Hox-4 genes and the morphogenesis of mammalian genitalia

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We examined the temporal and spatial expression patterns of the homeo box HOX-4 complex genes during the morphogenesis of the genitalia of mice. The results show that only those Hox-4 genes that are expressed very posteriorly in the trunk, or very distally in the limbs, seem to be involved in the patterning of the genital tubercle. This is consistent with the idea of “temporal colinearity”, which suggests that the very last structure to require patterning during vertebrate development will express Hox genes located at the 5′ extremity of the HOX complexes. We also show that genital tubercle mesenchyme can respecify pattern in the chicken wing bud. This finding reinforces the concept of the uniformity in the patterning mechanisms along the various axes of the body.

[Key Words: Pattern formation; homeo box; development]

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The mouse genome contains four clusters of Antennapedia-like homeobox-containing genes: the HOX-1, HOX-2, HOX-3, and HOX-4 complexes (Kessel and Gruss 1990). This family of transcription factors has been generated, during evolution, by duplications and diversification of an ancestral homeo-box-containing gene complex. Within each cluster, the expression of these genes during development is regulated according to the same rules. First, each gene displays a spatially restricted expression domain along the anteroposterior (A-P) axis of the mouse embryo, in neural tissues and various mesoderm derivatives. These expression domains are related to the position of the genes in the Hox complexes so that genes located 3′ have expression domains that extend more anteriorly than the domains of 5′-located genes (the structural colinearity, Gaunt et al. 1988; Akam 1989; Duboule and Dollé 1989; Graham et al. 1989). Second, transcripts of 5′-located genes appear slightly later than those encoded by more 3′-located genes (the temporal colinearity, Dollé et al. 1989, Izpisúa-Belmonte et al. 1991a). Five adjacent Hox-4 genes, which are related to the Drosophila Abdominal-B (AbdB) homeotic gene, are specifically expressed in progressively more posteriordistal domains in the limb bud mesenchyme as well as in the posterior-most areas of the embryo (Dollé et al. 1989, 1991; Izpisúa-Belmonte et al. 1991a). Hence, the Hox-4 gene products are probably used as positional cues during pattern formation at different times and along different axial structures.

During fetal development of mammals, the last structure to develop along an independent axis is the genital tubercle (GT). This bud will ultimately give rise to the external genitalia, namely the penis in males and the clitoris in females. In the penis of adult mice, different structures are arranged along the proximodistal (P-D) axis such as the proximal erectile tissue or corpus cavernosum penis (c.c.), and the distal os penis (also termed baculum, os priapi, see Fig.1). An os penis is found in certain groups of mammals, including rodents and some primates (Ruth 1934), where it generally consists of a proximal segment (p-segment) and a distal segment (d-segment), which differ in their mechanisms of ossification and times of differentiation (Murakami and Mizuno 1984).

In mice, the tubercle appears as a bud of proliferating mesenchyme around day 11 of gestation. The development of the GT is initially identical between males and females, roughly until birth. At first, three cellular condensations appear in the immature mesenchyme at day 16, which are the rudiments (or blastemas) of the c.c., the p- and d-segment of the os penis (Fig.1). These condensed cells, located above the urethra, do not differentiate before birth. In males, the proximal rudiment of the os penis differentiates first and begins to ossify by day 2 after birth (Glucksmann et al. 1976). The p-segment ossifies directly as a membrane bone, although some cartilage is formed at its proximal extremity, allowing bone growth by endochondral ossification [the same mechanism as for long bones of the limb]. The d-segment ossifies several weeks after birth through a fibrocartilage intermediate. These three rudiments fail to differentiate in females, mainly because of the absence of androgen hormones. Only a tiny os clitoridis, analogous to the p-segment of the os penis, is formed in rodents by direct ossification without any cartilage precursor. It has been shown that the prenatal stage of mesenchymal condensations is independent of androgens, whereas the differentiation of chondrocytes and osteocytes is caused by...
androgens and occurs only in males and androgen-treated females [Glucksman and Cherry 1976; Murakami and Mizuno 1984].

