal) ulna and radius, and the posterior four

digits. *Hoxd12* expression was detected in the posterior four digits, but not in digit one. *Hoxd13* was expressed throughout the autopod. Of special note was the darker staining (longitudinal stripes) around the perichondrium (Morgan and Tabin, '94) near the sites of future growth plate formation in the metacarpal heads/phalanges of all specimens (note dark "rings" in Fig. 6). Expression in these positions accords well with a role for *Hoxd11–13* in regulating anabolic activity within these regions. Of equal import is the lack of detectable *Hoxd* expression in the proximal regions of the autopod, in the region of the short bones of the wrist. This is an important observation with respect to our earlier proposed demarcations of Hox module boundaries as deduced from an examination of misexpression maps (Fig. 3).

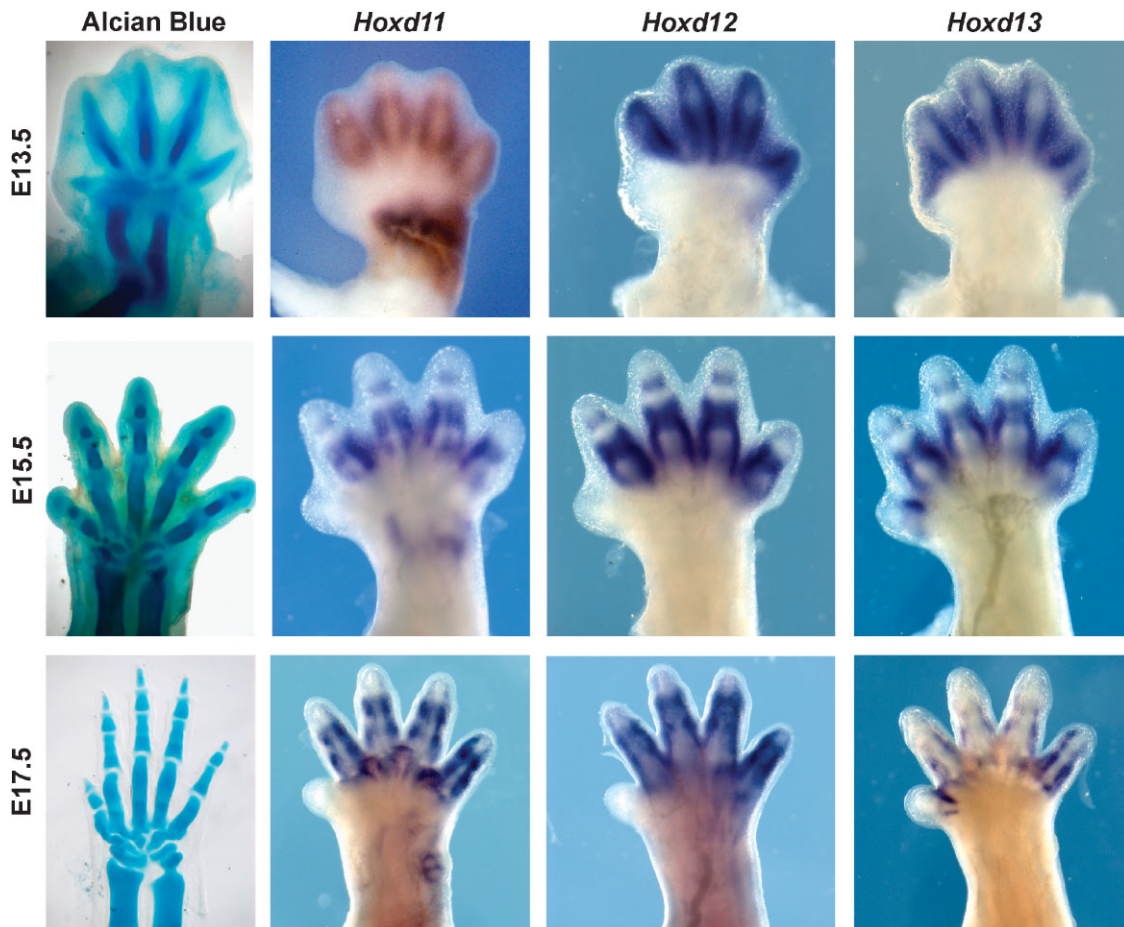


Fig. 6. Whole-mount in situ hybridization showing murine *Hoxd11*, *Hoxd12*, and *Hoxd13* expression at E13.25, E15.25 and E17.25. Alcian blue stained specimens shown to collocate Hox staining with cartilaginous elements. Anterior is to the left in all specimens. *Hoxd11* expression is detected in the developing ulna, distal (but not proximal) radius and the posterior four digits (but not digit one). *Hoxd12* expression is detected in the posterior four digits, but not in digit one. *Hoxd13* is expressed throughout the long bones of the autopod, but not the bones of the wrist. Note darker staining near the perichondrium (longitudinal stripes) and the dark "rings" around the presumptive growth plates of the metacarpal heads/phalanges. Expression in these positions is consistent with a role of *Hoxd11–13* in program(s) that regulate growth plates.

The latter reflect changes produced during pattern formation as well as later Hox expression within growth plates. The later stage appears to be much more spatially restricted, that is, may be limited only to the growth regions themselves, and is apparently not manifested in the postnatal cartilage of short bones of the wrist (which lack growth plates).

Also of special note is the fact that E15.25 and E17.25 expression differed from that at E13.25 for all three genes of the posterior Hoxd complex. In the more mature handplates, for example, two bands of *Hoxd12* expression surround each of the posterior four digits. The proximal band colocalized with the joint between the metacarpal and proximal phalanx, whereas the distal band colocalized with the joint between the proximal and intermediate phalanges. Thus, at this later stage of development *Hoxd12* expression was found to be restricted to only the regions of the growth plates of the posterior metacarpals, and the proximal and intermediate phalanges of digits two to five. Similar patterns are observed in *Hoxd11* and *Hoxd13*.

