Complementary homeo protein gradients in developing limb buds

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A new human homeo box-containing gene designated *Hox-5.2* was cloned and mapped to human chromosome 2. This homeo box is related in sequence to *Abdominal-B*, a *Drosophila* homeotic gene that specifies identity of posterior segments. An antibody probe was made using a human *Hox-5.2* fusion protein and was found to stain posterior regions of mouse, chicken, and *Xenopus* embryos. Unexpectedly, when the distribution of *Hox-5.2* antigen was compared with that of *XlHbox 1* antigen, a non-overlapping and mutually exclusive pattern was detected (e.g., in developing limb buds, intestine, and somites). Regions expressing *Hox-5.2* do not express *XlHbox 1* protein, and vice versa. *Hox-5.2* antigen is detected strongly in developing fore- and hindlimb buds, where it forms a gradient of nuclear protein throughout most of the mesenchyme. This gradient is maximal in distal and posterior regions. *Hox-5.2* expression is activated in *Xenopus* limb regeneration blastemas, as expected for any gene involved in pattern formation. As described previously, a gradient of *XlHbox 1* protein can be detected in the forelimb. The latter gradient has the opposite polarity to that of *Hox-5.2*, i.e., maximal in anterior and proximal mesoderm. These two opposing gradients (and possibly others) could be involved in determining positional values in developing limb buds.

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How is genetic information translated into the spatial patterns of tissues that determine, for example, the differences between an arm and a leg? In vertebrate development it has been argued that pattern is established by combinations of mechanisms such as positional information, developmental fields, and morphogenetic gradients (Weiss 1939; Huxley and De Beer 1934; Child 1941). It has been suggested that gradients of morphogens (presumably small molecules or growth factors) provide cells with positional values (Wolpert 1969). The type of cellular response depends on the differentiation program of the responding tissue (perhaps provided by particular combinations of nuclear transcription factors). The vertebrate embryo is subdivided into fields of cells that respond to positional information signals by forming specific organs. In salamander embryos, fields for the formation of the forelimb, hindlimb, balancer, eye, and ear have been reported (Weiss 1939; Child 1941). Homeo box genes are expressed in defined bands along the antero-posterior (A-P) axis of *Drosophila* embryos, and in vertebrates similar patterns of position-specific expression have been reported (reviewed by Holland and Hogan 1988; Wright et al. 1989). It has been proposed that these regions of homeo box expression could provide a molecular explanation for the establishment of developmental fields in the vertebrate embryo (Oliver et al. 1988b).

The existence of a gradient in vertebrates with all the expected properties of a diffusible morphogen is suggested by the finding that retinoic acid is 2.5 times more concentrated in the posterior region than in the anterior region of the developing chicken limb bud (Thaller and Eichele 1987). A gradient of nuclear protein in forelimb mesenchyme has been described for the *Xenopus laevis* Homeo box 1 (*XlHbox 1*) gene. This gradient is maximal in the anterior and proximal portion of the forelimb buds in both *Xenopus* and mouse embryos, and is absent in the hindlimb buds (Oliver et al. 1988b). The forelimb arises from a region contained within the early field of *XlHbox 1* expression, whereas the hindlimb does not (Oliver et al. 1988b). Expression of the homologous newt gene (*NvHbox 1*) also has been studied during limb regeneration (Savard et al. 1988). Another homeo box gene, expressed in interdigital regions, has been implicated in limb morphogenesis (Benoit et al. 1989; Hill et al. 1989). Limb bud development is an interesting model for the study of pattern formation because positional values can be changed by simple experimental manipulations, resulting in malformations such as duplications or deletions of digits (Bryant and Muneoka 1986). Gradients of other proteins have been reported in vertebrates, but their function in development is unclear (Constantine-Paton et al. 1986; Rosa et al. 1988).

In *Drosophila* embryos, protein gradients have been

reported for the products of some development-controlling genes. Genes such as *caudal* (Macdonald and Struhl 1986; Mlodzik and Gehring 1987), *bicoid* (Driever and Nüsslein-Volhard 1988a,b), and *dorsal* (Steward et al. 1988) are expressed in gradients involved in establishing positional information in the embryo.

