

# Hox Genes and Mammalian Development

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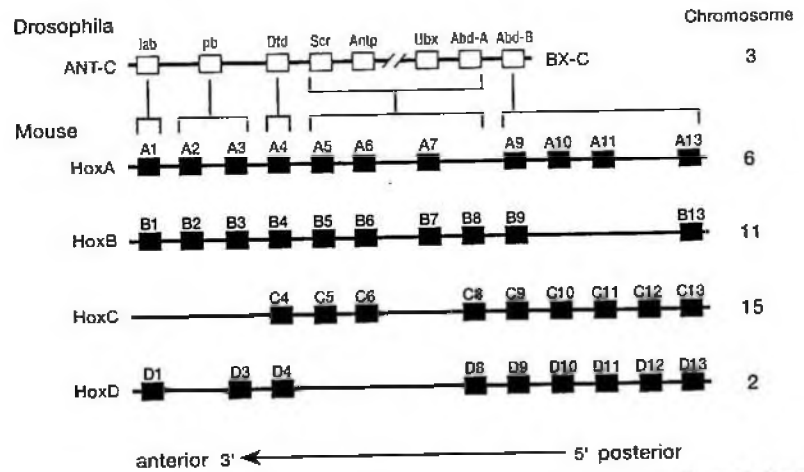
Phylogenetic relationships among all animal and plant species are characterized by the themes of unity and diversity, an observation brilliantly synthesized and explicated by Darwin in his *Origin of the Species*. However, only following the last decade and a half of intense molecular and genetic analyses, principally of three species, *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Mus musculus*, has it become apparent that the foundation for these two themes is deeply rooted in the molecular circuits used to guide the development of all species. Unexpectedly, parallel molecular circuits have been discovered that guide the formation of the basic body plan, the eyes, the heart, and neural circuits, to name just a few systems, in both vertebrates and invertebrates. From such analyses, it has become apparent that structures previously viewed as being disparate, such as the invertebrate and vertebrate eye or the invertebrate trachea and vertebrate lungs, may have common origins. More importantly, such discoveries reemphasize that investigators of invertebrate and vertebrate development can communicate with one another through a common language. Whatever is learned about the molecular circuits guiding development in one species is likely to have direct relevance to revealing similar circuits in other species. Finally, these recent discoveries of developmental parallelism underscore the wisdom of using model organisms, such as bacteria, yeast, *C. elegans*, *Drosophila*, and the mouse, to study all biological phenomena, human or non-human.

In no genetic system have the above principles been more clearly illustrated than with the *Hox* complexes (McGinnis et al. 1984b; Scott and Weiner 1984). The genes within these complexes encode transcription factors of the *Antennapedia homeodomain* class and may be used to establish the body plans of all metazoans of the animal kingdom (Carrasco et al. 1984; McGinnis et al. 1984a; Slack et al. 1993). It was the discovery of the structural and functional similarities between the *Drosophila* and mouse *Hox* complexes that sparked the current revolution in our ability to appreciate the common threads that weave through the molecular fabric that guides development in all species. Figure 1 shows a comparison of the *Hox* complexes from *Drosophila* and the mouse. *Drosophila* has 8 *Hox* genes present in two sub-complexes, the *Antennapedia* and *Bithorax* complexes, whereas the mouse has 39 genes distributed on four linkage groups designated Hox A, B, C, and D. Humans and

mice, and perhaps all mammals, have the same set of 39 *Hox* genes. The mammalian organization is believed to have arisen early in vertebrate phylogeny by quadruplication of an ancestral complex common to vertebrates and invertebrates (Pendleton et al. 1993; Holland and Garcia-Fernandez 1996). From Figure 1 it is apparent that the order of *Hox* genes on the chromosomes of these two species has not altered significantly since the lineages of insects and vertebrates diverged approximately 530 million years ago. On the basis of DNA sequence similarities and the position of the genes on their respective chromosomes, individual members of the four mouse linkage groups have been classified into 13 paralogous families (Scott 1992). Members of a paralogous family share both DNA sequence similarities and similarities in their patterns of expression. As first demonstrated by Ed Lewis for the *Drosophila Bithorax* complex, the position of a homeotic gene within a complex correlates with the expression of that gene along the embryo's anteroposterior axis, a phenomenon termed spatial colinearity (Lewis 1978; Duboule and Dollé 1989; Graham et al. 1989). Furthermore, in vertebrates, there is also a temporal colinearity (Duboule 1994) in which the position of a gene on the chromosome correlates with the time that the gene is activated in the embryo. Thus, a 3' *Hox* gene is activated prior to and in a more anterior region of the mouse embryo than its 5' neighbors.