Correspondence of the forelimb and Hox modules

Combining morphological and Hox expression data provides a potential model of modular evolution of the anthropoid forearm. Its validity can be assessed by Mantel's test. The correlation analyses and PCA suggest a module linking the posterior digits and distal radius (particularly in hominoids). It corresponds with the expression pattern of *Hoxd11*. In addition, a second module is suggested by the common correlations between elements of digit one and the generally poor correlations of each thumb element with other bones of the hand and forearm. The putative competitive relationship between *Hoxd11* and

Hoxd13 in specifying growth in territories in which they overlap (i.e., the posterior digits, for further discussion see below) suggests that *Hoxd13*, which along with *Hoxa13* are the only Hox genes expressed in the thumb, could define a separate digit one module. This model agrees with our Hypothesis 3.

To test the correspondence between these proposed Hox-defined modules and the morphological data, we constructed a Hox expression matrix in which hypothesized correlations between the distal radius and bones of the posterior digits were coded as one (1), as well as the expected correlations among the elements within digit one (bold entries in Table 2). All other hypothetical correlations were coded as zero (0). For each taxonomic group the morphological and Hox module matrices were compared using both the full data set and again using a partial dataset where MC2, 4 and 5 were removed to ensure that common correlations among the four posterior metacarpals were not over-represented in the analysis. This approach is similar to that of Nemeschkal ('99) except that (1) the correlations in our morphological matrices were calculated using species means (as opposed to pooled species samples) and (2) we tested the targeted hypothesis that morphological covariation significantly corresponds with posterior Hox expression.

In all cases the morphological correlation and Hox expression matrices were correlated positively (Table 4). All but one correlation fell outside the range of the 10,000 permutations performed in their respective Mantel's test. The one exception is the NWM with the partial sample. Exclusion of the three metacarpals resulted in a comparison between 4 × 4 matrices, yet the intermatrix correlation still bordered on significance (Table 4). These results demonstrate a very close correspondence between the patterns of morphological correlation and Hox expression modules in anthropoid forelimbs.