The development of the GT shares some striking similarities with the developing limbs. As in the limb, the GT epithelium has an inductive effect, although no discrete structure resembling an apical ectodermal ridge has been described for the GT. Because of these apparent ontogenetic similarities between the genital bud and the limb buds, we searched for evidence supporting a possible identity of signaling mechanisms, via homeo gene products, along this late-developing "axis." We therefore analyzed the expression of six homeo genes of the HOX-4 complex in the developing mouse GT and genitalia, from its earliest appearance (gestational day 10.5) until day 7 after birth. We show that the Hox-4 genes are expressed in a tissue-specific manner in the GT. Their expression is coordinately regulated, since 5' genes constantly display a higher transcript abundance than 3' neighboring genes in the GT mesenchyme. A decrease in the transcript abundance occurs during late gestation, such that only 5' genes remain significantly expressed shortly after birth. Hox-4 gene expression patterns are identical between male and female individuals in prenatal, undifferentiated GT. During the first week postpartum, transcripts remain associated in males with the differentiating c.c. and perichordium of the p-segment of the os penis. The observed absence of spatially distinct expression domains along the GT axis supports the idea that the penis is not, in the strict sense, a segmented structure. However, some basic morphogenetic mechanisms must be conserved between the "genital axis" and other body axes, as GT mesenchyme cells express a polarizing activity when grafted in chick wing buds. These results are discussed in relation with the expression logic of the Hox genes along the other body axes, and a general scheme is proposed to account for a possible function of this gene network during development.

Results

Hox-4 expression during outgrowth and early development of the GT

Hox-4 gene transcripts are expressed to different extents in the immature mesenchyme of the GT prior to formation of the blastemas [Fig. 2A,B; day 11.5 postcoitum (p.c.) and 13.5 p.c., respectively]. We could not detect any Hox-4.4 transcripts and the 5'-located neighboring gene, Hox-4.5, is expressed only weakly in the GT. In contrast, genes located in more 5' positions (Hox-4.6, Hox-4.7) show increasing expression levels culminating with the Hox-4.8 gene, which displays very strong labeling. This gradient in labeling intensities can be appreciated when one compares the labeling in the GT versus that seen in hindlimb mesenchyme [Fig. 2A]. The same pattern is seen earlier, at day 10.5 p.c., in the presumptive area of the GT, that is, in ventral cells surrounding the cloacal epithelium [not shown]. Expression in the GT mesenchyme is tissue-specific, as 5' genes (Hox-4.7, Hox-4.8) have a well-defined boundary of expression and are not expressed in more dorsal cells outside the GT [Fig. 2A,B]. In contrast, more 3'-located genes are expressed in cells at the same level along the body axis but not in the GT, as exemplified by Hox-4.4 transcripts in the neural tube and prevertebrae [Fig. 2A,B]. At these stages and despite strong differences in labeling intensities [discussed later], there are no clear differences in the spatial extent of the expression domains in the GT. The maximal expression of Hox-4.8 is also visible in transverse sections of a 13.5 p.c. GT [Fig. 3A,B, cf. the signal intensities for each probe in the GT vs. the tail]. In sections of the proximal part of the GT, Hox-4.8 is again expressed predominantly in the anlage of the c.c. [Fig. 3B, visible in the bright-field view]. Hox-4.2 (an anterior gene from the same complex) transcripts are not detected in the GT mesenchyme but are specifically expressed in various epithelial components [see below].

The ratios of various Hox-4 transcripts remain similar during the following gestational days, which are characterized by the appearance of mesenchymal condensations, the rudiments of the c.c and os penis. Hox-4.8 expression is strong and the abundance of RNAs encoded by more 3'-located genes in the GT decreases (Figs. 2C and 3C,D). In the 15.5-day-old GT, labeling is homogeneously distributed in the distal extremity of the GT [Fig. 3C], although no signal is detected in the prepuce cells. In more proximal sections, cells of the c.c. rudiment are preferentially labeled by the Hox-4.8 probe. By day 17.5 p.c, the signal is seen in the rudiments of the c.c. and the p-segment of the os penis [Fig. 2C]. On the basis of differences in the histology of the gonads, the fetal sex can be determined, at least by day 14.5 p.c. Throughout all of these developmental stages, no difference in the hybridization patterns could be detected between male and female individuals.