So far, I have stressed the unity among *Hox* complexes in different species. It is equally important to understand how modulations of these molecular circuits can be responsible for generating the enormous diversity of body plans that enrich our planet. Modifications of this system could occur in a number of different ways, including, for example, simple changes in the number and type of *Hox* genes retained within the complex. In this context, it is interesting that the puffer fish, *Fugu rubripes*, has recently been shown to retain only 31 *Hox* genes, having lost, relative to the mammalian complexes, 5 genes from the Hox D complex alone (Aparicio et al. 1997). Other possible variations that could contribute to the formation of vastly different body plans are changes in *Hox* gene expression patterns, changes in the transcriptional cofactors that interact with *Hox* genes, and qualitative and quantitative changes in the target genes controlled by these transcriptional regulators.

The two homeotic complexes that have been most extensively analyzed are those belonging to *Drosophila* and



**Figure 1.** Comparison of the mouse *Hox* complex with the *Drosophila Homeotic Complex (HomC)*. The *Drosophila HomC* is depicted at the top, and the four mouse linkage groups Hox A, B, C, and D are depicted below. (Open and closed boxes) Genes belonging to the *Drosophila HomC* and mouse *Hox* complex, respectively (Scott 1992). The relationships based on DNA and protein sequence similarities, between the eight *Drosophila HomC* genes and the mammalian *Hox* genes, are denoted by solid lines. Thus, the *Drosophila labial (lab)* gene is most closely related to the mouse *Hoxa1* gene and its paralogous family members *Hoxb1*, *Hoxd1*, and so on. Mice and humans have the identical network of *Hox* genes. All of the mammalian *Hox* genes are transcribed from the same DNA strand. The polarity of transcription is indicated at the bottom of the figure. In the mouse, a 3' *Hox* gene is expressed before and in a more anterior region of the embryo than its 5' neighbors. (A, B, reprinted, with permission, from Chisaka and Capecchi 1991; C-F, reprinted, with permission, from Condie and Capecchi 1994 [copyright Macmillan].)

the mouse. The *Drosophila HomC* genes are used to establish the identity of parasegments through specification of cell identity (Akam 1987). A mutation in *HomC* genes does not change the number of parasegments in the *Drosophila* embryo, but rather the identity of the parasegment. The situation in the mouse is much more complex. First of all, quadruplication, of perhaps the entire genome, appears to have preceded many of the innovations and elaborations that characterize more complex vertebrates, such as an internal skeletal system, cranial ganglia, expansion of the brain with the concomitant remodeling of the entire head, the acquisition of teeth, and so on. In fact, expansion of this gene complex may have contributed directly to the progression from invertebrates to vertebrates by supplying the complexity in this genetic network required to accommodate the development of the more complex vertebrate body plan. In this context, it is important to determine whether paralogous *Hox* genes provide merely redundant functions and thus serve only to increase the fidelity of the system or whether, following the expansion of the complex, novel regulatory mechanisms arose, utilizing multiple *Hox* genes, that directly contributed to the explosion of innovations that characterize vertebrates.

To address such issues, we have initiated a systematic genetic analysis of the mouse *Hox* complex. A critical comparison of *Hox* gene function in *Drosophila* and the mouse may shed light on these broader issues and perhaps reveal whether combinations of *Hox* genes are used in novel ways in vertebrates to modulate the amplitudes,

times, and sites of expression of downstream target genes. The first phase of this project was to generate loss-of-function mutations in the individual *Hox* genes. This phase is nearing completion. We have generated mice with targeted disruptions in 37 of the 39 *Hox* genes. These mice have been used not only to define the individual functions of *Hox* genes during development, but also to explore the interactions among *Hox* genes by introduction of multiple mutations into the same mouse. From this analysis, an understanding of how multiple *Hox* genes cooperate to direct the formation of the myriad of structures under their guidance is emerging. It is anticipated that, in mammals, *Hox* genes will be found to function not as individual entities, but rather as members of a highly integrated network with paralogous genes, adjacent genes in the same linkage group, and even nonparalogous genes in separate linkage groups, interacting positively, negatively, and in parallel with each other to orchestrate the morphological regionalization of the embryo.

In this paper, I describe three stories involving interactions among *Hox* genes in development of the mouse. Each story has been chosen to illustrate particular features of mammalian *Hox* gene function that contrast with the more familiar roles of the *Homeotic* complex in *Drosophila* development. The first story involves the interactions among group-3 paralogous genes in forming the cervical vertebrae, the second involves potential roles of *Hox* genes in the formation and/or maintenance of the segmental paradigm in the hindbrain, and the third involves the role of *Hox* genes in forming the limbs.