TABLE 4. Pearson correlation between morphological correlation matrix and hypothetical Hox module matrix for each taxonomic group

Sample	Full matrix	P	MC2, 4 and 5 omitted	P
New World monkeys	0.849	0.0001	0.756	0.083
Old World monkeys	0.851	0.0001	0.721	0.0001
All hominoids (GMEAN)	0.694	0.0001	0.660	0.0001
Great apes and humans (GMEAN)	0.796	0.0001	0.736	0.0001
All hominoids (humerus)	0.785	0.0001	0.721	0.0001
Great apes and humans (humerus)	0.834	0.0001	0.783	0.0001

Significance values calculated using Mantel's test (10,000 random permutations).
MC, metacarpal.

DISCUSSION

Identification of growth modules in the anthropoid distal forelimb

Results support the existence of at least two distinct growth modules that are partially determinant of anthropoid distal forelimb segment lengths. A distal zeugopod/posterior digit module is indicated by the significant correlations among distal radius, posterior digit metacarpals and phalanges observed in all three anthropoid groups (particularly in hominoids) (Fig. 5; Table 2). A developmentally independent distal thumb module is indicated by the common correlation between MC1 and PP1 in hominoids, as well as the general failure of the proximal thumb phalanx to correlate positively with the posterior digit elements (Fig. 5; Table 2) and its strong loading on a principle component independent from that defined by posterior digit lengths in both OWMs and hominoids (Table 3). We propose that these constitute modules within which growth plates are regulated in a coordinated manner. Our finding that the distal zeugopod/posterior digit module localizes to a domain defined and regulated exclusively by *Hoxd11* suggests that this gene is a candidate for regulating growth plate proliferation within this morphogenetic module. The thumb module, on the other hand, could be defined and regulated by *Hoxa13* and *Hoxd13* (see below).

That *Hoxd11* should play a fundamental role in determining posterior digit length in anthropoid primates is consistent with what is currently known of the function of this gene in forelimb development. In the autopod, *Hoxd11* is expressed along with *Hoxa13*, *Hoxd10*, *Hoxd12* and *Hoxd13* in the posterior digits, but only *Hoxd11*, *Hoxd12* and *Hoxd13* are known to have an effect on posterior digital morphology (reviewed in Zákány and Duboule, '99). Of these genes, *Hoxd12* is believed to have only a moderate effect on digital

morphogenesis as mice carrying targeted mutations in the *Hoxd12* gene (but normal copies of both *Hoxd11* and *Hoxd13*) display only a slight reduction in the length of the metacarpals and phalanges and a slight modification of a distal carpal element (Davis and Capecchi, '96; Kondo et al., '96). In addition, expression of *Hoxd12* in the *Hoxd13* pattern (resulting from a deletion of *Hoxd13*, which brought *Hoxd12* closer to the Global Control Region) only moderately affected digit length (Kmita et al., 2002, but see below). In comparison, altering the expression of either *Hoxd13* or *Hoxd11* has dramatic effects on digit morphology. *Hoxd13* mutant mice display severely truncated metacarpal lengths, absence of some phalanges and abnormal carpal elements (Dolle et al., '93; Davis and Capecchi, '96). *Hoxd11* mutant mice show significant reductions in the length of most posterior digit metacarpals and several posterior digit phalanges, in addition to severe malformations of the distal radius, distal ulna and carpals (Davis and Capecchi, '96). Significantly, deleting both *Hoxd13* and *Hoxd12*, which causes *Hoxd11* to be expressed in a *Hoxd13* fashion, produces myriad malformations of the wrist and hand, including the development of supernumerary chondrogenic condensations in the wrist and metacarpus, chaotic, partially fused metacarpals and dramatically truncated phalangeal lengths (Kmita et al., 2002).

The different phenotypes that result when *Hoxd* genes are either disrupted through mutation or deleted altogether underscore that the "function" of a given gene cannot be simply defined, but instead must be integrated into the larger "genomic landscape" and functional context of the entire cluster (Kmita et al., 2002). In this respect, data gathered from *Hoxd* misexpression in the limb buds of chicks are especially revealing. Misexpression of *Hoxd11* in the anterior digit of the chick hindlimb autopod results in the

TABLE 5. Summary of hypothesized effects by *Hoxd* expression or downstream target action on the anthropoid metacarpals, phalanges and distal forearm