We quantified the intensities of expression of the different Hox-4 genes by measuring the gray levels of selected areas on neighboring histological sections. For each probe, the values obtained were corrected with respect to the different background levels measured in negative (anterior) parts of the same embryo. In the GT it appears that the amount of transcripts encoded by the Hox-4 genes increases progressively when moving toward the 5' part of the complex [Fig. 4]. This observation is also true when the distal part of a limb at day 12.5 p.c. is analyzed similarly [Fig. 4].
Pattern formation in genitalia

**Figure 2.** Comparative analysis of Hox-4 gene transcripts during the development of the GT. In situ labeling obtained with riboprobes specific for Hox-4.2, Hox-4.4, Hox-4.5, Hox-4.6, Hox-4.7, and Hox-4.8 on parallel adjacent sections of GT is photographed under dark-field illumination. (Left) Bright-field illumination of one section for the histology. (A) Transverse horizontal section of a GT at day 11.5 p.c. At this stage, shortly after budding, the GT consists of a small mesenchymal mass surrounding the urogenital sinus (us). Compare the differential labeling obtained with various probes in the GT vs. the hindlimb bud (hlb) mesenchyme, or the neural tube (nt). (B) Sagittal section through a 13.5-day p.c. GT. The corresponding area is boxed in the fetus scheme of Fig. 3. (pv) Prevertebra. (C) Sagittal section through a 17.5-day p.c. GT. (u) Urethra; (r) Rectum. (D) Sagittal section through the penis of a newborn mouse at the time of blastema formation. Note the specific expression of Hox-4.2 in the prepucial epithelium at the tip of the GT.

**Expression of the Hox-4 genes in sexually differentiated genitalia**

An overall decrease in the abundance of Hox-4 transcripts in the genitalia is observed between day 17.5 of gestation and birth. In males, Hox-4.5 and Hox-4.6 signals are barely above background in the penis at birth, whereas Hox-4.8 is still expressed more strongly than Hox-4.7 [Fig. 2D]. During the first week after birth, major morphological changes appear in the GT as a result of sexual differentiation. In newborn males, Hox-4.8 labeling is diffuse in the distal mesenchyme of the penis but is reinforced proximally in cells of the p-segment of the os penis and of the c.c. [Fig. 5A]. Four days later, Hox-4.8 expression remains weak and diffuse in distal mesenchyme [Fig. 5B]. However, the signal is clearly reinforced in the c.c. and the perichondrium surrounding the cartilaginous nodule in the differentiating os penis [Fig. 5C,D, arrows]. In newborn females, the expression of Hox-4.8 is weak and diffuse in the clitoris anlagen [not shown].

During these various stages, Hox-4.2 transcripts are still absent from the mesenchyme cells but are expressed in numerous epithelial structures along the genitoexcretory apparatus. This epithelial-specific expression of Hox-4.2 is observed throughout development, early in the epithelia of the urogenital sinus, Wolffian, and Müllerian ducts [not shown], as well as later in the epithelia of the ureters, the urinary bladder [Fig. 6A], the proximal urethra [Fig. 3B], and the epididymis [Fig. 6D]. Hox-4.2 transcripts are also expressed in a very limited group of cells at the base of the immature GT [Fig. 6B; day 12–14.5 p.c.]. These cells could be precursors of the prostate, as Hox-4.2 transcripts are found later in a limited area at the base of the urinary bladder that corresponds to the prostate primordium [Fig. 6C]. Hox-4.2 is also expressed in the prepubic epitelium and in the developing prepubic glands [Figs. 3C and 6E]. In contrast, Hox-4.2 is not expressed in the most distal urogenital sinus epithelium, which gives rise to the distal (penian) urethra. This limited region is the only urogenital epithelium that expresses the Hox-4.8 gene at various developmental stages [e.g., Fig. 2A,C, 3D].

