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Two gene members of the murine HOX-5 complex show regional and cell-type specific expression in developing limbs and gonads

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This study reports the expression domains of two murine HOX gene members of the HOX-5 complex (*Hox-5.2, Hox-5.3*). These two genes have very similar homeodomain sequences, as well as temporal and spatial specificities of expression. They are both expressed at very posterior levels in the central nervous system, in sclerotome derivatives and in a few internal organs. In addition to these expression domains which are shared with other HOX genes, transcripts from both *Hox-5.2* and *Hox-5.3* are present at high levels in developing limbs. After an early homogeneous expression in mesodermal limb bud cells, transcription becomes restricted to cartilage-differentiating cells. In addition, *Hox-5.2* is a marker for gonadal development. The possible involvement of such genes during inductive processes or organogenesis is discussed.

Key words: mouse embryos/homeobox/*in situ* hybridization/morphogenesis/development

Introduction

More than 20 murine genes containing an Antp-like homeobox have been characterized so far. These so-called '*Hox*' genes are clustered in four complexes, HOX-1 (Colberg-Poley *et al.*, 1985; Duboule *et al.*, 1986), HOX-2 (Hart *et al.*, 1987; Graham *et al.*, 1988), HOX-3 (Awgulewitsch *et al.*, 1986; Breier *et al.*, 1987) and HOX-5 (Featherstone *et al.*, 1988; Duboule and Dollé 1989), located on chromosomes 6, 11, 15 and 2 respectively. Recent work carried out with *Drosophila* genes encoding comparable homeodomain sequences suggests that such homeoproteins can bind DNA and thus probably play a regulatory function at the molecular level (Desplan *et al.*, 1988; Hoey and Levine, 1988; Müller *et al.*, 1988). However, if it is probable that these proteins are involved in important developmental processes in the vertebrates, their biological function remains unclear. One of the possible ways to gain insight into this problem is to analyse the distribution of their transcripts at various stages of development in wild-type animals. The expression patterns of several murine *Hox* genes have been extensively studied by *in situ* hybridization (Dony and Gruss, 1987; Holland and Hogan, 1988a,b; Gaunt *et al.*, 1988) and the following general observations can be made: the transcription of *Hox* genes probably starts early in development, since spatially restricted expression domains have been reported as early as 7.5 days post-coitum (p.c.), at the primitive streak stage prior to somitogenesis (Gaunt *et al.*, 1986; Gaunt, 1987; B.Galliot, P.Dollé and D.Duboule, unpublished work). With respect to cell lineages and organogenesis, all studied genes are expressed in the foetal central nervous system (CNS) and most of them have additional expression domains restricted to mesoderm derivatives. These latter include somitic mesoderm, particularly the sclerotome-derived 'pre-vertebrae' and structures derived from the intermediate segmented mesoderm (mesonephros) and from the lateral plate unsegmented mesodern whose cells are found in intestinal and pulmonary parenchyma and, more generally, in connective tissues. No expression of *Hox* genes has been reported in the epidermal ectoderm so far, and only two genes show a very specific expression in the primitive intestine endoderm at early stages of development (*Hox-1.6*, Duboule and Dollé, 1989; and *Hox-3.1*, Le Mouellic *et al.*, 1987). Thus, *Hox* genes are expressed during ontogeny in identical structures but in distinct and partially overlapping domains along the antero-posterior axis. Several homeobox-containing genes have sharp anterior boundaries of expression in the CNS and pre-vertebral column, distinct from one gene to another, and the expression generally decreased posteriorly (Holland and Hogan, 1988b). In fact, the position of a given *Hox* gene expression domain along the rostro-caudial axis correlates with the position of this gene within its cluster (Gaunt *et al.*, 1988), and we and others have recently proposed that both *Drosophila* and vertebrate *Antp*-like homeobox-containing gene complexes show a comparable structural and functional organization (Duboule and Dollé, 1989; R.Krumlauf, personal communication). These different observations strongly suggest that vertebrate homeoproteins serve as positional cues during the development of neurectoderm and mesoderm structures. Other functions of homeoproteins have been discussed, such as a possible inductive role during organogenesis (Dony and Gruss, 1987a; Holland and Hogan, 1988a; Gaunt, 1987).

The fourth major murine *Hox* gene complex (HOX-5) was recently cloned (Featherstone *et al.*, 1988) and shown to contain at least three homeogenes: *Hox-5.1*, *Hox-5.2* and *Hox-5.3* (Duboule and Dollé, 1989). In this paper, we describe the expression patterns of two members of the HOX-5 complex – *Hox-5.2* and *Hox-5.3* – as detected by *in situ* hybridization using antisense RNA probes. Both of them show unusual tissue specificities when compared to previously reported *Hox* gene expression domains. We show that both *Hox-5.2* and *Hox-5.3* expression domains are very posteriorly restricted in the foetal CNS and pre-vertebral column. However, *Hox-5.2 and Hox-5.3* transcripts were also found in mesenchymal cells of the developing limb buds at early stages of development as well as in the growth regions of the limb cartilage of late gestation foetuses. In addition, *Hox-5.2* is specifically expressed in male and

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Hox-5.1 CCCAAGCGCTCCCGGACGGCCTACACCAGACAGCAAGTCCTAGAA
Hox-5.2 A--CG-AAAAAG--CTGTC---------A-TAC--GACG-----G
Hox-5.3 GG--GAGAGAAGA--TGCC-T-------AG--C---ACG--G---
          * *  * *** * ** ** ********   *** ***** **

Hox-5.1 CTGGAAAAGGAATTTCATTTTAACAGGTATCTGACCAGGCGCCGT
Hox-5.2 -----G--------C-TC--C----T---C--C---C--GA---G
Hox-5.3 T-------A--G--CTTG--C--T-T---C--C---C-CGAG--C
         **** ** ** *** * ***** ************** ** **

Hox-5.1 CGGATTGAAATCGCTCACACCCTGTGTCTGCCTGAGCGCCAGATC
Hox-5.2 --CTAC--GG-G---AGG-TT---AAC--TA-A---A-A--AG--
Hox-5.3 --CC-A--G---AG-A-G-G-G-TAAC--CA-C--CA-G---G--
        ***   *** *   ** **   * ***** ** ** ** ** ***

Hox-5.1 AAGATCTGGTTCCAGAACCGGAGGATGAAGTGGAAAAAAGACCAC
Hox-5.2 --A-----------------T--------AATG-----GATGAGC
Hox-5.3 -----T-----T--A-----CC-A-----ACTC--G--GATGAGC
        ** ** ***** ** *****  * ****** * ** *********
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Hox-5.1 ProLysArgSerArgThrAlaTyrThrArgGlnGlnValLeuGlu
Hox-5.2 ThrArgLysLys   CysPro      LysTyr   Thr
Hox-5.3 GlyArgGluLys   CysPro      LysHis   Thr

Hox-5.1 LeuGluLysGluPheHisPheAsnArgTyrLeuThrArgArgArg
Hox-5.2                Leu      Met           Asp
Hox-5.3                Leu      Met           Glu

Hox-5.1 ArgIleGluIleAlaHisThrLeuCysLeuSerGluArgGlnIle
Hox-5.2    Tyr   Val   ArgIle   Asn   Thr         Val
Hox-5.3    Leu      SerLysSerValAsn   ThrAsp      Val

Hox-5.1 LysIleTrpPheGlnAsnArgArgMetLysTrpLysLysAspHis
Hox-5.2                                Met      MetSer
Hox-5.3                                Leu      MetSer
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Fig. 1. Nucleotide and amino acid sequences of the *Hox-5.2* and *Hox-5.3* homeoboxes. The sequences are compared with that of the other member of the HOX-5 complex (*Hox-5.1*; Featherstone *et al.*, 1988). Only those nucleotides (amino acids) different from the *Hox-5.1* sequence are indicated. These two novel sequences are divergent from the 'reference' *Antennapedia* sequence (McGinnis *et al.*, 1984) but very similar to each other (stars). This similarity is further enhanced when protein sequences are considered.