Hox action	MC2-5	P2-5	MC1	P1	Distal forearm	Taxa
↑ <i>Hoxd11</i>	Long	Long	No effect	No effect	Long	Chimpanzees, orangutans
↓ <i>Hoxd11</i>	Short	Short	No effect	No effect	Short	Gorillas
↑ <i>Hoxd13</i>	Short	Short	Long	Long	No effect	<i>Australopithecus afarensis</i>
↓ <i>Hoxd13</i>	Long	Long	Short	Short	No effect	<i>Ateles</i> , <i>Colobus</i>
↑ <i>Hoxd11</i> and ↑ <i>Hoxd13</i>	Intermediate	Intermediate	Long	Long	Long	Gibbons, <i>Presbytis frontata</i> , <i>Erythrocebus patas</i>
↓ <i>Hoxd11</i> and ↑ <i>Hoxd13</i>	Short	Short	Long	Long	Short	Modern humans

MC, metacarpal; P, phalanges.

production of a larger than normal digit one condensation, which then undergoes an additional segmentation event, producing a toe with an additional phalanx (Morgan et al., '92). In comparison, ectopic expression of *Hoxd13* in the distal zeugopod of chicks results in a reduction in the length of the tibia/fibula and radius/ulna (Goff and Tabin, '97). Ectopic expression of *Hoxd13* in the forearms of mice produces a similar phenotype (van der Hoeven et al., '96). In the chick studies, the *Hoxd13* misexpression phenotype was interpreted as having resulted from a dominant-negative interference with the growth-promoting activity of other Hox genes expressed contemporaneously in the hindlimb growth plates. This implies that *Hoxd13* promotes growth less strongly than does *Hoxd11*. Our morphometric analysis reveals growth patterns that correspond tightly with these domains, which supports the idea that *Hoxd* gene expression domains may underlie the coordinated scaling of long bone length within these territories. That these gene functions are similar in mice is indicated by both the clear (but unreported) elongation of at least forelimb rays two and three in mice in which *Hoxd13* is deleted (Kmita et al., 2002: note in their Figure 2 the relatively proximal positions of the strong lateral interosseous ridges present on these elements in comparison with those of controls—such morphology implies more robust growth plate activity in response to the loss of *Hoxd13*) as well as significant elongation of the posterior metacarpals and phalanges when the normal dosage of *Hoxd11* is experimentally increased in the distal forelimb (Boulet and Capecchi, 2002). In addition, ectopic expression of *Hoxa13* in the zeugopod of mice shortens their radius and ulna, implying that, as with *Hoxd13*, this gene's targets yield a substantially reduced growth rate when in competition with *Hoxd11* (Yokouchi et al., '95; Zhao and Potter, 2001).

As noted above, other than *Hoxd11*, the only other Hox gene capable of producing a phenotypic effect in the distal radius and ulna is *Hoxa11* (Nelson et al., '96; Boulet and Capecchi, 2002). In these elements there seems to be little difference in the ability of either gene to promote growth at the distal growth plates. Instead, as indicated by the ability of extra *Hoxd11* transcripts to compensate for an absence of *Hoxa11* protein (Boulet and Capecchi, 2002), *Hoxa11* and *Hoxd11* may be functionally equivalent in the murine zeugopod. Unlike its expression in the autopod, therefore, *Hoxd11* expression in the zeugopod occurs in the

absence of competition from other functionally diverse Hox proteins for access to growth-promoting targets. The finding that the radius and ulna of experimental mice with an increased dosage of *Hoxd11* in the forelimb were not significantly longer than those of their wild type counterparts suggests that subtle changes in the amount of transcript produced are unlikely to yield significant changes in the growth observed in the distal zeugopod (Boulet and Capecchi, 2002). Thus, elongation of the distal forearm in mammals requires more than a simple augmentation of the *Hoxd11* signal and instead likely involves modification of downstream Hox response elements and/or target genes (note that such mechanisms would still produce modular changes defined by Hox expression).