In this paper we report the isolation of two new human homeo box genes that belong to a gene cluster located in chromosome 2. One of these genes, called human *Hox-5.2*, has a homeo domain similar to that of the *Drosophila Abdominal-B* gene. *Abdominal-B* is expressed in posterior segments of the fly embryo. In vertebrates, several genes with this type of homeo domain have been reported (Boncinelli et al. 1988; Scott et al. 1989) and one of them has been shown to have a posterior domain of expression (Sharpe et al. 1987). An antibody against a human *Hox-5.2* fusion protein was prepared and found to immunostain the posterior region of the vertebrate body. The antigen recognized by this antibody is expressed in a transient gradient in hindlimb buds of mouse, chicken, and frog embryos. This gradient is maximal over the distal and posterior part of hindlimb bud mesenchyme. In the forelimb bud a similar gradient is present, and is complementary to the previously reported distribution of *XlHbox 1* protein (Oliver et al. 1988b). In other words, regions that express high levels of *Hox-5.2* antigen are devoid of *XlHbox 1* protein. This suggests that one gene may repress expression of the other, as is known to occur in interactions between certain *Drosophila* homeotic genes. *Hox-5.2* antigen is activated during *Xenopus* limb regeneration, as would be expected for a gene involved in transmitting positional information. The data suggest that interaction of these two opposing gradients could be involved in determining positional values in developing limb buds.

Results

Two new human homeo box genes from the *Hox-5* cluster

A 350-bp cDNA clone was isolated by screening a human neuroblastoma cDNA library at low stringency with a mixture of *Xenopus* homeo box probes. Its DNA sequence revealed a new human homeo box, which was then used as a probe for screening a human leukocyte genomic library at high stringency. The genomic clone isolated contained a homeo box corresponding to that of the cDNA used as probe, as well as a second homeo box located 5' to it (Fig. 1A). This defines two new genes that are transcribed in the LAN 5 human neuroblastoma cell line (in Northern blots two transcripts of about 2.6 kb and of 2.1 kb were detected for the first gene, and a single band of about 2 kb was found for the second one in this cell line, data not shown). The direction of transcription indicated in Figure 1 was deduced from DNA sequence analysis.

The mammalian genome contains several clusters of genes containing homeo boxes of the *Antennapedia* type (Fienberg et al. 1987; Boncinelli et al. 1988; Dressler and Gruss 1988) that presumably arose by large duplications

Figure 1. Genomic organization of the region of the human *Hox-5* cluster containing *Hox-5.2* and *Hox-5.4* homeo boxes and evolutionary relationships with other homeo domain proteins. (A) Map of the genomic clone containing the new human homeo box-containing genes *Hox-5.2* and *Hox-5.4*: (E) *EcoRI*, (B) *BamHI*, (H) *HindIII*. Arrows indicate direction of transcription. (B) Comparison of related homeo domain sequences of the subfamily comprising *Hox-5.2* (human), *Hox-3.2*, *Hox-1.7*, *Hox-2.5* (mouse), *XlHbox 6* (frog), and *Abdominal-B* (*Drosophila*). (C) Subfamily of vertebrate genes including the homeo domains of *Hox-5.4* (human), *Hox-3.1*, *Hox-2.4* (mouse), and *XlHbox 7* (frog).

during evolution. Chromosome mapping is the simplest approach for determining to which cluster a new human homeo box gene belongs. Probes prepared from both homeo box genes were hybridized separately to a panel of 17 mouse-human somatic cell hybrids (Fig. 2A). The results unambiguously assigned both genes to human chromosome 2. By in situ hybridization to metaphase

Figure 2. Segregation of human *Hox-5* genes in somatic cell hybrids and chromosomal localization by in situ hybridization. (A) Southern analysis of human–mouse somatic cell hybrids using the human *Hox-5.4* cDNA as a probe (for description of the cell lines, named at the top of the panel, see Mohandas et al. 1986). The lanes contain representative hybrids; GM 0347A is the mouse and IMR 91 is the human control. A 14.2-kb band in the human cell line can be distinguished from an 11.5-kb band in the mouse genome. The presence of the human band in two cell lines allows us unambiguously to assign *Hox-5.4* to human chromosome 2. The same analysis was carried out with a human *Hox-5.2* probe. (B) In situ hybridization distribution of silver grains on a diagrammed G-banded human chromosome 2. In this analysis the 1.8-kb *Bam*HI restriction fragment corresponding to human *Hox-5.2* homeo box was used as probe. The main concentration of grains is over region 2q31–2q32, with a peak at 2q32.3, indicating that this is the site of the *Hox-5* locus. Similar results were obtained using a human *Hox-5.4* probe.