### INTERACTIONS AMONG *Hoxa3*, *Hoxb3*, AND *Hoxd3*

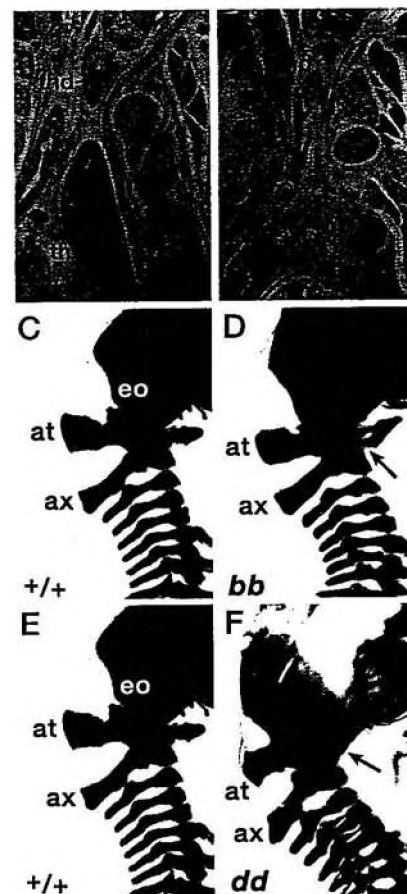
The mutant phenotypes resulting from targeted disruption of *Hoxa3*, *Hoxb3*, and *Hoxd3* are distinct (Chisaka and Capecchi 1991; Condie and Capecchi 1993; N.R. Manley and M.R. Capecchi, in prep). Disruption of *Hoxa3* causes defects in tissues derived from mesenchymal neural crest, whereas *Hoxd3* mutant mice show abnormalities in somitic mesoderm-derived structures. Thus, *Hoxa3* mutant homozygotes are athymic, aparathyroid, have reduced thyroid tissue, and malformations in throat cartilages, whereas *Hoxd3* mutant mice show transformations of the cervical vertebrae, C1 and C2, which acquire characteristics associated with more anterior structures (Fig. 2). In contrast, mice homozygous for a *Hoxb3* mutation have milder defects at lower penetrance both in somitic-mesoderm-derived structures and in neural-crest-derived tissues. Although *Hoxb3* mutant homozygotes also show malformation in the first and second cervical vertebrae, the set of defects is distinct from those observed in *Hoxd3* mutant mice (Fig. 2).

Mice mutant for either *Hoxa3* or *Hoxd3* alone do not show overlapping defects, suggesting that although these two paralogous genes operate in the same region of the embryo, they function in tissues of separate embryonic origins. However, analysis of mice mutant for both genes reveals a more complex picture. In the double mutant, it is clear that the presence of *Hoxd3* mutant alleles exacerbates the *Hoxa3* defects and, conversely, that the *Hoxa3* mutation enhances the *Hoxd3* mutant phenotype (Fig. 3) (Condie and Capecchi 1994). Furthermore, the degree of exacerbation is proportional to the total number of disrupted alleles carried by the mutant mice; i.e., mice heterozygous for the *Hoxa3* mutation and homozygous for the *Hoxd3* mutation, or vice versa, show intermediate mutant phenotypes compared with mice homozygous for a mutation in either *Hoxa3* or *Hoxd3* alone, and the double mutants. These results suggested that both *Hoxa3* and *Hoxd3* are functional in neural-crest-derived and somitic-mesoderm-derived tissues but that the role of *Hoxa3* in forming the atlas and axis only becomes apparent in the absence of *Hoxd3* function. Similarly, the role of *Hoxd3* in the development of neural-crest-derived tissues was only evident in *Hoxa3* mutant homozygotes. In addition, the interactions between the *Hox* genes are very sensitive to the concentration of Hox protein present within these cells, since intermediate genotypes showed intermediate phenotypes.

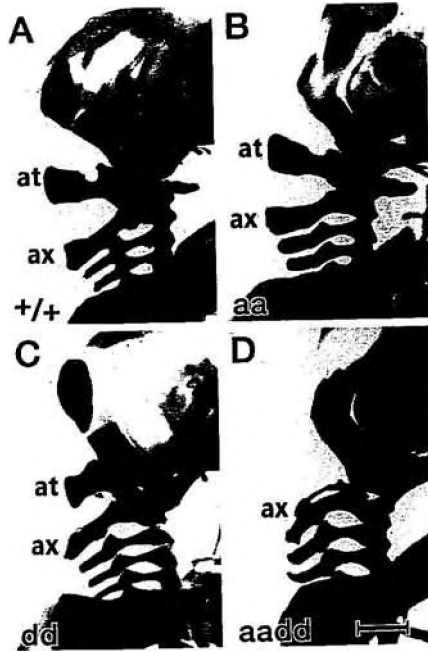
Analysis of *Hoxa3/Hoxb3* and *Hoxb3/Hoxd3* double mutants extended this concept by showing that not only do these paralogous genes operate in multiple tissues in the same region of the embryo, but they also often appear to be performing equivalent functions within these tissues. This is particularly evident in the formation of the cervical vertebrae, where *Hoxa3/Hoxd3* and *Hoxb3/Hoxd3* double mutants have indistinguishable defects, the deletion of the entire atlas (Fig. 4). Similar examples of equivalent functions among these paralogous genes are apparent in other tissues (N.R. Manley and M.R. Capecchi, in prep).