With respect to the first metacarpal, our results demonstrate the unique behavior of the length of the first metacarpal in all anthropoid taxa (Fig. 5; Tables 2 and 3 and see below) suggesting the presence of an additional growth module influencing this element. As noted above, the mammalian MC1 has a unique structure. Unlike its posterior counterparts, its physis is proximal in mammals rather than distal (Reno et al., 2007), and therefore lies in the immediate proximity of the carpus. The first ray always exhibits a maximum of two phalanges, but very often in those cases in which it suffers distinct involution (e.g., *Ateles*, *Colobus* and hallux of *Pongo*), the effects of such reduction are far more dramatic in its phalanges than its metacarpal—which can sometimes remain large and robust (even though subcutaneous) within the palm, despite the loss of its phalanges. Such behavior is consistent with some other substantial effects on its growth other than those of the *Hoxa13/Hoxd13* (see below). Histological sectioning of our in situ specimens confirms that *Hoxd11* and *Hoxd12* are not expressed adjacent to the MC1 (data not shown). In fact, the lack of *Hoxd11/Hoxd12* expression is a necessary prerequisite for establishing digit one identity in the chick and mouse (Vargas and Fallon, 2005). This suggests the existence of a distinct module defined by a gene other than Hox that isolates the MC1 from both its own phalanges as well as the four posterior rays.

The evolution of the primate forelimb

Although much has yet to be learned regarding the regulation and action of Hox genes in the growth plates of the developing forelimb, it is

nevertheless clear that subtle modulation of the expression and/or the targeted actions of these genes can account for many prominent aspects of primate distal forelimb anatomy. For example, based on the elongated metacarpals and phalanges observed in mice in which the number of *Hoxd11* transcripts has been increased (Boulet and Capecchi, 2002), it is conceivable that the posterior digit elongation observed in ateline and colobine monkeys has been achieved through a simple upregulation of *Hoxd11* expression, or alternatively by the expression of *Hoxd12* or its targets, as the posterior digits uniquely express this gene. However, a third possibility is that posterior digit elongation in these taxa was achieved through downregulation of *Hoxa13* or *Hoxd13* or, more likely, the modification of their Hox response elements in *cis*, thereby reducing the ability of their gene products to compete with those of *Hoxd11* for growth-promoting targets (Goff and Tabin, '97). Such a mechanism is especially appealing because in addition to having an effect on posterior digit length, modification of *Hoxa13/Hoxd13* expression would also substantially modify digit one, but would have no impact on the distal zeugopod, which shows no evidence of any associated elongation (Reno et al., 2000). In fact, mice heterozygous for the semidominant hypodactyly (*Hd*) mutation, in which a large deletion is present in the first exon of *Hoxa13*, displays shortened first digits on all four limbs. In some cases this shortening is manifested by the absence of one or two phalanges (Morlock et al., '96); a phenocopy of the condition observed in *Ateles* and *Colobus*. Thumb reduction in atelines and colobines has traditionally been considered an adaptation that prevents this digit from becoming a hindrance during brachiation (Straus, '42) or running and leaping on pliable substrates (Tuttle, '75). An alternative, more conservative explanation is that the reduction in thumb length observed in these taxa is merely a developmental byproduct of posterior digit elongation through a *Hoxa13/Hoxd13*-modulated mechanism.

In comparison with atelines and colobines, the relative elongation (or reduction) of the posterior digits of gorillas, chimpanzees and orangutans occurs in association with what appear to be proportionally elongated distal radial segments (Fig. 5). The total morphological pattern displayed by these taxa therefore suggests that their posterior digit and distal radial elongation would be achievable through upregulation of *Hoxd11*-mediated growth. The dramatically elongated