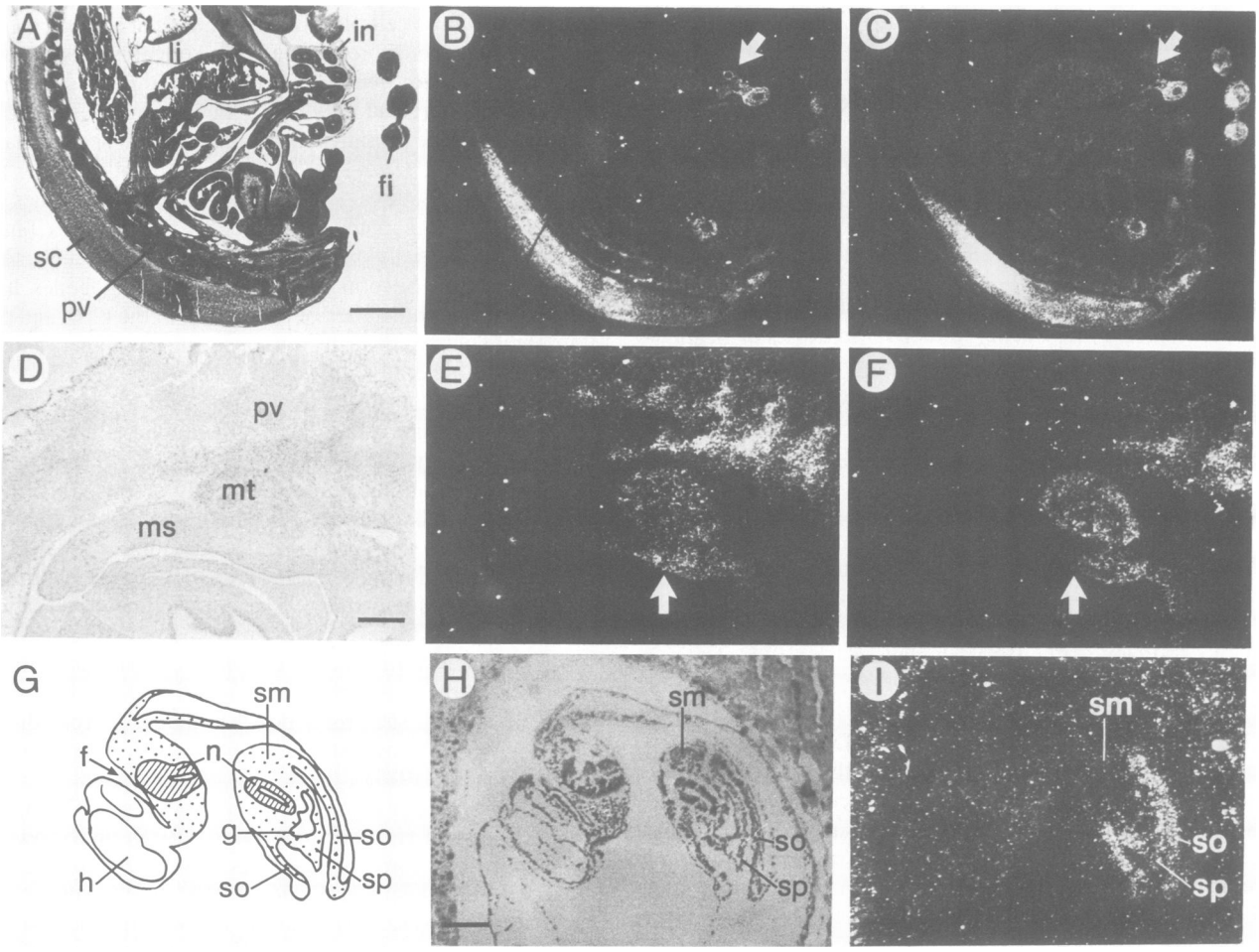

Fig. 2. The antero-posterior specification of the *Hox-5.2* and *Hox-5.3* expression domains. (**A**) Sagittal section of a 14.5 day old mouse embryo stained with haematoxylin–eosin. Only the posterior half is shown. Bar: 750 μm. (**B,C**) Adjacent sections of the same foetus hybridized with the *Hox-5.2* (**B**) or *Hox-5.3* (**C**) antisense RNA probes and viewed under dark-field illumination. The *Hox-5.3* hybridization signal is slightly more posterior in the spinal cord, the pre-vertebrae (very weak at this stage) and in the intestine (arrow). The *Hox-5.3* hybridization is stronger in the finger tips. (**D,E,F**) Two adjacent sagittal sections through a 12.5 day old foetus, hybridized with the *Hox-5.2* (**E**) and *Hox-5.3* (**F**) probes. An enlargement of the mesonephros region is shown. Again, the *Hox-5.3* expression domain is more posterior than that of *Hox-5.2*, both in the pre-vertebrate and the mesonephros (vertical arrows indicate identical landmarks). Bar: 200 μM. (**G,H,I**) Section through an 8.5 day old embryo (rotation underway) hybridized with the *Hox-5.2* probe. Hybridization is found in the posterior part of the embryo (right side of **I**) preferentially in the lateral mesoderm consisting of the somatopleure and splanchopleure layers. Bar: 150 μm. in, intestine; li, liver; fi, fingers; sc, spinal cord; pv, pre-vertebrae; mt, metanephros; ms, mesonephros; f, foregut; h, heart; n, neural tube; g, gut; sm, presomitic mesoderm; so, somatopleure, sp, splanchnopleure.