The observation that the contributions of *Hoxa3* and *Hoxb3* to the formation of the atlas and axis appears to be

equivalent in *Hoxd3* mutant homozygotes suggests that the identity of the *Hox* gene operating in the developing tissue is not as critical a factor as the number of *Hox*



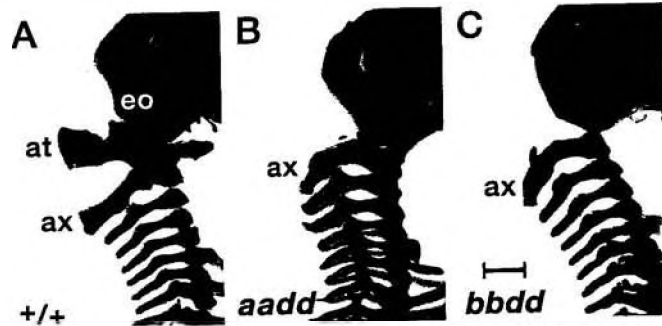
**Figure 2.** Defects observed in *Hoxa3*, *Hoxb3*, and *Hoxd3* mutant homozygotes. Disruption of *Hoxa3* results primarily in abnormalities of tissues derived from mesenchymal neural crest. Thus, *Hoxa3* mutant homozygotes are athymic (B). *Hoxb3* mutant homozygotes show milder and lower penetrant defects in tissues derived from either neural crest cells or somitic mesoderm. (D) Ossified fusion between the anterior arch of the atlas and the dens which is specific to *Hoxb3* mutant homozygotes. *Hoxd3* mutant homozygotes have defects in somitic mesoderm-derived structures where the first and second cervical vertebrae acquire shape characteristics normally associated with their immediate anterior structures. These transformations are shown in panel F. (+/+) Normal, wild-type controls; (aa, bb, and dd) *Hoxa3*, *Hoxb3*, and *Hoxd3* mutant homozygotes; (th, thd, svc, and h) thymus, thyroid, superior vena cava, and heart, respectively; (at and ax) atlas and axis, respectively. (Reprinted, with permission, from Condie and Capecchi 1994 [copyright Macmillan].)



**Figure 3.** Comparison of skeletal defects in mice homozygous for the *Hoxa3* or *Hoxd3* mutation with mice homozygous for both mutations. Lateral views of cleared skeletal preparations of newborn mice. (A) Wild-type newborn, the first cervical vertebra or atlas (at) and the second cervical vertebra or axis (ax) are labeled. (B) *Hoxa3* mutant newborn. The skeletons of *Hoxa3* mutant homozygotes are indistinguishable from wild type. (C) *Hoxd3* mutant newborn displaying the characteristic homeotic transformation of the atlas and axis. (D) *Hoxa3/Hoxd3* double-mutant homozygotes. In these mutant newborns, the complete atlas is deleted and the axis is transformed in shape to resemble a more typical cervical vertebra, C3-C5. +/+, aa, dd, and aadd denote wild type, *Hoxa3* mutant homozygotes, *Hoxd3* mutant homozygotes, and *Hoxa3/Hoxd3* double-mutant homozygotes, respectively.

genes functioning within this tissue. In further support of this concept, mice that are heterozygous for both the *Hoxa3* and *Hoxb3* mutations and homozygous for the *Hoxd3* mutation have the same mutant phenotype as *Hoxa3/Hoxd3* or *Hoxb3/Hoxd3* double-mutant mice. Thus, the perspective changes from a qualitative one to a quantitative one. It is not critical which *Hox* genes are operating in a given tissue, but rather the number of paralogous *Hox* genes functional within that tissue. A molecu-

lar interpretation of this phenomenon would suggest that individual *Hox* genes may not be individually responsible for implementing unique developmental programs (as is observed in *Drosophila*), but rather that multiple *Hox* genes physically function together to mediate a program by controlling common target genes through common *cis* elements, and that for proper development, it is the stoichiometry of paralogous *Hox* genes operating within a developing tissue that is critical.



**Figure 4.** *Hoxa3/Hoxd3* and *Hoxb3/Hoxd3* double mutants show identical cervical vertebral defects, the deletion of the entire atlas. This suggests that in *Hoxd3* mutant homozygotes, *Hoxa3* and *Hoxb3* have equivalent functions. +/+, aadd, and bbdd denote wild type, *Hoxa3/Hoxd3* double-mutant homozygotes, and *Hoxb3/Hoxd3* double-mutant homozygotes, respectively.