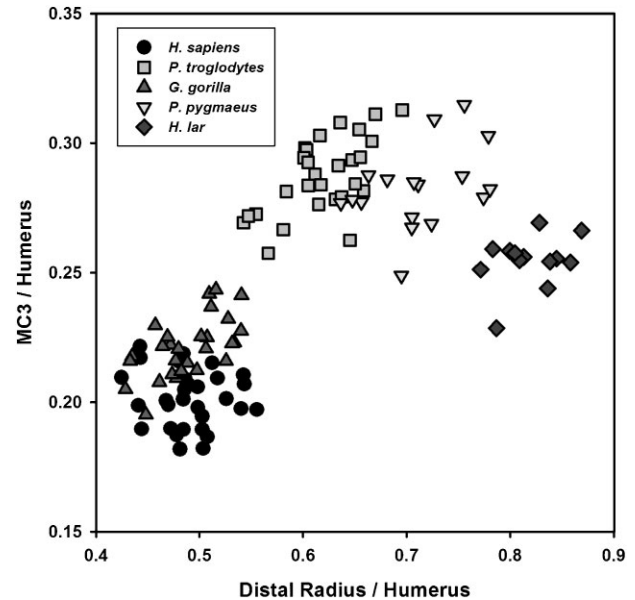


Fig. 7. Humerus-normalized metacarpal three (MC3) length compared with distal radius. Note the reduced length of MC3 relative to the degree of forearm elongation in humans and gibbons compared with the other apes.

distal radius of hylobatids also suggests upregulation of the growth targets of *Hoxd11*. However, if the posterior digits of hylobatids were elongated proportional to that of the zeugopod, they would be so long as to be virtually useless in manipulation, which is critical to terminal branch feeding. This is reflected by the weaker correlations between humerus-corrected posterior digit length and distal radius across hominoids, which are the result of relatively shorter posterior digits than predicted by the distal radius in gibbons and humans (Fig. 7). Such reduction of digits two to five could result from upregulation of *Hoxd13* and/or *Hoxa13* targets and thereby ameliorate some of *Hoxd11*'s effect on growth without any reduction of the gain in radial length where neither *Hoxd13* nor *Hoxa13* is expressed. Such a mechanism would also account for the elongated first digit (both MC1 and phalanges; Figs. 5 and 8) characteristic of both gibbons and humans. It is possible that such a mechanism also underlies forearm modifications seen in *Presbytis frontata* and *Erythrocebus patas*. In both these species, as in gibbons, the distal radius is substantially elongated (Reno et al., 2000) and accompanied by both more moderate elongation of the posterior metacarpus as well as the presence of a more robust thumb (when compared with other similar taxa; Fig. 5). A summary of these various forms of primate

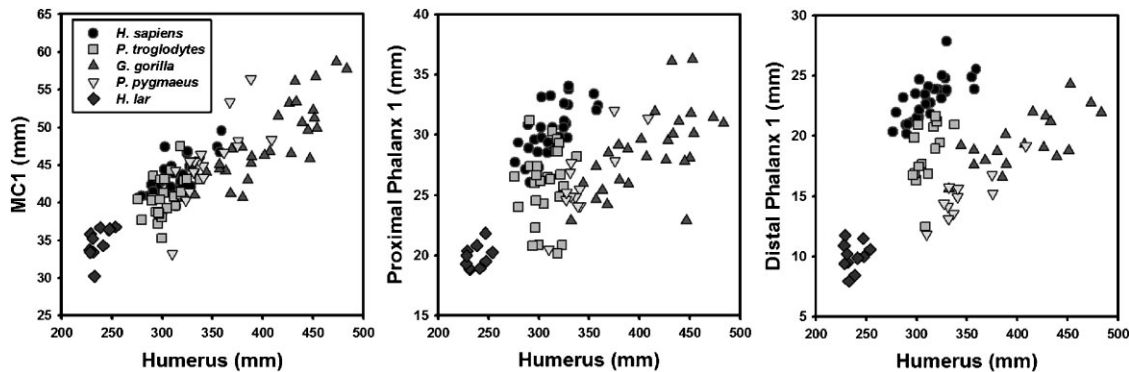


Fig. 8. Scatterplots of the three elements of the first ray compared with the length of the humerus. Note the much more consistent relationship of metacarpal one (MC1) with the humerus in all taxa, compared with the much greater fluctuation in length in its two phalanges. These data suggest that the genic basis of growth differs in the metacarpal and phalanges of digit one, and that MC1, resides in a module defined by a gene other than Hox. Note that it is the phalanges of *Homo sapiens* (especially the distal phalanx) that distinguish it from other taxa, rather than its MC1.

morphological expression and the Hox genes that potentially underlie them as discussed above is presented in Table 5.