chromosomes, it was determined that both genes map in the region 2q31–2q32 (Fig. 2B). A human homeo box gene, called *c13*, had been mapped previously to this region (2q31–2q37; Mavilio et al. 1986; Cannizzaro et al. 1987), defining the human *Hox-5* complex (Boncinelli et al. 1988). The murine homolog of *c13* also has been cloned and called *Hox-5.1* (Featherstone et al. 1988). Recently, a murine homeo box gene named *Hox-5.2* has been isolated and determined to be linked physically to the *Hox-5.1* gene (D. Duboule, pers. comm.). Another mouse homeo box gene (*Hox-5.3*) was identified 6 kb 5' to *Hox-5.2* (D. Duboule, pers. comm.). On the basis of sequence similarities and following the recommended nomenclature (Martin et al. 1987), we consequently named our human genes *Hox-5.2* and *Hox-5.4* (Fig. 1A). The human *Hox-5.2* is the homolog of mouse *Hox-5.2*. The murine homolog of the human *Hox-5.4* has not yet been identified.

Homeo domains can be grouped into subfamilies according to their sequences (Boncinelli et al. 1988; Scott et al. 1989). Human *Hox-5.2* belongs to a subfamily containing murine genes *Hox-1.7*, *Hox-2.5*, and *Hox-3.2* as well as *Xenopus XlHbox 6* (Fig. 1B). These genes are located towards the 5' end of homeo box complexes and are most related in sequence to the *Drosophila Abdominal-B* gene, which is the most posteriorly expressed gene of the *bithorax* complex. Human *Hox-5.4* belongs to a subfamily containing murine homeo boxes 2.4 and 3.1, and *Xenopus XlHbox 7* (Fig. 1C). This type of vertebrate homeo box is always located immediately 3' to a member of the *Abdominal-B* family (human *Hox-5.4* is 3' to *Hox-5.2*; *Hox-2.4* is 3' to 2.5; *Hox-3.1* is 3' to 3.2; and *Xenopus XlHbox 7* to 3' to *XlHbox 6*; Boncinelli et al. 1988; Breier et al. 1988; Fritz et al. 1989). In the mammalian genome, therefore, these two types of

homeo box gene are present at least three times (in the *Hox-2*, *Hox-3*, and *Hox-5* complexes).

Hox-5.2 is expressed posteriorly

Antibodies were generated against a fusion protein containing the last 73 amino acids, including the homeo domain, of human *Hox-5.2*. Antigens recognized by this antiserum were analyzed in paraffin sections of mouse embryos. To facilitate analysis, in every case we stained the adjacent section with anti-*XlHbox 1* antibodies, whose staining pattern had been described in detail previously (Oliver et al. 1988a,b). Comparison of Figure 3, A (*XlHbox 1*) and B (*Hox-5.2*), shows that *Hox-5.2* antigens are detected only in posterior regions of the embryonic spinal cord, somites, and mesoderm of the digestive tract. In contrast, *XlHbox 1* is detected in more anterior regions of the body, which, in general, do not express *Hox-5.2* antigen. Similar staining patterns were found with both antibodies in sections of frog and chicken embryos (data not shown), consistent with the finding that antibodies against homeo proteins cross react between different vertebrate species (Oliver et al. 1988a). The apparent overlap in central nervous system staining seen in Figure 3, A and B, is due to staining of two different types of neuronal populations by each antibody (data not shown).

Because the human *Hox-5.2* fusion protein was derived from a genomic DNA fragment containing the entire homeo box region, it was necessary to test the specificity of the antibody. When anti-human *Hox-5.2* antiserum was depleted over affinity columns containing either *XlHbox 1* or *XlHbox 2* homeo domain proteins, so that all cross-reacting antibodies were removed (tested by Western blots of fusion proteins), the staining pattern