In the absence of *Hoxd3*, the roles of *Hoxa3* and *Hoxb3* in forming the cervical vertebrae appear to be equivalent. Nevertheless, *Hoxd3* is more important than its paralogs in mediating the formation of the vertebrae. Similarly, *Hoxa3* predominates over *Hoxb3* and *Hoxd3* in directing the formation of neural-crest-derived structures. These rankings of normal functional roles might derive from either qualitative or quantitative differences. A qualitative difference would postulate that *Hoxd3* and *Hoxa3* proteins physically interact with different transcriptional cofactors, conferring on one Hox protein specificity for directing somitic mesodermal function and on the other mesenchymal neural crest function. Alternatively, a quantitative explanation would be based either on the production of more *Hoxd3* or *Hoxa3* protein in somitic mesoderm or neural crest cells, respectively, or on a higher affinity of *Hoxd3* and *Hoxa3* proteins for common vertebral or neural crest tissue target gene *cis* elements, respectively.

In summary, we have demonstrated extensive genetic interactions among all three group-3 paralogous *Hox* genes in forming multiple tissues in the throat region. The concentration of Hox proteins operating in this region must be very tightly regulated since twofold differences in the concentration of these proteins show marked differences in mutant phenotypes. In many of the genetic interactions exhibited by these paralogous genes, the identity of the mutated gene appears to be less critical than the total number of normal alleles that remain functional within the affected region.

#### ROLES OF *Hoxa1* AND *Hoxb1* IN HINDBRAIN DEVELOPMENT

The mouse hindbrain is a particularly attractive target for molecular genetic analysis. Although it is an enormously complex structure controlling numerous autonomic and voluntary functions, its complexity is generated during development by a rather simple and commonly used paradigm: the generation and diversification of repeated units. Early in development, the mouse hindbrain anlage is transiently subdivided along its rostrocaudal axis into eight metamereric segments called rhombomeres (Vaage 1969; Lumsden and Keynes 1989). Rhombomeres can function as compartments that limit cell movement and thereby create centers capable of independent development and diversification through localized gene activity and cell interactions (Fraser et al. 1990). However, it is also apparent that through inter-compartmental communication, these units can function collectively to form a scaffold upon which a coherent neural network is built (Glover and Petrusdotir 1991; Clarke and Lumsden 1993).

Mouse hindbrain development has become particularly amenable to molecular genetic analysis following identification of members of the *Hox* complex as major components of the molecular network that specifies cell identity within rhombomeres. I will also argue that *Hox* genes are involved in the establishment and/or maintenance of hindbrain segmentation itself (Chisaka et al. 1992; Car-

penter et al. 1993; Dolić et al. 1993; Mark et al. 1993). This is a major departure from what is observed in *Drosophila* development, where the *gap*, *pair* rule, and *segment polarity* genes are used to establish and maintain segmentation, whereas the *Hom C* genes are subsequently used to determine the parasegmental identities (Akam 1987).

Suspicion that *Hox* genes were involved in hindbrain development arose from an examination of their expression patterns (for review, see Krumlauf 1994). The expression boundaries of the rostral (3') set of *Hox A* genes are illustrated in Figure 5. *Hox* gene expression commences early in embryogenesis, typically during the formation of the primitive streak (mouse gestation day 7.5, E7.5). From this posterior position, expression moves rostrally to a specific anterior boundary, characteristic for each *Hox* gene, thus forming a nested set of transcripts extending along the anteroposterior axis. From Figure 5, it is evident that *Hox* gene expression respects rhombomere boundaries. However, the anterior limits of *Hox* gene expression are established prior to the formation of rhombomere boundaries. Thus, the timing of their ex-

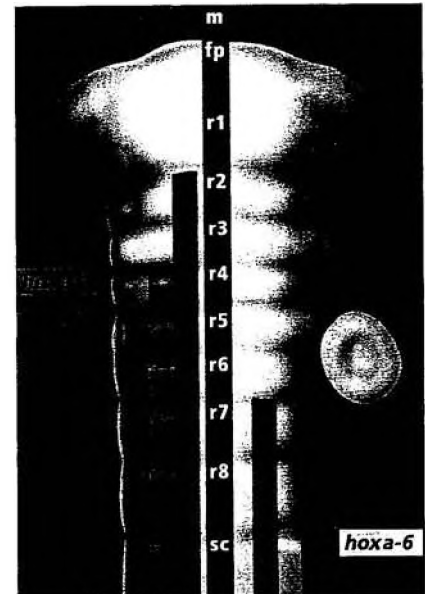


Figure 5. The rostral limits of expression in the murine hindbrain for the 3' members of the *Hox A* linkage group. The hindbrain anlage is transiently subdivided into eight segments called rhombomeres (r). The rhombomere boundaries are depicted by darker shading. The otocyst is positioned lateral to the hindbrain adjacent to rhombomeres 5 and 6. The position of major neural crest production in the hindbrain is depicted by red shading. Note that the anterior boundaries of expression of these *Hox* genes respect rhombomere boundaries. (mfp) Midline floor plate; (sc) spinal cord.