Evolution of hominid forearm and hand proportions

The mechanisms for concurrent changes in the forearm and hand suggested for extant hominoids also provide a conciliant explanation for the evolution of human hand proportions. The posterior digits of the human hand are shorter than those of any other higher primate (Fig. 5). In addition, humans have very long thumb phalanges (Fig. 8) and a very short radius. On the basis of the spatial relationship of the growth modules to the Hoxd expression domains, this complex of traits could be achieved by the combination of (1) upregulation of the targets of *Hoxd13* and/or *Hoxa13* (thereby reducing growth of the posterior digits, but simultaneously lengthening the thumb phalanges) and (2) downregulation of *Hoxd11* targets (reducing the length of the distal forearm and posterior digits). A lack of complete specimens prevents detailed tracking of such changes in the hand and/or antebrachium of Plio-Pleistocene hominids. However, a recently discovered *Australopithecus afarensis* specimen (A.L. 438) provides a glimpse at the possible timing of these changes. A.L. 438 consists of an exquisitely preserved ulna (with an identifiable nutrient foramen), MC2 and MC3 (Drapeau et al., 2005). As seen in Figure 9, a comparison of posterior digit length with that of the distal ulna yields, as expected, a pattern similar to that seen with the distal radius: gibbons and humans are separated from gorillas, chim-

panzees and orangutans by their strongly reduced posterior digits. In addition, the species are also sorted by distal forearm length relative to limb size (with orangutans and gibbons to the right on the graph and humans and gorillas to the left). Interestingly, A.L. 438 falls closest to the gibbons indicating that it may have already undergone posterior digit reduction (and attendant thumb enlargement), whereas not yet having undergone the antebrachial reduction seen in modern humans.

Such a pattern fits with what is known for early hominid forelimb proportions. Studying the composite hand skeleton from A.L. 333, Alba et al. (2003) determined that *A. afarensis* had already evolved thumb proportions more similar to modern humans, likely under selection for improved manipulative behavior in an animal that had abandoned significant arboreal activity (Lovejoy, 2005a,b; 2007). However, although the first digit of *A. afarensis* appears robust and the posterior digits somewhat shortened, they still maintain features (such as phalangeal curvature) that indicate relatively longer posterior digits than obtain in modern humans. It is also demonstrated that *A. afarensis* and its likely descendants (*A. africanus* and BOU-VP-12/1: Clarke, '99; Asfaw et al., '99; Reno et al., 2005) maintained elongated forearms. The Narokotome *Homo erectus* skeleton (KNM WT-15000) establishes that human-like forearm proportions were, however, attained by 1.8 million years ago (mya) (Walker, '93). Thus, forearm reduction (and likely final reduction of the posterior digits) occurred in the time period between 2.5 mya and 1.8 mya. An age of 2.5 mya marks the appearance of butchery of large animals