Figure 3. *Hox-5.2* antigen in mouse embryos is expressed posteriorly and exhibits a pattern complementary to that of *XlHbox 1*. (A) Day-10.5 mouse embryo sectioned parasagittally and stained with an antibody raised against the last 106 amino acids of the human homolog of the *XlHbox 1* gene (Oliver et al. 1988a). The protein is detected in anterior regions of the spinal cord beginning in the hindbrain (HB), in somites, and in mesoderm of the digestive tract. (B) The adjacent mouse section was immunostained with anti-*Hox-5.2* antibody (raised against the last 73 amino acids of the protein). Nuclear staining is detected over the same tissue derivatives as *XlHbox 1*, but its distribution is in more posterior regions of the embryo (e.g., this protein is absent in hindbrain and only the posterior digestive tract is stained). The mutually exclusive expression pattern of both proteins is better seen in the two following panels. (CNS) Central nervous system. (C) Transverse section through an intestinal loop of a mouse embryo stained with *XlHbox 1* antibody. Staining is detected in the mesoderm of an anterior intestinal loop. (D) Adjacent section immunostained with anti-*Hox-5.2* antibody; note that only the posterior intestinal loop is positive. These mutually exclusive staining patterns strongly support the specificity of the antibodies used in this analysis.

on embryo sections was unaffected. Antibodies depleted in this way were used interchangeably with the undepleted antibody throughout the study. In addition, fusion proteins were added as competitors during the immunostaining reaction itself. As expected, *Hox-5.2* protein abolished staining, while addition of either *XlHbox 1* or *XlHbox 2* proteins containing the entire homeo domain did not result in any change. The best argument in favor of *Hox-5.2* antibody reacting in a very specific way is that it stains different body regions than other antibodies raised against fusion proteins containing other homeo domains of the *Antennapedia* type (Fig. 3). However, because there are other genes very related to

human *Hox-5.2* in the mammalian genome (*Hox-1.7*, *Hox-2.5*, *Hox-3.2*, Fig. 1B), we cannot exclude the possibility that the antibody might cross-react with them. Consequently, the *Hox-5.2* antigens to which we refer throughout this paper could include other genes of the *Antennapedia-B* subfamily or very related, still undiscovered, genes. This antibody, however, has proved to be a very useful marker for studying limb development.

Hox-5.2 and XlHbox 1 are expressed in non-overlapping regions

Figure 3 shows that the *Hox-5.2* and *XlHbox 1* antigens

are expressed in mutually exclusive regions of the mouse embryo. This is particularly striking if one compares Figure 3, C and D. The mesoderm of an anterior intestinal loop stains strongly with *XlHbox 1* antibody, whereas that of a more posterior one does not (Fig. 3C). Conversely, *Hox-5.2* antigen is expressed in the posterior intestinal loop, but not in the anterior one (Fig. 3D). This differential staining of the anterior and posterior digestive tract can also be observed in Figure 3, A and B.

Expression in non-overlapping domains of the body was also found in frog and chicken embryos. In the spinal cord both antibodies stain different subsets of neurons; a similar type of complementarity was also noted in mesonephric tubules (data not shown). The mutually exclusive nature of the expression of these two

antigens was also manifested in developing limb buds (see below). This complementary expression is reminiscent of the behavior of certain *Drosophila* homeotic genes, in which expression of one gene represses that of another (Hafen et al. 1984).

A homeo box protein expressed in developing hindlimbs

In developing hindlimbs of a 10.5-day mouse, *XlHbox 1* is expressed only in the ectoderm (Fig. 4A), whereas *Hox-5.2* antigen is expressed in mesodermal cells (Fig. 4B). Expression is strongest in the distal part of the hindlimb bud, forming a gradient of nuclear protein. The *Xenopus* embryo displays similar staining patterns (Fig. 4C,D), including the hindlimb bud gradient of *Hox-5.2*

Figure 4. Immunolocalization of *Hox-5.2* antigens in developing hindlimb buds. (A) Day-10.5 mouse embryo hindlimb buds (HL) sectioned transversely and stained with the *XlHbox 1* antibody raised against the human homolog (Oliver et al. 1988b). As described previously, staining is detected only in the surrounding ectoderm (indicated by arrows). (B) Staining of an adjacent section with anti-*Hox-5.2* antibody. Mesodermal cells exhibit a distal (Dist) to proximal (Prox) gradient of nuclear protein. (C) Transverse section at the level of the hindlimb buds of a *Xenopus* stage-50 tadpole (2-week) stained with *Xenopus* antibody B (Cho et al. 1988; Oliver et al. 1988a). As previously reported (Oliver et al. 1988b), no mesodermal staining is detected in hindlimbs, while the medial region of the ectoderm shows expression (arrows). (D) An adjacent section was stained with anti-*Hox-5.2* antibody and, as in mouse, the staining is observed in the mesoderm in a distal to proximal gradient. (CNS) Central nervous system.

antigen. The 'progress zone,' a distal region where most limb growth takes place (Tickle et al. 1975), strongly expresses *Hox-5.2* antigen at early stages. Intense mesodermal staining is only present in early hindlimb buds, with antigen loss starting at the distal tip by day 12 in mouse embryos. As the limb develops, *Hox-5.2* staining becomes weaker and is restricted to nuclei of cells that are differentiating into cartilage (day 13 in mouse, stage 53 in tadpoles, data not shown).