**Structural colinearity along the genitoexcretory tractus**

Analysis of serial histological sections revealed distinct A-P domains of Hox-4 gene expression in mesenchyme
derivatives of male and female urogenital tracts during late gestation and in newborn animals. In males, Hox-4.8 is expressed in the mesenchymal walls of the urinary bladder, posterior ureteres, and vas deferens [Fig. 7A]. However, its expression does not extend anteriorly up to the epididymis. Hox-4.7 transcripts overlap with those of Hox-4.8 [although Hox-4.7 is expressed very weakly in the urinary bladder mesenchyme] but extend more anteriorly in the vas deferens. Hox-4.5 expression extends even more anteriorly into the posterior parts of the epididymis and testis while more posterior organs are poorly labeled [e.g., the bladder in Fig. 7A]. In females, Hox-4.8 is expressed in the urinary bladder and in the posterior regions of the uterus mesenchyme [Fig. 7C], whereas Hox-4.7 transcripts are detected in the whole uterus wall but do not extend more anteriorly in the oviduct. Hox-4.6 and Hox-4.5 transcripts are found in the oviduct, the latter being also expressed in a limited part of the ovary [Fig. 7B]. These two genes are not expressed significantly in the urinary bladder and uterus [Fig. 7C]. Hox-4 genes are therefore expressed in spatially restricted domains in both male and female developing urinary and genital organs [schemes in Fig. 7].

Polarizing activity of the GT
As Hox genes seem to have similar functions in patterning along the trunk and limb axes [Dollé et al. 1989] and because of the particular distribution of Hox transcripts in the developing GT, we analyzed the GT for its polarizing activity in the chicken wing bud system. Polarizing activity is defined as the potential of some cells [e.g., cells at the posterior margin of the early limb bud] to respecify

Figure 3. Comparative analysis of Hox-4 gene transcripts on cross sections of GT. Labeling obtained with three probes is shown, including a 3' gene (-4.2) and a 5' gene (-4.8). (A,B) Two cross sections of the GT and tail of a 13.5-day p.c. fetus. A is more distal than B. Hox-4.2 is not significantly expressed in the GT mesenchyme but is strongly expressed in tail structures. The signal intensity is quite similar in GT vs. tail for Hox-4.5, whereas Hox-4.8 is expressed very strongly in the GT. Note the specific expression of Hox-4.2 in the urethra epithelium. [tl] Tail, [nt] neural tube, [u] urethra. (C,D) Two cross-sections of a 15.5-day p.c. GT. Only Hox-4.8 transcripts are predominant in the GT mesenchyme [gm]. Hox-4.2 is expressed in the prepucial epithelium. [pr] Prepuce, [u] urethra, [a] anus.

Figure 4. Silver grain densities in selected areas of either a day-13.5 p.c. GT [solid columns] or the distal tip of a day-12.5 p.c. developing limb [hatched columns]. Numbers at left design the mean gray value of the selected area. The background level [adjusted to -50 for all probes] is shown by the broken line.
The grafts appeared to have given rise to a swathe of muscle in addition to the soft tissue bump. When tissue from the proximal part of the GT was tested, no positive results were obtained (three cases). When the entire GT was disaggregated, reaggregated, and used as a graft, one of five limbs had the pattern 2 2 3 4. In another series of experiments, mesenchyme was taken from the dorsal tip of tubercles of 16−17 day p.c. mouse embryos and one out of five grafts gave an extra 2. Thus, at day 12.5 p.c., the cells at the tip of the genital bud possess a weak polarizing activity that is functionally similar to that present in the posterior part of the limb buds (the polarizing region; Saunders and Gasseling 1968), in the floor plate (Wagner et al. 1990), and in Hensen’s node (Horn-...
bruch and Wolpert 1986). This activity appears to be retained until at least day 16-17 p.c.