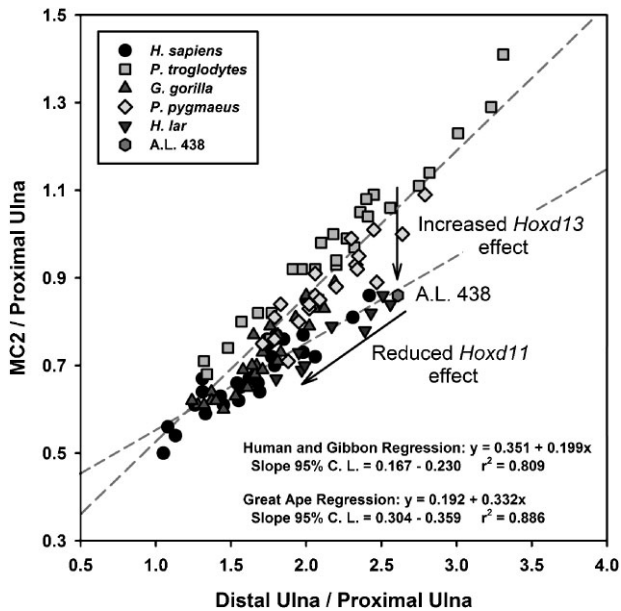


Fig. 9. Comparison metacarpal two (MC2) versus distal ulna corrected by proximal ulna in extant hominoids and *Australopithecus afarensis* (A.L. 438) illustrating possible effects of altered *Hoxd11* and *Hoxd13* signaling during human evolution. MC2 was used to allow direct comparison of the relative size of A.L. 438 with modern humans because the latter uniquely possess a styloid process on their metacarpal three (MC3). Size correction here must be by proximal ulna because there was no associated radius or humerus for A.L. 438. Note that it has the unfortunate effect of increasing variation of relative distal ulna length because individual variation in the location of the ulnar nutrient foramen affects both the numerator and denominator of the index (in particular note the range of chimpanzees). However, the taxa can still be discriminated and show the same pattern as in Figure 7: humans and gibbons show reduced posterior digit length compared with the great apes. We hypothesize that modification of *Hoxd13* produces a shift in elevation because it shortens posterior digit length but has no effect on the distal zeugopod. In contrast, reducing *Hoxd11* action shortens both the metacarpal and distal forearm simultaneously and therefore causes a diagonal translation. Analysis of covariance demonstrates a significant difference in least-squared slopes between those taxa hypothesized to have altered *Hoxd13* signaling (humans and gibbons) compared with that of the other hominoids ($P > 0.001$). Note that although A.L. 438 lies on the line defined by taxa to have undergone modification of *Hoxd13* signaling, it falls within the range of gibbons and outside the range of humans. This suggests that A.L. 438 has reduced its posterior digit length (and possibly to have increased its pollex length) relative to our common ancestor with chimpanzees, but has yet to reduce its forearm length as in modern humans, implying that the further reduction of digit and forearm lengths results from reduced action of *Hoxd11*.

(de Heinzelin et al., '99). The initiation and progressive intensification of this activity may have placed a further selective premium on digital proportions more conducive to the manufacture and effective use of tools used in extractive

foraging and provisioning (Lovejoy, '81), thus leading to further reduction of posterior digit and distal radius reduction via decreased promotion of growth effects of *Hoxd11*.

CONCLUSIONS

Although the morphometric data obtained and presented here are consistent with the spatial patterns of *Hoxd* expression territories during long bone growth, we note that all of the above explanations for the role of Hox domains in determining zeugopod/autopod relationships are essentially post hoc, that is, the proposed roles of these genes require conjecture based on previously acknowledged morphology, rather than being predictive tests of hypotheses. Furthermore, the data presented here and inspection of various primate forelimbs make it equally clear that other modular response systems must also be involved in the determination of final autopod form. For example, some species have altered only one or more of the four posterior digits with relatively little effect on the remainder (e.g., *Daubentonia*—the “aye-aye” and *Perodicticus*—the “potto”)—clearly illustrating that mechanisms are available which can also be employed to further “refine” (in the selective sense) individual elements of the autopod. It should be noted that, at least in primates, such modifications of the posterior rays appear to be primarily consequences of the phalangeal portion of the ray—an observation that correlates well with the morphology of *Daubentonia* and *Perodicticus*. Such changes imply that, as expected, a variety of alternative anatomical dispositions of gene expression become available as morphogenesis becomes increasingly individuated. The fundamental question is the degree to which such ontogenetic hierarchies can be correlated with observed patterns of progressively more local gene expression. Indeed, can selector gene expression patterns be predicted anatomically from repetitively observed patterns of morphological correlation?

Unfortunately at this time there are insufficient data with which to resolve most of these kinds of questions. Nevertheless, despite the existence of these other developmental mechanisms and sample limitations imposed by the vagaries of evolutionary history, it is quite remarkable that morphological changes between hominoids in particular, and anthropoids in general, so closely correspond to the developmental modules defined by murine Hox expression. We therefore believe

that these domains should be considered judiciously during any analysis of primate (or other mammal) limb evolution, along with a continued search for greater mechanistic detail in the regulation of Hox gene expression, and of their downstream target genes and domain boundaries. Moreover, our results suggest that Hox expression and function should be integrated with morphological data to construct parsimonious models with which to account for developmental changes that underlie specific evolutionary transformations. Although a wide variety of potential modular relationships must be carefully considered in the analysis of mammalian autopod evolution, the data presented here strongly suggest that the growth of limb elements is intertwined with Hox genes and their upstream *cis*-regulation and/or downstream targets. Consideration of such facts will certainly aid what promises to be laborious, but highly rewarding examinations of the genetic basis of mammalian autopod evolution.

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