Two opposing gradients in forelimb buds

Hox-5.2 antigen was detected not only in hindlimb buds, but it also stained forelimb bud mesoderm intensely, as can be seen in Figure 5B. As reported previously (Oliver et al. 1988b), *XlHbox 1* protein is expressed in the forelimb bud mesoderm as a gradient that is maximal in the anterior and proximal regions (Fig. 5A). *Hox-5.2* antigen expression is complementary to that of *XlHbox 1*: It is strongly expressed in mesodermic regions negative for *XlHbox 1*, and is absent from the ectoderm (Fig. 5B).

In the forelimb, *Hox-5.2* antigen is also expressed in a gradient, as can be seen in the stage-25 chicken wing bud shown in Figure 6 (which was sectioned in an oblique plane, so that the left side of the photograph is distal as well as more posterior, while the right side is proximal as well as more anterior). A distal-to-proximal gradient (highest at the tip of the limb) can be observed clearly. Serial sections of this and other chicken limbs reveal that the gradient is also posterior to anterior (higher in the posterior limb bud, i.e., the region that will bear the little finger). The same type of gradient was observed in forelimb buds of *Xenopus* and mouse embryos. As was the case in the hindlimb, the *Hox-5.2* gradient in the forelimb is transient and starts disappearing from the distal end of the mouse forelimb bud by day 12.

Figure 6. Section through a stage-25 chicken forelimb showing a clear nuclear gradient of *Hox-5.2* antigen. In this oblique section *Hox-5.2* gradient is maximal in the distal (and posterior) region, and minimal in the proximal (and anterior) region of the limb bud. The arrow indicates the apical ectodermic ridge, a thickening of the ectoderm involved in limb growth.

We conclude that in the forelimb *Hox-5.2* is expressed in a gradient with the opposite polarity to that of *XlHbox 1*. The *Hox-5.2* gradient has an orientation similar to that of the proposed retinoic acid gradient (Thaller and Eichele 1987).

Homeo protein induction in regenerating limb buds

The mechanisms that control pattern formation during amphibian limb regeneration are similar to those operating during embryonic development (Bryant and Mun-eoka 1986). Following amputation of a limb, a regeneration blastema is formed from dedifferentiated stump

Figure 5. Anti-*Hox-5.2* antibody staining in developing day-10.5 mouse forelimbs. (A) Longitudinal (frontal) section at the level of the forelimb (FL) bud stained with the human *XlHbox 1* antibody. Staining is detected in the anterior (Ant) mesoderm and in the surrounding ectoderm. (Post) Posterior; (Prox) proximal; (Dist) distal. (B) Staining of an adjacent section with anti-*Hox-5.2* antibody detects this antigen in a complementary pattern to that of *XlHbox 1*, i.e., negative in the ectoderm and positive in those mesodermal cells that do not show staining for *XlHbox 1*.

tissues; it eventually produces a replica of the lost part. Transplantation studies have shown that positional values in the blastema can be respecified experimentally. Therefore, any gene product involved in pattern formation should also be active during limb regeneration. We analyzed *Hox-5.2* antigen expression in *Xenopus* hindlimbs amputated at stage 52 (3 weeks of larval development). In contrast to salamanders, which can regenerate limbs even as adults, *Xenopus* is only able to regenerate limbs during the larval period. In stage-52 tadpoles, almost complete regeneration can be obtained (Dent 1962). Figure 7 shows sections of the hindlimbs of a tadpole from which the distal half of one leg was amputated 8 days previously. While at this late stage only very weak anti-*Hox-5.2* staining is detected on the nonamputated side (Fig. 7A), strong expression is detected in nuclei of the regeneration blastema (arrows in Fig. 7B), consistent with a possible role of this gene product in pattern specification.