**Discussion**

**Genital vs. limb buds: is the penis a true axis?**

Morphogenesis of the GT is comparable, to some extent, to that of the limbs. In both cases, the initial mesenchyme proliferation [budding] is initiated by local epithelium–mesenchyme induction. Limb growth is maintained by a discrete epithelial thickening, the apical ectodermal ridge [AER], which has no visible counterpart in the GT epithelium. However, removal of the GT epithelium provokes a loss of tissue differentiation [Murakami and Mizuno 1986]. The basic sequence of differentiation along the proximodistal axes of the limb and GT is quite similar, in that some condensations of mesenchymal cells [blastema] appear. However, the GT does not appear like a segmented structure, in the strict sense, because its development involves only the correct P-D differentiation of several cell types such as the erectile tissue of the c.c., the cartilage, and bone of the os penis rather than the differential fate of reiterated structures such as those in limbs [the penis has no articulation]. In limbs, proximal “segments” are determined earlier than distal ones [Saunders 1948]. There is no significant delay in the appearance of the genital mesenchyme blastemas, but evidence for a successive P-D timing in the differentiation potential of the blastemas has been reported [Murakami and Mizuno 1986]. In contrast to the similarity between the limb and the external genitalia along the P-D axis, the penis [and clitoris] have no particular A-P pattern but, rather, a sort of imperfect radial symmetry.

The expression of genes of the HOX-4 complex in both the developing limb and GT suggests that patterning may have a common basis. In both limb buds and GT, Hox-4 transcripts are first expressed throughout the mesenchymal component, and the expression persists until
The AbdB-like Hox-4 genes are sequentially activated, in a 3' → 5' direction, between day 8 and 10 of gestation (the temporal colinearity; Izpisúa-Belmonte et al. 1991a). We proposed earlier that the proper establishment of their progressively overlapping domains of expression (the structural colinearity) is dependent on this precise timing of activation. We now show that the last gene to appear during this process is always the one that is expressed most strongly. This feature, the quantitative colinearity, had already been observed in the distal parts of developing limbs where genes located at 5' positions are expressed systematically stronger than their 3'-located neighbors, culminating with Hox-4.7 and Hox-4.8 (Dollé et al. 1989, 1991). It is thus possible that the different outcomes obtained from various combinations of homeo proteins might not rely only on subtle differences in protein content but might rather be the result of the massive presence of one novel homeo protein species in a given structure at a given time. Alternatively, protein concentrations may not be critical in setting up combinations and quantitative colinearity may be the result of some intrinsic properties of the system, without a real functional significance.

The Hox-4.4, Hox-4.5, Hox-4.6, Hox-4.7 and Hox-4.8 genes are phylogenetically related to the Drosophila AbdB homeotic gene (Izpisúa-Belmonte et al. 1991a and references therein), which specifies the identities of posterior parasegments in Drosophila embryos (references in Peifer et al. 1987) and is thought to be controlled by a set of parasegment-specific regulatory units defined by the infra-abdominal groups of mutations tab-5→tab-9. The recent observation that more posterior parasegments contain progressively higher amounts of AbdB proteins (DeLorenzi and Bienz 1990) suggests that quan-
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Constitutive colinearity might have an important function. In *Drosophila*, the genitalia are thought to derive from abdominal segment 8 (A8) and the analia from A9 and/or A10 (Dubendorfer and Nöthiger 1982; Sanchez-Herrero et al. 1985; discussed in Simcox et al. 1991), and a loss-of-function mutant in the extreme posterior AbdB regulatory sequences [i.e., *iab-8, iab-9*] result in strong truncations or absence of genitalia [Karch et al. 1985]. The strong expression, in vertebrates, of the last two *Hox*-4 genes in the GT emphasizes the parallel between the functions of AbdB and those of the AbdB-related murine genes in specifying the posterior parts of vertebrate and invertebrate bodies.