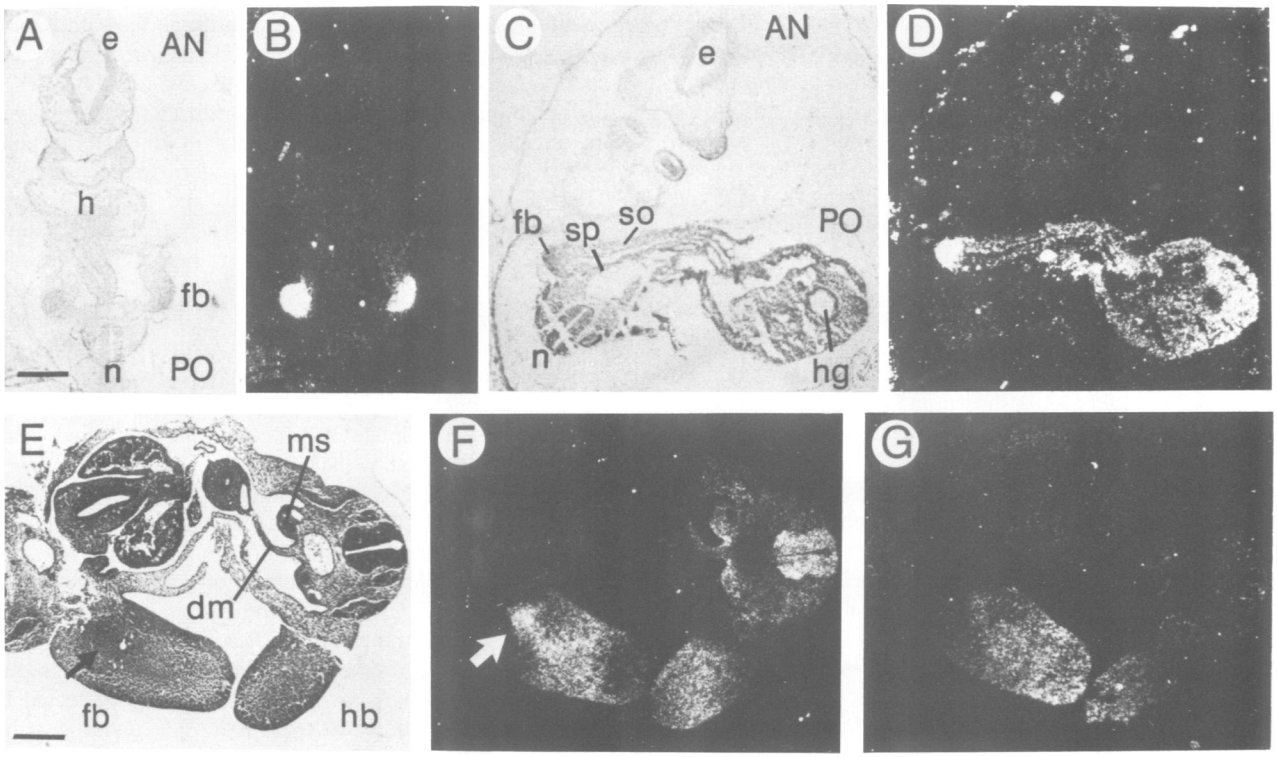

Fig. 3. *Hox-5.2* and *Hox-5.3* expression in limb buds and developing limbs. (**A,B**) Section of a 9.5 day old embryo at the level of both forelimb buds and first branchial pouch, hybridized with the *Hox-5.2* probe. A strong and highly restricted signal is detected in limb buds. Bar: 250 μm. (**C,D**) Section of the same embryo at a more posterior level hybridized with the *Hox-5.2* probe. An uninterrupted signal is detected in both the somatopleural and splanchnopleural mesoderm, from the left forelimb bud to the more posterior widely labelled regions. (**E,F,G**) Adjacent sections through both anterior and posterior developing limbs from a 10.5 day old embryo, hybridized with either the *Hox-5.2* (**F**) or *Hox-5.3* (**G**) probes. The arrow in (**E–F**) points to the proximal condensation of *Hox-5.2*-specific hybridization gains in the forelimb bud, whereas *Hox-5.3* is more distally expressed (**G**). *Hox-5.2* hybridizes strongly in the ventral edge of the mesonephros and around the dorsal mesenteric attachment, whereas *Hox-5.3* does not. Bar: 250 μm. AN, anterior; po, posterior; e, encephalon; h, heart; fb, forelimb bud; n, neural tube; sp, splanchnopleure; so, somatopleure; hg, hindgut; ms, mesonephros; dm, dorsal mesentere; hb, hindlimb bud.

female developing gonads. In both limb buds and gonads, *Hox-5.2* and *Hox-5.3* expression extends anteriorly, beyond the metameric boundary of expression visualized in the sclerotomic column and mesonephros. These observations suggest that the *Hox-5.2* and *Hox-5.3* proteins, in addition to their probable role as positional cues along the rostro-caudal axis, might be involved in processes such as cellular regionalization or cell-type specification.

Results

The mouse HOX-5 complex contains at least three *Hox* genes. The *Hox-5.2* and *Hox-5.3* genes are located 30 and 35 kb upstream from *Hox-5.1* respectively, and possess very similar homeobox sequences (Figure 1). Both DNA sequences are relatively divergent from the *Hox-5.1* sequence (61 and 55% respectively). At the protein level, the similarities with *Hox-5.1* increase slightly (65 and 60% respectively), both sequences being quite divergent from the reference *Antp* sequence (70 and 60%). In fact, the *Hox-5.2* amino acid sequence is almost identical to that of *Hox-1.7* (97%; Rubin *et al.*, 1987), *Hox-2.5* (96%; A.Graham, personal communication) and *Hox-3.2* (95%; Breier *et al.*, 1988). These four *Hox* genes are the members of the *Hox-1.7*-like subfamily (Duboule and Dollé, 1989; Graham *et al.*, 1989) and show significant similarities with the *Xenopus XHBox-6* gene (Sharpe *et al.*, 1987). They also share divergent amino acids with the *Drosophila AbdB* homeotic gene (Regulski *et al.*, 1985). The amino acid sequence of the *Hox-5.3* homeo-domain is virtually identical to that of *Hox-5.2* in its N-terminal part (90% along the first 28 amino acids) but becomes divergent in the region of the Helix 2 motif and so may represent a novel subfamily located upstream from all those reported so far.

Expression in the central nervous system and the trunk mesodermal derivatives

In situ hybridization was performed using ^{35}S-labelled antisense RNA probes synthesized from the 3′ flanking regions of the *Hox-5.2* and *Hox-5.3* homeoboxes. Hybridization on serial sections of embryos and foetuses from 8.5 days p.c. to 17.5 days p.c. revealed that both *Hox-5.2* and *Hox-5.3* are expressed in the developing CNS (neural tube, spinal cord), and in populations of mesodermally derived cells of somitic (sclerotomic derivatives) or non-somitic (mesonephros; mesodermal component of the intestine) origin. In these structures, *Hox-5.2* and *Hox-5.3* display a very posterior restriction, *Hox-5.3* being even slightly more posteriorly expressed than *Hox-5.2*. In fact, *Hox-5.2* is expressed in 12.5 day old foetuses posterior to the first lumbar pre-vertebra, whereas *Hox-5.3* expression starts one metameric unit more posteriorly, a comparable difference being observed in the spinal cord (Duboule and Dollé, 1989). These slightly different posterior expression

domains can still be seen on sagittal sections of 14.5 day p.c. foetuses, as shown in Figure 2 (compare panels B and C), though the expression in the pre-vertebral column is very weak and restricted to the most peripheral regions of the vertebral body primordia. Similarly, expression of both genes is also restricted to the most posterior part of the 12.5 day mesonephric tissue. Here again, the *Hox-5.2* and *Hox-5.3* expression boundaries are slightly displaced and coincide with the boundaries found in the sclerotomes (Figure 2; compare panels E and F). The expression in the mesodermal layer of the intestine is also restricted to posterior regions and, again, the expression domains of the two genes are spatially delayed since part of the gut sectioned in the 14.5 day old foetus shown in Figure 1 hybridizes to the *Hox-5.2* probe and not to the *Hox-5.3* probe (compare panels B and C, arrows).

This posterior specificity of expression is established early during embryogenesis, since it is already present in sections of 8.5 and 9.5 day p.c. embryos hybridized with the *Hox-5.2* antisense probe. At 8.5 day p.c., the hybridization signal is detected in posterior parts of the neural tube, the axial presomitic mesoderm and lateral plate mesoderm including the splanchnopleure and somatopleure (not shown). The precise limit of hybridization is somewhat difficult to determine since it lies in a region where somites have not yet condensed. However, it is clear that the expression of the *Hox-5.2* gene in such an early developmental stage is already restricted to the posterior-most parts of the embryo, and that in a region (the presomitic mesoderm) which has not yet acquired its segmented organization. Interestingly, the hybridization extends more anteriorly, solely in the lateral plate mesoderm in both intra-embryonic splanchnopleural and somatopleural layers (Figure 2, panels G–I). This signal in the lateral mesoderm extends until an anterior limit located near the recently condensed somites. In more anterior parts, all structures are negative.