Discussion

We report here the isolation of two new homeo box genes, designated human *Hox-5.2* and *Hox-5.4*. An antibody reacting with *Hox-5.2* antigen was prepared, and the protein was found to be distributed as a nuclear gradient in fore- and hindlimb buds in mouse, chicken, and frog embryos. The gradient reported here has a polarity opposite to that described previously for *XlHbox 1* protein in developing forelimb mesoderm (Oliver et al. 1988b). The *Hox-5.2* gradient encompasses most of the limb bud mesoderm and is maximal at the distal tip and the posterior border (Fig. 6). At the height of its expression *Hox-5.2* antigen is very strongly stained, but the gradient is transient and starts decreasing in the mouse limb bud by day 12.

The early embryonic mesoderm is subdivided into

fields expressing different homeo box genes, which are transiently reactivated later when organogenesis occurs (reviewed by Wright et al. 1989). In the case of *Hox-5.2*, this reactivation occurs during intestinal and limb bud differentiation. This antigen is also activated during regeneration of amputated *Xenopus* limbs. The latter finding is consistent with a possible role of *Hox-5.2* in the transmission of positional information in the regeneration blastema (Stocum and Fallon 1982; Bryant and Muneoka 1986).

Antibody probes against homeo domain proteins are particularly useful because they permit one to study the expression of homologous genes in a wide variety of vertebrate embryos. As described above, a number of competition studies showed that anti-*Hox-5.2* antibodies are rather specific and do not cross-react with other homeo domains of the *Antennapedia* type. Since there are a number of related genes within the *Abdominal-B* subfamily in the mammalian genome, we cannot exclude that our antibody might cross-react with some of them. In situ hybridization to transcripts on embryo sections will establish whether the protein patterns we describe here are due exclusively to *Hox-5.2* expression. However, in situ hybridization across species boundaries is not feasible (because it would require reduced stringency) and these studies would be difficult in the homologous (human) system. The main conclusion of this paper, i.e., that opposing gradients of homeo domain proteins exist during limb bud development, is valid whether the antibody recognizes solely *Hox-5.2* or not.

Lewis (1978) has suggested that the linear order of homeotic genes in the chromosome is associated with their regions of expression along the anterior–posterior axis of the *Drosophila* embryo. Genes at the 5' end of a cluster are expressed in more posterior segments of the fly (e.g., *Abdominal-B*, which is the most 5' gene of the *bithorax* complex is also the one expressed most posteri-

Figure 7. Activation of *Hox-5.2* protein during limb regeneration. (A) Normal hindlimb bud from a stage-54 *Xenopus* tadpole (oblique section). Very weak staining is detected with *Hox-5.2* antibody. (B) Contralateral hindlimb bud amputated distally 8 days previously (stage 52). Note that *Hox-5.2* antigen is strongly expressed in the regeneration blastema (arrows).

orly). It has been suggested that a similar correlation between position in a cluster and regions of expression may also exist in vertebrates (Boncinelli et al. 1988; Gaunt et al. 1988; Graham et al. 1988). *Hox-5.2* antigens were found to be expressed in posterior regions of the embryonic mesoderm and central nervous system (Fig. 3B), as expected for a gene belonging to the *Abdominal-B* subfamily. Members of this subfamily in general are located in 5' positions within vertebrate homeo box clusters (Boncinelli et al. 1988; Wright et al. 1989).

During the course of this study it was found unexpectedly that expression of a second homeo domain protein, *XlHbox 1*, is excluded from areas strongly positive for *Hox-5.2*. This results in embryos expressing the two antigens in non-overlapping subsets of cells. This is particularly obvious in Figure 3, in which the mesoderm of an anterior intestinal loop expresses *XlHbox 1* while a posterior loop reacts with anti-*Hox-5.2* antibodies, and also is valid for other embryonic regions such as the limb bud (Fig. 5). In *Drosophila*, homeotic genes are expressed in complementary and mutually exclusive regions of the embryo (Akam 1987). These patterns are thought to arise from interactions between homeotic genes in which expression of one gene represses that of the other (Lewis 1978; Hafen et al. 1984). If a similar mechanism exists in vertebrates, one might predict that microinjection of synthetic *Hox-5.2* mRNA could, for example, repress transcription from *XlHbox 1* promoters.