**Patterning is not sex-dependent**

The early development of the GT proceeds identically in males and females until about birth. After birth, the mesenchymal blastemas [for the c.c., the p-, and d- segment of the os penis] fully differentiate only in males. In females, a small focus of ossification is seen in the clitoris, which will further differentiate into a tiny clitoridian bone in the adult mouse. The sexual dimorphism in the structures deriving from the GT is known to depend on the influence of androgen hormones [Glucksmann and Cherry 1972]. During the prenatal period, we did not detect any difference between the expression patterns of *Hox*-4 genes in the GT of male versus female fetuses, neither qualitatively nor quantitatively. Some differences between sexes are detected after birth at stages when only *Hox*-4.8 shows significant labeling. In the penis, *Hox* transcripts are proximally restricted to cells of the c.c. and cells surrounding the cartilage of the p-segment of the os penis. In females, no restriction of *Hox*-4.8 transcripts is observed, rather, a weak and diffuse distribution in the mesenchyme of the clitoris anlage is seen. It is therefore probable that the patterning information encoded by these genes is identical in both males and females but is interpreted differently depending on the presence or absence of androgen hormones.

*Hox*-4 genes are expressed in different spatial domains in the mesenchyme associated with the urinary and genital tract. This spatially restricted expression is seen in males [along the vas deferens, epididymis, and testis] and females [along the uterus, oviduct, and ovary]. In all cases, 3′-located genes have more anterior boundaries of expression than 5′ genes, which are most strongly expressed in posterior structures [e.g., *Hox*-4.8 in the urinary bladder mesenchyme]. Different spatial domains of *Hox* gene expression have been described previously in several mesoderm-derived structures. Such tissues have either a segmented organization, such as the sclerotomes or the mesonephros, or have no apparent segmentation, such as the gut mesenchyme [Gaunt et al. 1988; Holland and Hogan 1988]. We show here that the colinearity between the arrangement of *Hox* genes in their complex and their respective spatial expression domains is also true for the posterior *Hox*-4 genes along the developing urogenital system. These genes remain significantly expressed during late gestation and even after birth. Such well-defined transcript domains may have some relevance for correct determination of genital structures according to A-P position and be related to the ability of mesenchyme from the genital tract to control epithelial differentiation in organ culture [Cunha et al. 1991].

**Uniformity of patterning mechanisms along the body axes**

The observation of the *HOX*-4 complex gene expression patterns during limb and trunk development led us to propose a molecular basis for the uniformity of patterning mechanisms along the different body axes. Such a uniformity had been postulated by Hornbruch and Wolpert [1986], who showed that chicken Hensen's node has polarizing activity when grafted at the anterior margin of developing wing buds. Other regions of the embryo such as the floor plate [Wagner et al. 1990] have since been reported to contain this activity. We show here that the genital bud also has polarizing activity in the chicken wing. However, we could obtain the duplication of a digit 2 only [the most anterior digit] even though the grafted cells express only the very posterior genes [these genes are probably required for the specification of digits 3 and 4; see Izpisúa-Belmonte et al. 1991b]. The presence of an extra digit 2 could be related to an incomplete resetting of the chicken *HOX*-4 complex [Izpisúa-Belmonte et al. 1991b; Duboule 1991], as a result of, for example, a low amount of polarizing factor or to a lower efficiency in the wing bud of the factor present in the GT. From these data, we conclude that the mechanisms of positional signaling along all embryonic axes involve the same regulatory network of *Hox* genes. Temporal colinearity implies that different *Hox* genes will be active in different structures if these structures develop at different times. Thus, anterior (early) genes such as *Hox*-2.9 or *Hox*-2.8 will exert their functions in anterior (early) structures like the hindbrain [Wilkinson and Krumlauf 1990 and references therein], intermediate genes such as *Hox*-4.4 or *Hox*-4.5 will be involved later in the patterning of the nascent limbs [Dollé and Duboule 1989], and very posterior (late) genes will be patterning the last, most posterior, structure, the genitalia. This system could thus be seen as the superimposition, in phase, of two processes: one controlling cell proliferation and migration during the appearance and growth of embryonic axes, and the other one patterning the newly formed structures through the activation, in parallel, of the *HOX* gene network.