Expression in the developing limb buds

The condensation of the forelimb bud appears in the 9th day p.c. at the level of the 8th–12th somites. The hindlimb bud appears only several hours later, as a condensation at the level of somites 23–28. The first morphological landmark of limb formation is a transient longitudinal thickening of the ectoderm located on the lateral side of the somitic column, the Wolffian (or apical ectodermal) ridge. The medial part of the Wolffian ridge rapidly disappears while it becomes thicker in the region of the developing limbs. The limb buds develop by lateral mesenchymal proliferation under the epidermal ridge. Thus, each limb bud is at first a mass of undifferentiated mesenchyme. The contributions of somitic mesoderm and lateral somatopleure mesoderm to the limb bud mesenchyme are not clearly elucidated (Hinchliffe and Johnson, 1980). Both *Hox-5.2* and *Hox-5.3* probes detect a strong hybridization signal in the mesodermal cells of the forelimb and hindlimb buds of embryos at various stages of development. Hybridization to 9.5 day p.c. embryos reveals *Hox-5.2* transcripts in the mesenchymal cells of the forelimb bud (Figure 3A and B). Labelling seems to be homogeneously distributed among the mesodermal cells of the forelimb bud and stops abruptly so that cells lying outside the prominent bud are not labelled. The apical ectodermal layer is not labelled either. Interestingly, specific labelling is found, at that stage, in lateral mesoderm continuously from the level of the forelimb buds until most posterior regions where, in addition, neuroectoderm and somitic mesoderm are labelled (Figure 3C and D). This is likely reminiscent of the 8.5 day hybridization pattern where the signal extends more anteriorly in the lateral plate mesoderm than in the presomitic mesoderm and neural tube.

By day 10.5 p.c., both forelimb and hindlimb buds, consisting of undifferentiated mesenchymal cells, show *Hox-5.2* expression. The signal is rather homogeneously scattered throughout the hindlimb bud (Figure 3F), whereas a higher grain density is found in the central regions of the forelimb bud, limited, in its most proximal part, to a node of highly labelled cells which may correspond to the presumptive humeral epiphysis (arrow in Figure 3F). By day 12.5 p.c., the handplate and footplate have formed. This stage is characterized by the appearance, in a proximo-distal sequence, of local condensations of mesodermal cells, presumably corresponding to precartilaginous blastemas (Hinchliffe and Johnson, 1980). In both limb buds, *Hox-5.2* transcripts are clearly restricted to central regions of condensed cells (Figure 4B). The presence of a crown-shaped signal around the humeral head (Figure 4E) suggests that expression may occur in the blastemas of cartilaginous differentiating cells. A restricted signal is also detected in more anterior superficial mesenchymal tissue underlying the epidermis on the latero-ventral side of the posterior part of the head (Figure 4E, arrow).

At both 10.5 and 12.5 days p.c. comparative *in situ* hybridization with the *Hox-5.2* and *Hox-5.3* probes reveals very similar hybridization patterns in limb buds (Figures 3F,G, and 4B,C). However, the *Hox-5.3* signal is repeatedly denser in the most distal extremities of the limb buds (compare Figures 4B to 4C and 2B to 2C). In contrast, the *Hox-5.3* signal is barely detectable in proximal segments (e.g. in the humeral epiphysis primordium; Figure 4F). No signal is found with the *Hox-5.3* probe in the superficial head mesenchyme (Figure 4; compare panels E and F).

In 14.5 day limbs, the muscular and skeletal anlage are clearly identifiable. The bone primordia consist of cartilage cells which undergo progressive maturation: their hypertrophy towards the centre of the proximal bones anlage is characteristic of endochondral ossification centres. Ossification will proceed from the centre towards the ends of the bone, while the apposition of newly cartilaginous cells at the ends of the cartilaginous model will allow bone growth (Hinchliffe and Johnson, 1980). At this stage, the *Hox-5.2* probe hybridizes to both fore- and hindlimbs in very specific cell subsets surrounding zones of cartilaginous skeletal primordia. The strongly labelled zones are found at the extremities of the long bones of both proximal and distal segments including the proximal epiphysis of the humerus (at the level of the future scapulo-humeral joint; Figure 4K), the head of the femur, the distal extremities of the humerus and femur, the proximal and distal extremities of the radius and ulna, the tibia and fibula cartilaginous models and the periphery of the anlage of the bones of the proximal row of the carpus (Figure 4H) and tarsus. Labelled cells are mesenchymal cells with a fascicular organization neighbouring more roundish unlabelled chondroblasts (Figure 4M). It is therefore probable that *Hox-5.2* is expressed at that stage in the regions where growth of the cartilaginous skeleton takes place. This hybridization to the perichondrium is no longer found in the regions of the hand-