pression is such that *Hox* genes could be involved in the establishment and/or maintenance of rhombomeres, as well as in the specification of cell identities within rhombomeres. What is not evident from Figure 5 is that *Hox* gene expression is very dynamic, changing rapidly during development. For example, expression of *Hoxa1* reaches the presumptive boundary between rhombomere 3 (r3) and rhombomere 4 (r4) by 8 days of gestation and promptly recedes caudally. By E8.5, *Hoxa1* expression is no longer detectable within the hindbrain. In addition, the level of *Hox* gene expression is not uniform from one rhombomere to another. Each rhombomere is characterized by the expression of a unique set of highly expressed *Hox* genes, suggesting that combinations of Hox proteins may be used to specify cell identities.

Targeted disruption of *Hoxa1* in mice results in severe defects in the formation of the hindbrain and associated cranial ganglia and nerves (Chisaka et al. 1992; Carpenter et al. 1993; Dollé et al. 1993; Mark et al. 1993). From the analysis of molecular markers expressed within specific rhombomeres of *Hoxa1* mutants, it is apparent that defects extend from r3 through r8, but, most importantly, r5 is absent. Thus, disruption of this gene affects not only the identity of cells within rhombomeres, but also the integrity of segmentation itself. Whether this is a result of a defect in the specification or in the outgrowth and/or maintenance of specific rhombomeres remains to be determined. Whatever the mechanism, the outcome is that a mutation in a *Hox* gene alters the number of hindbrain rhombomeres present in the mutant embryos.

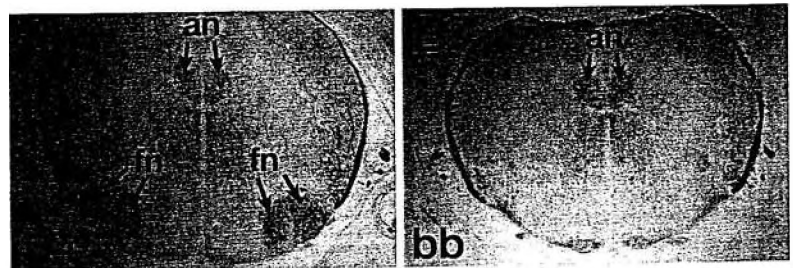
In contrast, disruption of the paralogous *Hox* gene, *Hoxb1*, does not alter the number or pattern of rhombomeres, but rather the properties of neurons within r4 (Goddard et al. 1996; Studer et al. 1996). Specifically, there is a loss of the motor component of the VIIth nerve, which normally innervates the muscles of facial expression (Fig. 6). Normally, the cell bodies of these neurons arise medially in r4 and then migrate caudally into r5. On reaching the r6 boundary, these cell bodies abruptly change course and migrate laterally to their final destination in the pons. In *Hoxb1* mutant homozygotes, this population of migrating neurons is not present, resulting in

paralysis and subsequent degeneration of the muscles of facial expression. Instead, there is a more lateral population of cell bodies in r4, the identity of which needs further characterization. By these criteria, *Hoxb1* function is more similar to that of a *Drosophila Hox* gene than is *Hoxa1*. However, as with the group-3 paralogous *Hox* genes described above, in *Hoxa1/Hoxb1* double mutants both *Hoxa1* and *Hoxb1* mutant phenotypes are exacerbated, demonstrating that both genes are involved in rhombomere specification and/or maintenance, as well as in decisions involving cell identities (M. Rossel and M.R. Capecchi, unpubl.).

In the hindbrain, each rhombomere is characterized by the expression of specific sets of *Hox* genes. This relationship further supports the hypothesis that *Hox* genes are involved in the establishment and/or maintenance of rhombomere compartments. Such an intimate relationship is not observed between *Hox* gene expression patterns and somitic mesoderm compartments. Thus, *Hox* genes do not appear to be involved directly in the establishment and/or maintenance of compartments within the somitic-derived cell lineages. However, it is interesting that in sclerotome-derived tissues, the boundaries of *Hox* gene expression do respect the boundaries between different classes of prevertebrae (i.e., the transitions between the anlage that will form the cervical and thoracic vertebrae, thoracic and lumbar vertebrae, and so on; Burke et al. 1995). The latter observation suggests a role for *Hox* genes in mediating distinction between different vertebral classes.