The idea that gradients may provide a mechanism for specifying position in the embryo has been favored for many years, (Weiss 1939; Huxley and De Beer 1934; Child 1941; Wolpert 1969). In the limb bud, interactions of the two opposing protein gradients described here (and possibly others) could be involved in determining positional values. An interesting question is how does a gradient of nuclear protein arise in a seemingly homogeneous population of mesenchymal cells. Presumably intercellular signaling mechanisms involving multiple gene products exist. A large number of mutations affecting limb development are known in both the chick (Abbot 1983) and mouse (Green 1981). Some of these mutations may affect such signaling mechanisms.

Methods

Isolation and characterization of the *Hox-5* cluster

A human cDNA clone corresponding to *Hox-5.4* was isolated by screening a human neuroblastoma cDNA library (purchased from Clontech) with homeo box probes isolated from genes *XlHbox 1* and *XlHbox 4* of *X. laevis* (Fritz and De Robertis 1988). A genomic clone carrying human *Hox-5.2* and human *Hox-5.4* genes was isolated by screening an EMBL3 human leukocyte genomic library (purchased from Clontech) with the human *Hox-5.4* cDNA clone. Restriction fragments containing both homeo boxes were then subcloned into BlueScribe (Stratagene) and their DNA sequences were determined by double-stranded sequencing (Zagursky et al. 1985).

Chromosome mapping and in situ hybridization

A panel of 17 human-mouse somatic cell hybrids was derived from the fusion of normal human male fibroblasts (IMR 91) and

thymidine kinase-deficient mouse cells (B82, GM 0347A), as described previously (Mohandas et al. 1986). Cytogenetic analysis was carried out on a minimum of 30 Q-banded metaphases per hybrid clone.

DNA from the parental and the somatic cell hybrid clones was purified from nuclei (Fodor and Doty 1979), digested with *HindIII*, and subjected to Southern blot analysis at high stringency as described by Sparkes et al. (1987), using as probe either the 350-bp insert of the human *Hox-5.4* cDNA clone or a 1.8-kb *BamHI* fragment of human *Hox-5.2* which carries the homeo box (Fig. 1A).

The in situ hybridization analysis of normal human chromosomes was performed as described (Harper and Saunders 1981; Cannizzaro and Emanuel 1984) using the same two DNA probes. Slides were exposed for 1 week and silver grains located on the chromosomes or touching them were scored.

Construction of β -galactosidase fusion protein and antibody preparation

The 1.8-kb *BamHI* DNA fragment (shown in Fig. 1A) containing the human *Hox-5.2* homeo box was fused in-frame to the carboxyl terminal of β -galactosidase in the vector *pTRB 2* (Burglin and De Robertis 1987). This construct contains the first 5 amino acids upstream from the homeo box, the homeo box, and 8 amino acids downstream from the homeo box (Fig. 1A).

Fusion proteins were induced and isolated as described previously (Oliver et al. 1988a). A 6-week-old female New Zealand White rabbit was immunized with affinity-purified fusion protein. Each boost contained 2 mg of protein. Rabbits were bled (50 ml) via the marginal ear vein 10 days after each inoculation following the second boost. Antisera purification was as described (Oliver et al. 1988a). The two *XlHbox 1* antibodies used in this study have been described previously and were antibody B (used in all *Xenopus* sections) and a second antibody raised against the human *XlHbox 1* homolog (Oliver et al. 1988a). The latter antibody gives stronger signals in mouse and chicken embryos.

Immunostaining

Xenopus laevis tadpoles were staged according to Nieuwkoop and Faber (1967). Pregnant BALB/c mice were purchased from Simmons Labs Inc. (California) and their embryos were staged according to Rugh (1968). The day of the vaginal plug was designated as day 0.5 post coitum. Chicken embryos were kindly provided by Dr. James Paulson and staged according to Hamburger and Hamilton (1951).

Embryos were fixed in Bouin's solution (75 parts saturated picric acid, 25 parts formaldehyde, and 5 parts glacial acetic acid), embedded in paraffin, and sectioned by microtome. (8 μ m). Sections were dewaxed by passage through xylene and graded ethanol baths (2 min each). Immunostaining was carried out as described previously (Oliver et al. 1988a,b) using as second antibody anti-rabbit IgG conjugated with alkaline phosphatase (Promega Biotec).

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Note added in proof

The murine *Hox-5.2* gene has been isolated recently by Dolle and Duboule (*EMBO J.*, 1989, in press). When its transcript distribution was analyzed by in situ hybridization, the results were in complete agreement with the antibody data shown in this paper. This suggests that our antiserum is indeed directed mainly against the *Hox-5.2* antigen.

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