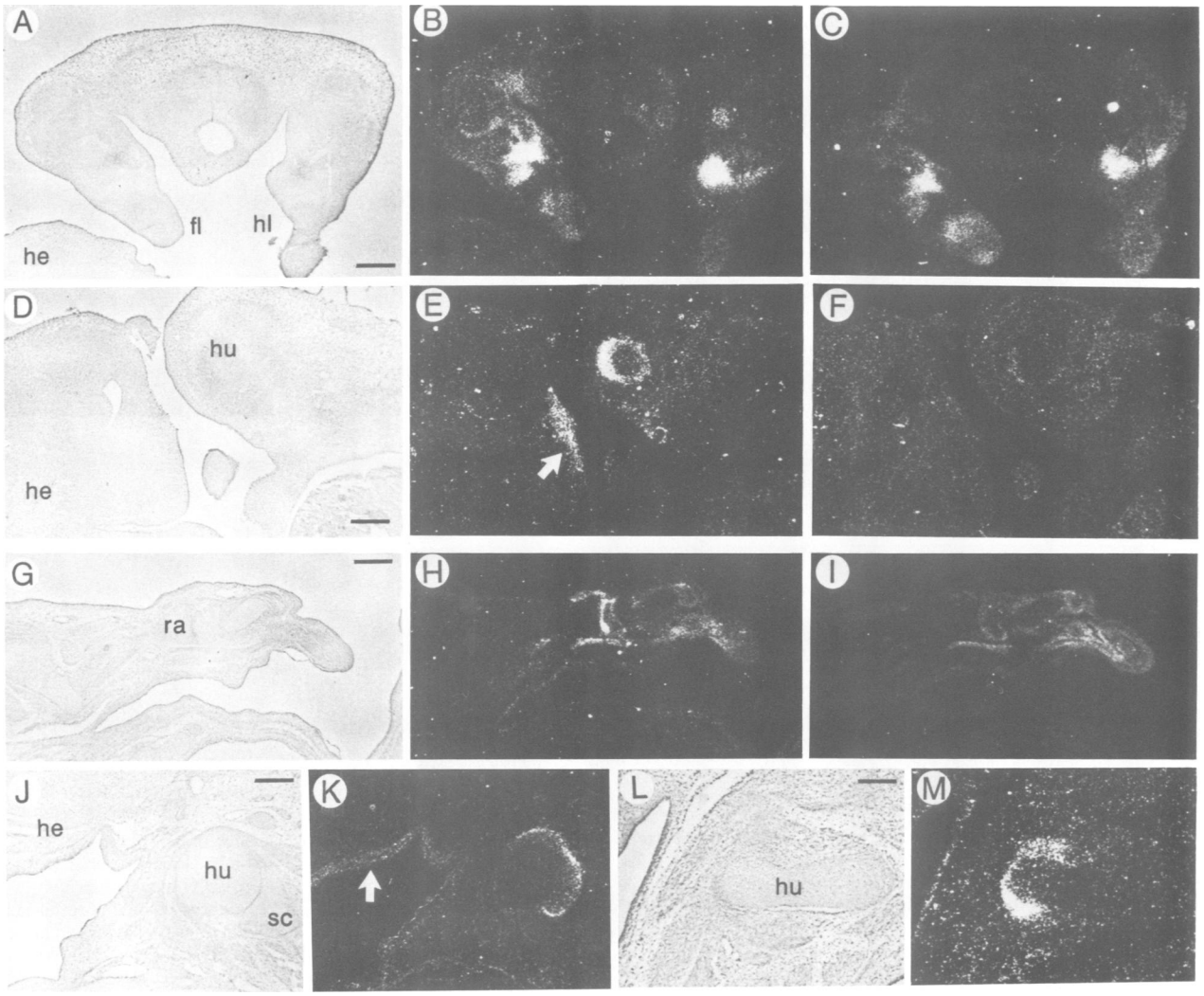

Fig. 4. *Hox-5.2* and *Hox-5.3* expression during later stages of limb development. (**A,B,C**) Parasagittal sections through 12.5 day old limbs hybridized with the *Hox-5.2* (**B**) or *Hox-5.3* (**C**) probes. Both signals are similar to each other but with a slightly stronger intensity for the *Hox-5.3* probe in more distal areas. Bar: 310 μm. (**D,E,F**) Parasagittal sections through the same foetus in a more medial plane hybridized with the *Hox-5.2* (**E**) or *Hox-5.3* (**F**) probes. *Hox-5.2* transcripts are detected in the presumptive humeral head and the superficial mesenchyme of the base of the head (arrow). *Hox-5.3* is not expressed in either of these structures. Bar: 250 μm. (**G,H,I**) Parasagittal section of a 14.5 day old foetus, enlarged in the forelimb distal region, hybridized with the *Hox-5.2* (**H**) or *Hox-5.3* (**I**) probes. A fine difference is again observed with respect to the proximo-distal position of both expression patterns. Bar: 310 μm. (**J,K**) Parasagittal section of a 14.5 day old foetus crossing the scapulo-humeral junction and hybridized with the *Hox-5.2* probe. The signal is restricted to the interzone between the humeral and scapular chondrogenic primordia, and to the superficial mesenchyme (arrow). Bar: 250 μm. (**L,M**) Parasagittal section of a 14.5 day old foetus enlarged at the distal part of the humerus primordium, and hybridized with the *Hox-5.2* probe. The hybridizing cells closely surround the cartilaginous bone model in its more distal extremity. Bar: 150 μm. fl, forelimb; hl, hindlimb; he, head; hu, humerus; ra, radius; sc, scapula.

plate and footplate where a weak homogeneous signal is distributed among the whole mesenchyme with the exception of the cartilaginous blastemas (Figure 4H). No labelling is detected in muscular blastemas. A weak signal is found in a thin mesenchymal layer underlying the epiderm of both fore- and hindlimb, also present in the lateral thoraco-abdominal and ventro-lateral walls of the base of the head extending until the caudal side of the muzzle (Figure 4K; arrow).

A comparison between *Hox-5.2* and *Hox-5.3* probes at that stage shows that *Hox-5.3* is expressed in similar perichondral regions of cartilage growth, but in a topographically more restricted way than that of *Hox-5.2*. In fact, no signal is detected with *Hox-5.3* near the proximal epiphyses of the humerus and femur (not shown). As for *Hox-5.2*, *Hox-5.3* transcripts are found near the cartilage of the distal segment bones and proximal rows of the carpus and tarsus (see Figure 4I). In the hand- and footplate, however, the signal is clearly more intense than that seen with the *Hox-5.2* probe (compare Figure 4H to 4I).

Expression in the foetal and adult gonads

The genital ridges, a thickening of the coelomic epithelium on the ventro-medial side of the mesonephric column, are colonized between the 10th and 11th days of mouse gestation by the primordial germ cells which migrate on both sides of the dorsal mesenteric attachment. Prior to day 13 p.c., gonadal development is in an undifferentiated state with no morphological distinctions between males and females. The proliferation of the coelomic epithelium into the underlying mesenchyme will give rise to the primitive sexual cords (for review and refs; see McLaren, 1981). The undifferentiated