**Material and methods**

**In situ hybridization, probes, and counting**

In situ hybridization was performed with 35S-labeled riboprobes specific for *Hox*-4.2, *Hox*-4.4, *Hox*-4.5, *Hox*-4.6, *Hox*-4.7, and *Hox*-4.8. The DNA templates used to prepare the riboprobes are described elsewhere, together with the general pattern of tran-
Mouse fetuses were recovered from natural matings between ICR mice. The day of the vaginal plug was designated as day 0.5 of gestation. Fixation of the fetuses in 4% p-formaldehyde, paraaffin-embedding, sectioning, in situ hybridization, emulsion autoradiography, and toluidine blue staining were performed as described previously [Dollé and Duboule 1989], except that prehybridization was omitted. Sections of from 5 to 7 μm in thickness were cut. Six adjacent sections on the paraaffin ribbon were collected on six different slides to be hybridized to various probes. The following sections were systematically placed on the same six slides so that any differential labeling might not be caused artifically by the progression of the sectioning. Fetuses were analyzed at each gestational day from day 10.5–18.5 p.c., as well as newborn mice at day +1, +4, and +7. For fetuses older than day 15.5 p.c. and newborns, only the posterior part of the trunk was dissected and embedded. Several mice were analyzed for each developmental stage. In particular, GTs were sectioned along two different orientations: sagitally and transversely (cross section of the GT). From day 14.5 and later, the sex of the fetus can be deduced from the histology of the gonads, and male and female individuals were analyzed. Some sections of newborn mice were stained in parallel with alcian blue, which allows visualization of the chondrifying areas.

Gray levels were measured on serial-selected areas of the samples by means of an image analysis system [EMBL, Olivo et al. 1988]. For each case, a mean value and standard deviation were computed that reflect the grain densities. For each section, the numbers obtained were corrected for the background of the section, and the final numbers were expressed after homogenization of all background values to 50. The specific activities of the various probes were comparable. The observation reported here for the mouse GT and developing limbs [Fig. 4] is also valid for the expression of the chicken Hox-4 genes in developing wings [Izpisúa-Belmonte et al. 1991b; P. Dollé et al., unpubl.]. There is no direct correlation between the intensity of the signals and the length of the various probes used in all of these studies.

GT grafts in chicken wing buds

The GTs were dissected from 12.5-day p.c. mouse embryos (C57/BL6 or C57 black and tan). On one occasion, embryos were taken at ~16 days p.c. The GTs were placed in 2% trypsin at 4°C, for 45 min. The tissue was then transferred into minimal essential medium (MEM) plus 10% calf serum at 4°C, and the epithelium was removed. The tissue for grafting was cut out of the distal one-third of the GT and comprised of either the right or left side (200 μm²). In a small series of experiments, the tissue was taken more proximally and consisted of cells from the middle one-third. In addition, in another experiment, the mesenchymes of three entire GTs were disaggregated by rapid flushing through a pasteur pipette, and the resulting cell suspension was then centrifuged in an Eppendorf tube to form a pellet. The pellet was incubated at 38°C for 1 hr to consolidate and then cut into fragments for grafting. The grafts were placed at the anterior margin of wing buds of stage 20 chick embryos [Hamilton–Hamburger stages]. The site for the graft was prepared by cutting beneath the apical ridge and easing it away from the limb mesenchyme to form a loop. The graft was then placed beneath the loop, and the host embryos were reincubated at 38°C. The embryos were inspected on the day following grafting to check that the graft had remained in place.

Six days after the graft, the chick embryos were removed from the eggs, and the torso with attached limbs was fixed in either 5% trichloroacetic acid or formal saline. The tissue was then stained for cartilage matrix with either alcian green or alcian blue, dehydrated, cleared, and viewed as whole mounts to show the pattern of wing elements that developed from the operated bud. Wings that had developed an additional digit 2 were then embedded in wax, and sectioned at 7 μm. The sections were stained with Biebrich scarlet to reveal the position of the grafted mouse cells [Cairns 1965].

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