#### HOX GENES AND LIMB DEVELOPMENT

During development, nested sets of *Hox* gene transcripts are observed not only along the major anteroposterior (A/P) axis of the embryo, but also in subcomponents of the embryo such as the gut, gonadal tissues, and the limbs (Dollé et al. 1989, 1991; Izpisua-Belmonte et al. 1991; Yokouchi et al. 1991, 1995; Haack and Gruss 1993; Roberts et al. 1995). The nested set of transcripts in limbs suggested a role for *Hox* genes in limb patterning. This role was confirmed by the demonstration that loss-

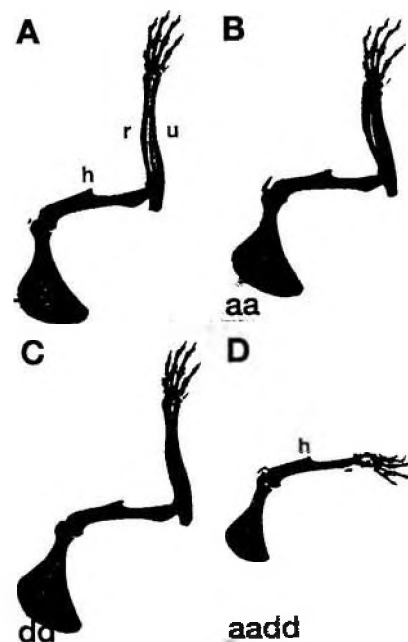


**Figure 6.** Absence of the facial motor nucleus in *Hoxb1* mutant homozygous mice. Transverse, 10  $\mu$ m paraffin sections of wild-type (A) and *Hoxb1* mutant homozygous (B) newborn mice. These sections were subjected to immunohistochemistry with the 2H3 antibody against neurofilament protein. Arrow indicates the abducens nuclei (an) and the facial motor nuclei (fn). (Reprinted, with permission, from Goddard et al. 1996.)

of-function mutations in 5' *Hox* genes cause limb malformations (Dollé et al. 1993; Small and Potter 1993; Davis and Capecchi 1994, 1996; Davis et al. 1995; Favier et al. 1995, 1996; Fromental-Ramain et al. 1996; van der Hoven et al. 1996; Zákány and Duboule 1996). However, the malformations are not readily interpretable in terms of simple patterning paradigms. For example, the set of defects does not exhibit a polarity with respect to the A/P axis. Thus, *Hox* genes do not appear to be used to interpret, in any simple way, a gradient of a morphogen, such as sonic hedgehog (*shh*), whose concentration varies along the A/P axis (Riddle et al. 1993). Instead, these *Hox* mutants exhibit delays in the timing of ossification of autopodal bones. In addition, reduction, complete absence, or improper segmentations occur, and localized reductions in growth of numerous limb bones are apparent. A correlation is also seen between the bones of the autopod that are most affected by the 5' *Hox* mutations and the order in which these bones are made during normal development (Shubin and Alberch 1986), with the most affected bones being those that are made last. The latter result suggests that one common consequence of 5' *Hox* gene mutations may be the reduction in the number of prechondrogenic precursor cells available for limb formation. This would result in the last bony components made being the ones most severely affected, since those cartilaginous elements would be left competing for the few remaining precursor cells.

An additional factor that contributes to the complexity of interpreting the phenotype in terms of patterning defects arises from the fact that the *Hox* genes do not affect formation of the limb at a single point in development but rather at multiple points. *Hox* genes are functional within the limb buds not only during the initial phases of limb development, when the prechondrogenic mesenchymal condensations are accruing, but also at later stages when the outgrowth of the long bones is taking place. Thus, *Hox* gene expression is detectable in the growth plates of all the long bones. The final phenotypic outcome of a mutation in these *Hox* genes results from a summation of defects accumulated at multiple steps in bone development.

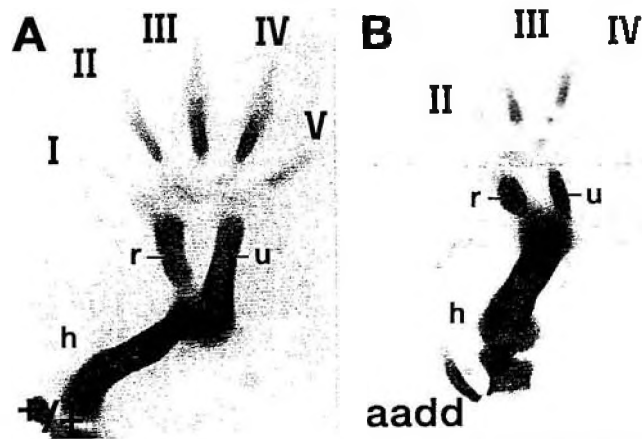
Nevertheless, combinations of *Hox* gene mutations that separately affect limb development have dramatically demonstrated that *Hox* genes do have a role in proximo-distal patterning of the limb. Disruptions of either *Hoxa11* or *Hoxd11* alone result in rather mild defects in the formation of the radius and ulna (Small and Potter 1993; Davis and Capecchi 1994). However, in *Hoxa11/Hoxd11* double mutants, only a vestige of the radius and ulna remains (Fig. 7) (Davis et al. 1995). The initial bifurcation of the radius and ulna anlage is still detected in these double mutants (Fig. 8), but the enormous subsequent outgrowth of these long bones does not occur. The apparently normal initiation of prechondrogenic condensations for radius and ulna in *Hoxa11/Hoxd11* double mutants argues either that *Hox11* genes are not involved in the initial specification of these bones or that the presence of functional *Hoxc11* genes in these mutant mice is sufficient to initiate this process. This latter hypothesis can be tested by generation of mice mutant for all three