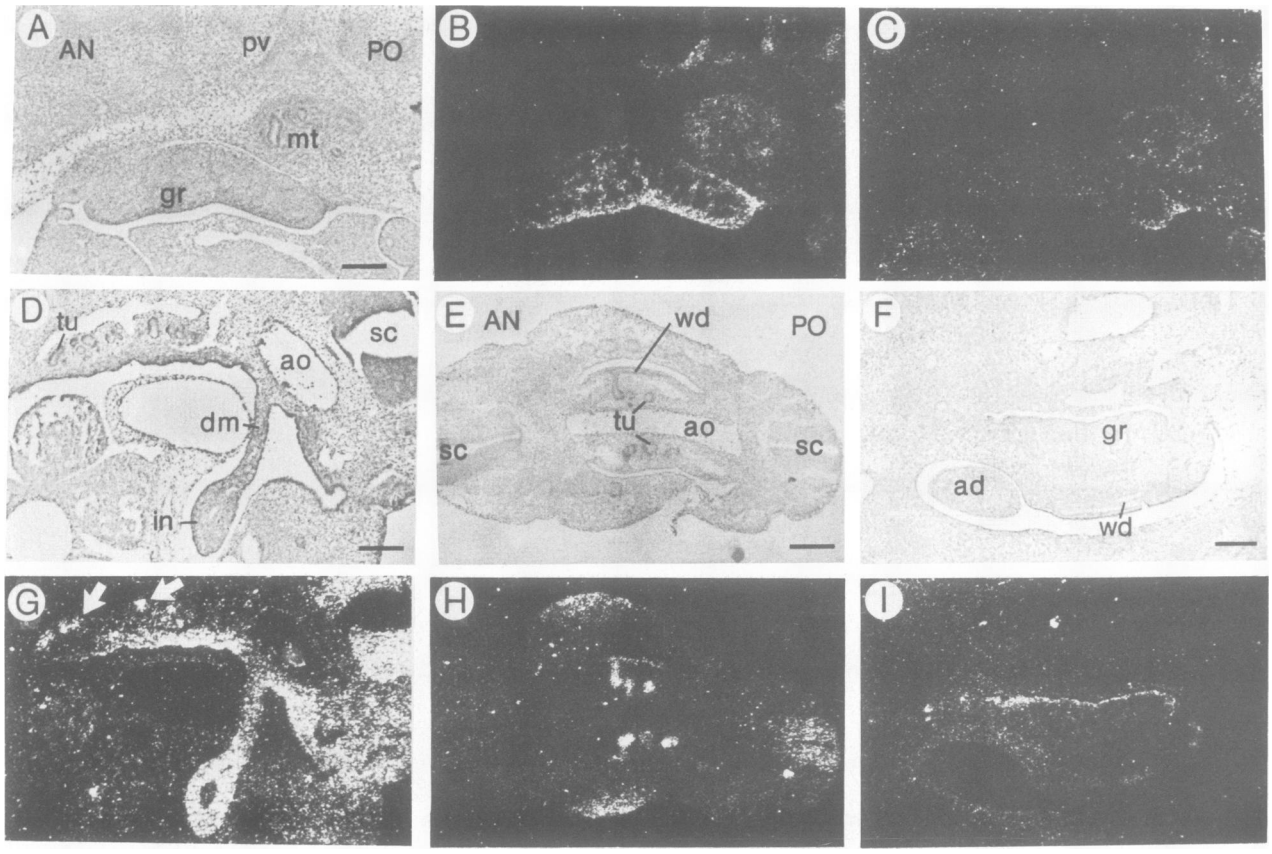

Fig. 5. *Hox-5.2* expression in the gonads, prior to sexual differentiation. (**A,B,C**) Sagittal sections through a genital ridge of a 12.5 day old foetus hybridized with the *Hox-5.2* (**B**) or *Hox-5.3* (**C**) probes. Only *Hox-5.2* is strongly expressed in the gonad anlage until a level more anterior (left side) than that seen in the pre-vertebrae. *Hox-5.3* hybridizes to the extreme posterior edge of the genital ridge. Bar: 150 μm. (**D,G**) Oblique section through a 10.5 day old foetus showing the mesonephros, the dorsal root of the mesentery and the presumptive genital ridges. *Hox-5.2* is specifically expressed in these presumptive areas as well as in mesonephric tubules (arrows in **G**). Bar: 150 μm. (**E,H**) Frontal section of a 10.5 day old embryo at the level of both mesonephros. At this level, only tubules and part of the Wolffian duct are *Hox-5.2* positive (**H**). In this last panel, both roots of the hindlimb buds are labelled. (**F,I**) Oblique sections showing the cortical distribution of *Hox-5.2* transcripts in the genital ridge. Bar: 150 μm. AN, anterior; po, posterior; pv, pre-vertebrae; mt, metanephros; gr; genital ridge; tu; mesonephric tubules; ao, aorta; sc, spinal cord; dm, dorsal mesentery; in, intestine; wd, Wolffian duct; ad, adrenal gland.

gonad thus consists of an external cortex and an internal medulla.

On both sagittal and transverse sections of 12.5 day old foetuses, *Hox-5.2* transcripts are found in the genital ridge region (Figure 5B and I). This strong hybridization extends more anteriorly than that seen in the dorsally adjacent mesonephros. In the latter, the anterior limit is at the level of pre-vertebra 21, which is in agreement with the limit in the sclerotomic column, whereas in the gonad anlage, the signal is absent until the level of metamere T5 (Figure 5B; compare hybridization in the gonad to the last labelled pre-vertebra). The hybridization is stronger in the cortical zone of the undifferentiated gonad, along the coelomic epithelium and in underlying cells, and is clustered in parallel bundles in the medulla, probably indicating the organization of the primitive sexual cords (Figure 5B). The *Hox-5.2* probe hybridizes to the presumptive gonadal region at 10.5 days p.c., when the genital ridge has not yet condensed. Strong hybridization is restricted to the ventral edge of the mesonephros, close to the coelomic epithelium, with a maximal intensity around the cleft separating this region and the dorsal mesenteric root (Figures 3F and 5G). Interestingly, at both developmental stages (10.5 and 12.5 days p.c.), labelling is found within each mesonephric tubular epithelium at all metameric levels where gonadal labelling occurs, while the remaining mesonephric tissue is negative (Figure 5G, arrow, and 5H). Comparative hybridization with the *Hox-5.3* probe fails to reveal any labelling in the indifferent gonad except in its very posterior edge (Figure 5C), in a level where hybridization is found in the adjacent mesonephros.

At 14.5 days p.c., sexual differentiation is clearly visible. In the testis, regularly arranged strands of seminiferous tubules (containing the gonocytes and the nourishing Sertoli cells) are separated by mesenchyme-derived Leydig cells. Hybridization with the *Hox-5.2* probe is intense, but is restricted to the cortex, whereas the seminiferous tubules are negative (Figure 6B). In females, the ovary forms via preferential development of the cortex in the undifferentiated gonad. The gonocytes are scattered throughout the ovarian cortex and surrounded by interstitial cells. The *Hox-5.2* hybridization pattern in the 14.5 day ovary is distributed in the whole ovarian cortex with a slight increase in the marginal layers (Figure 6D). The gonocytes do not seem to be significantly labelled. *Hox-5.2* expression persists in somatic cells of adult male and female gonads. Sections of adult testis after sexual maturation show that the signal is limited to the Leydig cells which are clustered between the seminiferous tubules (Figure 6F, arrows). In adult ovaries,

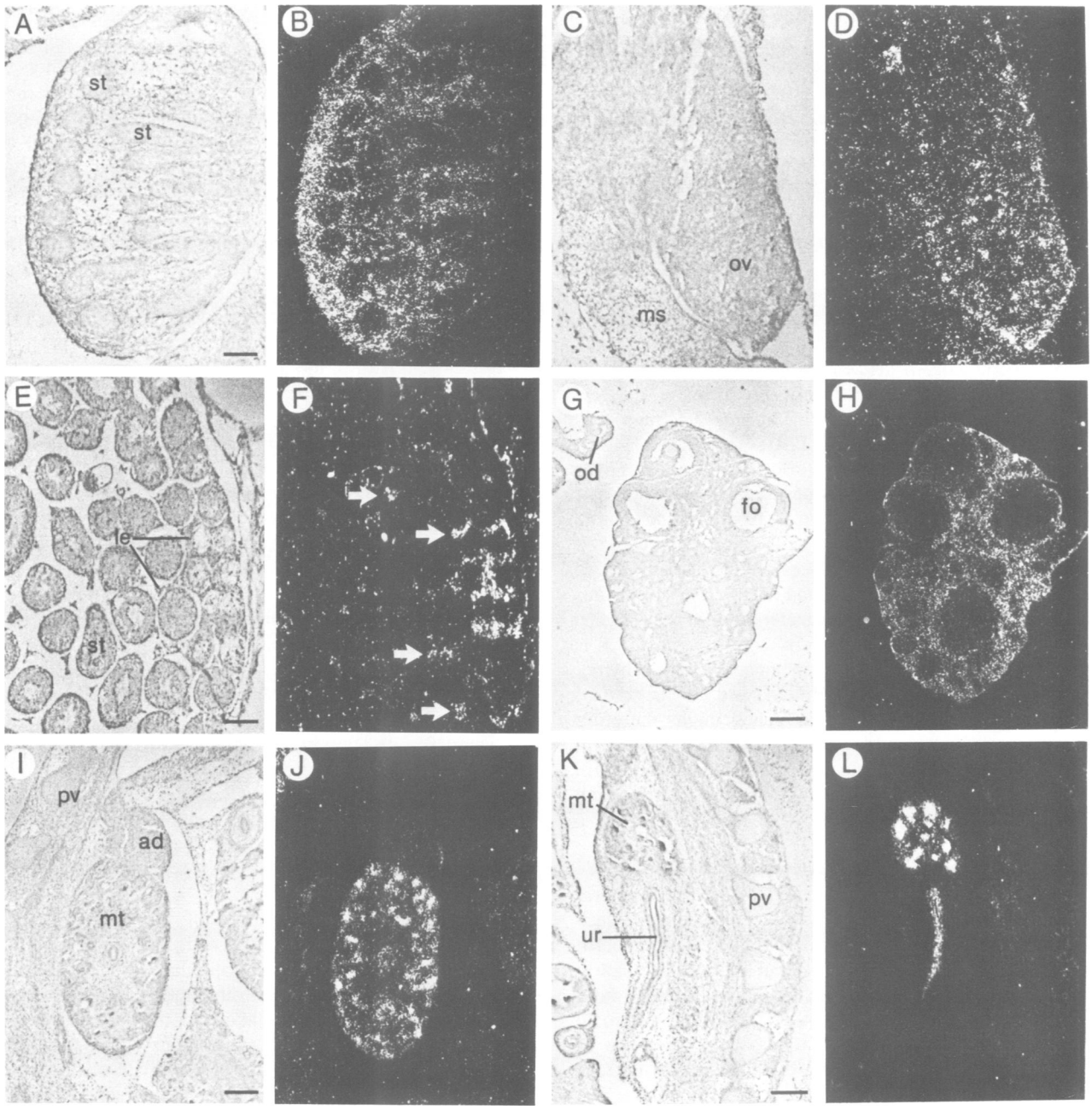

Fig. 6. *Hox-5.2* expression in foetal sexually differentiated and adult gonads. (**A,B**) Sagittal section through a 14.5 day old foetal testis hybridized with the *Hox-5.2* probe. Only interstitial cells are labelled, outside of the seminiferous tubules. Bar: 100 μm. (**C,D**) Section of an ovary at the same age as for (**A**) and (**B**). The *Hox-5.2*-specific signal is weaker than in testes and preferentially superficial. (**E,F,G,H**) Sections of an adult testis (**E−F**) and ovary (**G−H**) at sexual maturity. The *Hox-5.2* signal is found in the interstitial Leydig cells (arrows in **E**) and interstitial cells of the ovarian cortex respectively. Cells of the follicles, granulosa and thecae do not show significant labelling, neither does the neighbouring oviduct. (**I,J,K,L.**) Two different planes of sections, at 14.5 days, of the developing metanephros. The *Hox-5.2* transcripts are found in the whole blastema with reinforcements in tubular epithelial cells and in the ureter epithelium. Bar: 150 μm. st, seminiferous tubules; ms, mesonephros; ov, ovary; le, Leydig cells; od, oviduct; fo, ovarian follicle; pv, pre-vertebrae; ad, adrenal gland; mt, metanephros; ur, ureter.

hybridization is found in the interstitial cells of the ovarian stroma, whereas the follicular cells are not labelled (Figure 6H).

Expression in the developing metanephros

Both *Hox-5.2* and *Hox-5.3* are expressed in the metanephros, but with quite distinct specificities. The *Hox-5.2* probe hybridizes to the whole metanephric stroma, with particularly intense signals in the tubular epithelial structures including the ureter epithelium (Figure 6J and L). In contrast, the *Hox-5.3* probe displays a weak but homogeneous signal without any enhancement in epithelial structures.

Discussion

This paper describes the developmental expression pattern of two gene members of the mouse homeogene-containing HOX-5 complex as revealed by *in situ* hybridization. These two novel genes, *Hox-5.2* and *Hox-5.3*, lie close to each

other and far upstream from the previously described *Hox-5.1* gene (Featherstone *et al.*, 1988). *Hox-5.3* is located at the most upstream position so far described within the overall HOX network, whereas *Hox-5.2* belongs to the same subfamily as *Hox-1.7* (Rubin *et al.*, 1987), *Hox-2.5* (A.Graham and R.Krumlauf, personal communication) and *Hox-3.2* (Breier *et al.*, 1988). Because of the very upstream positions of these two related genes along the HOX network, we would have predicted their expression domains to be located in the posterior-most part of the developing organisms (Gaunt *et al.*, 1988). This is the case since the *Hox-5.2* and *Hox-5.3* transcripts are found in sclerotomes, posterior to the level of pre-vertebrae 21 and 22 respectively, and in the posterior part of the spinal cord (Duboule and Dollé, 1989). This restricted 'posterior specification' in sclerotomes and CNS is probably also true for the few internal organs containing *Hox-5.2* or *Hox-5.3* transcripts (Dony and Gruss, 1987a; Gaunt, 1988; Holland and Hogan, 1988a). Actually, only very posterior organs are positive, such as posterior parts of the intestine, the posterior part of the mesonephric column and the developing metanephros. In this last structure (the future adult kidney), *Hox-5.2* is expressed in all metanephric tissues but the signal is much stronger in the epithelial tubules and in the ureter itself, thus closely resembling the expression pattern of the *Hox-2.1* gene (Holland and Hogan, 1988b). In contrast, *Hox-5.3* is expressed homogeneously in the metanephric parenchyme, as are most of the Hox genes (Holland and Hogan, 1988a; Gaunt *et al.*, 1988). These two genes might therefore contribute to the metanephric blastema formation. In addition to these somewhat predictable expression domains, *Hox-5.2* is transcribed in gonads and both genes are highly active in limb buds.

Expression in limb buds and developing limbs

Hox-5.2 and *Hox-5.3* are expressed in a wide temporal period in mesodermal cells of the developing limbs. Since the forelimb buds are thought to derive from the mesoderm of metameres C4−T1, this regional expression clearly extends more anteriorly than the expression limits of both genes in the trunk structures. However, this anterior domain of expression could be related to the clear presence of *Hox-5.2* transcripts in the lateral plate mesoderm up to a very anterior position at earlier stages of development. The presence of *Hox-5.2* transcripts within mesenchymal cells at the time of limb bud formation suggests that these genes may also play a role in this crucial morphogenetic process. However, it is not yet clear whether the *Hox-5.2* product would actively take part in the inductive mechanism or be an early response to it. The dynamics of *Hox-5.2* expression in developing limbs is characterized by two periods. Early, from days 9 to 10 p.c., the expression is homogeneous in mesodermal cells of the limb buds. Later, a progressive restriction of *Hox-5.2* transcribing cells become apparent, which may proceed in a proximo-distal direction. Thus, the cells which continue to express *Hox-5.2* at later stages are cells engaged in a differentiation pathway leading to cartilage formation. These cells are found in the early pre-cartilaginous blastemal condensations (as early as the 10th day), and at a later stage in the zones of apposition of new cells in growth regions of the cartilaginous skeleton. In both cases, *Hox-5.2* transcripts are absent in cells showing a typical chondroblast morphology. Though *Hox-5.3* shows a similar expression pattern, some differences are systematically observed. First, while the expression of *Hox-5.2* seems to be more important in the proximal part of the developing limb (see Figures 3F or 4B), *Hox-5.3* is clearly expressed at high level more distally (see Figures 3G or 4C), suggesting that both gene products could be involved in the establishment of the proximo-distal axis of the limb (compare also the fingertips in Figure 2B and C). Secondly, some cellular subsets expressing *Hox-5.2* do not show any *Hox-5.3* expression, as exemplified by cells surrounding the humeral head (see Figure 4E and F). This might be due to the extreme 'proximal' position of the humeral head. Thus, the apparent restriction of *Hox-5.3* expression to more distal positions in developing limbs may be correlated with its more posterior expresion domain in the trunk (Duboule and Dollé, 1989; and Figure 2). This late expression of *Hox-5.2* and *Hox-5.3* in cartilage-differentiating cells is comparable to what is observed with other *Hox* genes in sclerotomic axial derivatives, i.e. the future vertebral bodies and ribs (e.g. *Hox-1.3*, Dony and Gruss, 1987a; *Hox-1.4*, B.Galliot and P.Dollé, unpublished results). Thus, *Hox-5.2* and *Hox-5.3* might play a role in the formation of the limb skeleton, via mechanisms similar to those involving *Hox* genes in the axial skeleton. Interestingly, *Hox-5.2* and *Hox-5.3* transcripts are disappearing from these cellular types by the time the proto-oncogene c-*fos* has been reported to be turned on (Dony and Gruss, 1987b). Comparative analysis underway will reveal whether a close relationship between their respective expression domains exists.