**Figure 7.** The loss of the radius and ulna in *Hoxa11/Hoxd11* double-mutant mice. Dorsal view of the right forelimbs from adult mouse skeleton preparations. (A) Wild-type mouse (+/+) with a normal humerus (h), radius (r), and ulna (u). (B) *Hoxa11* mutant homozygote (aa) with a slightly thicker and shorter zeugopod. (C) *Hoxd11* mutant homozygote (dd) with subtle defects in the zeugopod and autopod. (D) *Hoxa11/Hoxd11* double-mutant homozygote (aadd) showing an unexpected dramatic reduction of the radius and ulna. The forepaw is also malformed and rotated 90° compared with wild type. (Reprinted, with permission, from Davis et al. 1995 [copyright Macmillan].)

group-11 paralogous genes. Technically, this is a difficult experiment because loss-of-function mutations in each of the group-11 paralogous genes affect the fertility of the mutant mice.

To test for genetic interactions between adjacent genes on the same linkage group, a series of *trans*-heterozygotes was constructed, in which one chromosome has a mutation in one *Hox* gene and the other chromosome has a mutation in the adjacent *Hox* gene (i.e., *Hoxd11*<sup>-/-</sup>/*Hoxd12*<sup>-/+</sup>). Mice heterozygous for a mutation at only a single *Hox* locus usually show mild defects with low penetrance in the formation of the limb. In each case, the *trans*-heterozygote shows a much more severe and more highly penetrant set of defects (Davis and Capecchi 1996). The severity of the mutant phenotypes in these *trans*-heterozygotes increases from (*Hoxd11/Hoxd12*) through (*Hoxd11/Hoxd13*) to (*Hoxd12/Hoxd13*) *trans*-heterozygotes. The pattern of observed



**Figure 8.** Cartilage condensations in the developing right forelimb of wild-type (A) and *Hoxa11/Hoxd11* double-mutant homozygous embryos (B) at E13.5. In the double-mutant zeugopod, defects are already evident with the reduction of the radius and ulna anlage. However, initiation of the bifurcation of mesenchyme condensations that will give rise to the radius and ulna has occurred in the double-mutant embryos. (Reprinted, with permission, from Davis et al. 1995 [copyright Macmillan].)

defects suggests a dominant role for *Hoxd13* in forming the autopod. From an evolutionary standpoint, this hierarchy may have provided flexibility for modulating the autopod. A limb can be divided into three zones. The most proximal is the stylopod containing one large support bone. This is followed by the middle zeugopod with two similar bones that usually function as a hinge to allow rotation. Finally, at the most distal end is the autopod with several carpals, metacarpals, and phalangeal bones. The most distal end of the limb holds the greatest potential for exploitation by natural selection. Thus, it is advantageous that the last *Hox* genes (paralogous group-13 members) involved in producing the last structures, the autopod, have the greatest sensitivity to potential modulations that have been randomly accrued during evolution. Thus, changes in the expression pattern of the group-13 genes could dramatically alter the form of the autopod. This concept may underlie the well-documented paleontological observation of proximal stability/distal variability in tetrapod limb structures (Hinchliffe 1991).

In summary, we have examined the interactions of *Hox* genes in forming cervical vertebrae, hindbrain, and limbs. In each case, it is apparent that individual *Hox* genes are performing individual functions but that more profound roles are apparent when they act in combination with other *Hox* genes. The observed interactions suggest that multiple *Hox* genes function in concert to regulate overlapping sets of target genes. This suggestion is particularly strong in the interactions observed among the group-3 paralogous genes in formation of the cervical vertebrae and among *Hoxd11*, *Hoxd12*, and *Hoxd13* in formation of the autopod. In each case, the cumulative effect of combining multiple mutations is the deletion of structures, resulting from either lack of specification or lack of prolif-

eration of the precursor cells needed to form the structures. Similarly, the combination of *Hoxa1* and *Hoxb1* mutations results in more extensive deletions of anterior structures than is apparent in mice homozygous for either individual mutation. All of the results, both of single and combined mutations, are compatible with a role of *Hox* genes in the early regionalization of the embryo. In the absence of *Hox* gene function, formation of the axes and germ cell layers of the embryo still occurs. At this point, the *Hox* genes are activated to initiate the formation of multiple tissues and structures specific to each region of the embryo by conferring positional value along the major axes of the embryo. Perhaps the most primitive function of *Hox* genes is their innate ability, through their chromosomal organization, to convert a series of temporal signals into morphological direction, a conversion of time's arrow into a spatial vector.

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