These results support the observation made by Savard *et al.* (1988) concerning the possible involvement of *Hox* genes during amphibian limb specification and regeneration, and are in agreement with recently published work (Oliver *et al.*, 1988; G.Oliver and E.M.De Robertis, personal communication).

Expression in gonads

Hox-5.2 transcripts are detected with a high intensity in a subset of cells within the gonadal anlage. Strong labelling is seen on the 10th day p.c. around the ventro-medial side of the coelomic epithelium that covers the mesonephros, at the exact position where the genital ridge will appear. This suggests that *Hox-5.2* might be involved in inductive mechanisms specifying the fate of those mesonephric cells which will become part of the gonads. Though the colonization of the genital ridges by migrating gonocytes is underway, at this stage, *Hox-5.2* transcripts are clearly found in the somatic cells. Labelling persists until day 12.5 p.c. in the cortex of the genital ridges and presumably in the primitive sexual cords. This expression is not sex-specific, since it is found in the interstitial cells of both foetal testes and ovaries, until the latest stage studied (i.e. 17.5 day p.c.). Moreover, a weak expression is still restricted to the interstitial cells of the adult gonads (the Leydig cells of the testes, and the interstitial cells of the ovarian cortex). Whether this expression in adult tissues reflects a real function of the *Hox-5.2* protein in the adult gonads is not known. As in the case of the developing forelimbs, the expression in the foetal gonads is at a level more anterior than that observed in the surrounding somitic and mesonephric mesoderm (up to the level of metamere T5 by day 10.5 p.c.). However, a restricted hybridization in the anterior part of the mesonephros is detected only in the

Wolffian duct and mesonephric tubules close to it. The labelled tubules are found at the same metameric levels as the genital ridge. During development, most of the mesonephric tissue is thought to degenerate while only some tubules will differentiate into the genital excretory ducts. These data suggest that *Hox-5.2* could be a marker for those precise structures which will be involved in the formation of the genital excretory apparatus.

Hox-5.2 and *Hox-5.3* show similar homeo-domain sequences and expression domains though with subtly different anterior boundaries along the cranio-caudal axis (one or two metameres), reflecting their relative positions along the HOX-5 complex. However, *Hox-5.3* transcripts are not found in developing or adult gonads, at any stages analysed. Taken together, these observations may illustrate different functions for certain *Hox* genes. As well as providing positional cues along the body axis they could also act, for example, during inductive processes or cell lineage determination.

Materials and methods

Preparations of embryo sections

Embryos and foetuses were obtained from natural matings between F1 (B6 × B12 mice). Midday of the day of the vaginal plug was designated as day 0.5 p.c. Embryos up to 9.5 days p.c. were fixed, embedded and sectioned in their deciduae, while older foetuses were dissected out of their membrane prior to fixation. After fixation in 4% fresh paraformaldehyde in phosphate-buffered saline (12–24 h, 4°C), the embryos were dehydrated in ethanol, cleared in xylene and embedded in paraffin wax (m.p. 56°C). Sections (5–6 μm thick) were collected on 0.5% gelatine/0.5% chrome alum subbed glass micrsocope slides (adapted from Pardue, 1985), air-dried and occasionally stored at 4°C prior to hybridization. Foetuses at day 17.5 were frozen in liquid nitrogen and frozen sections were hybridized according to Dony and Gruss (1987).

Preparation of the RNA probes

The DNA templates used for probe synthesis are located 3′ to the *Hox-5.2* and *Hox-5.3* homeoboxes and were subcloned into the vector pGEM-1 (Promega Biotec). These probes have been previously reported together with the cloning of the HOX-5 complex (Duboule and Dollé, 1989). For *Hox-5.2*, the *Bam*HI–*Pvu*II fragment containing the homeobox was cloned and recut with *Fok*I, thus generating a ~750 nucleotide (nt) probe containing the 3′ most extremity of the homeobox and flanking sequences. For *Hox-5.3*, the *Bam*HI–*Hin*dIII fragment extending 3′ from the homeobox was cloned and linearized with *Hin*dIII. Three different *Hox-5.3* probes were tried which gave similar results but with a less favourable signal-to-noise ratio. ^{35}S-labelled antisense RNA probes with a specific activity of ~5 × 10^8 c.p.m./μg were synthesized using either T7 (*Hox-5.2*) or SP6 (*Hox-5.3*) polymerases (Promega Biotec), according to the manufacturer's recommendations and as previously described (Melton *et al.*, 1984). [α-^{35}S]CTP′ (850 Ci/mmol, Amersham) was used as substrate. The length of the probes was reduced to ~100 nt by limited alkaline hydrolysis with Na_2CO_3 at pH 10.2 (Cox *et al.*, 1984). The control sense RNA probes were simultaneously synthesized using the opposite strands as templates.

In situ hybridization

The *in situ* hybridization experiments were carried out essentially as described in Gaunt *et al.* (1986) with the following modifications. The slides were prehybridized for 2 h at 50°C, in 50% formamide, 0.3 M NaCl, 10 mM Tris–Cl, pH 6.8, 10 mM $NaPO_4$, pH 6.8, 5 mM EDTA, 1 × Denhardt's, 10 mM DTT, 500 mg/ml yeast RNA, 100 mg/ml salmon sperm DNA and 500 nmol/ml non-labelled α-thio-UTP (Du Pont). After the RNase A treatment the slides were washed for 1 h in the washing buffer. They were subsequently washed in 2 × SSC for 15 min, at room temperature, then in 0.1 × SSC for 15 min at 50°C, before a final wash in 3 l of 0.1 × SSC for 30 min at room temperature. After dehydration of the sections, they were coated with Kodak NTB-2 emulsion, dried and stored at 4°C. The exposure times were from 12 to 15 days. Kodak D19 developer was used for 2 min at 20°C. The sections were then stained in toluidine blue, dehydrated in ethanol and mounted under coverslips in Eukitt mountant.

For each development stage studied, at least two embryos or foetuses were sectioned and hybridized. Comparative *in situ* hybridization using the *Hox-5.2* and *Hox-5.3* probes were performed on serial sagittal or transverse sections, as follows: two sections were hybridized with the *Hox-5.2* probe, while the two adjacent sections on each side of the paraffin ribbon were hybridized with the *Hox-5.3* probe. In all cases, the overall hybridization pattern was similar for both sections.

As a negative control, adjacent sections were systematically hybridized with the sense probes. In addition, some sections were pretreated with RNaseA (50 μg/ml, 90 min) before hydridization with the antisense probes. In all cases, no specific signal was found.

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