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Developmental expression of the mouse *Evx-2* gene: relationship with the evolution of the HOM/Hox complex

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SUMMARY

The mouse *Evx-2* gene is located in the immediate vicinity of the *Hoxd-13* gene, the most posteriorly expressed gene of the HOXD complex. While the *Evx-1* gene is also physically linked to the HOXA complex, it is more distantly located from the corresponding *Hoxa-13* gene. We have analysed the expression of *Evx-2* during development and compared it to that of *Evx-1* and *Hoxd-13*. We show that, even though *Evx-2* is expressed in the developing CNS in a pattern resembling that of other *Evx*-related genes, the overall expression profile is similar to that of the neighbouring *Hoxd* genes, in particular with respect to the developing limbs and genitalia. We propose that the acquisition of expression features typical of Hox genes, together with the disappearance of some expression traits common to *Evx* genes, is due to the close physical linkage of *Evx-2* to the HOXD complex, which results in *Evx-2* expression being partly controlled by mechanisms acting in the HOX complex. This transposition of the *Evx-2* gene next to the *Hoxd-13* gene may have occurred soon after the large scale duplications of the HOX complexes. A scheme is proposed to account for the functional evolution of *eve*-related genes in the context of their linkage to the HOM/Hox complexes.

Key words: homeobox, *even-skipped*, development, HOX complex phylogeny

INTRODUCTION

The mouse genome contains 38 Hox genes which are clustered in four genomic loci (the HOXA,B,C and D complexes). These complexes have similar features of structure and organization as they arose by large-scale duplication of an ancestral complex (reviewed in McGinnis and Krumlauf, 1992; Dollé and Duboule, 1993). During development, Hox genes are activated during gastrulation, while the embyo establishes its anteroposterior axis. *Hox* transcripts are found in partially overlapping domains along the embryonic axis, with genes located towards the 5′ extremity of one complex being expressed in progressively more posterior (caudal) areas of the embryo (Gaunt et al., 1988). All these genes are transcribed from the same DNA strand so that each complex can be assigned with a general 5′ (posterior) to 3′ (anterior) orientation. The importance of this gene family to the establishment and realization of the vertebrate body plan has been recently demonstrated by gene inactivation experiments (LeMouellic et al., 1992; Ramirez-Solis et al., 1993; Dollé et al., 1993; Condie and Capecchi, 1993). The results of such inactivations suggest that the function of Hox genes could be mediated through the control of either the local growth or patterning (or both) of structures of neurectodermal and/or mesodermal origin.

In their 5′ regions, the HOXA, C and D complexes contain 4-5 Hox genes that are related to the *Drosophila Abdominal-B* homeotic gene (Izpisúa-Belmonte et al., 1991). The tandem duplication of these Hox sub-groups is thought to have occurred before large scale HOX cluster duplication and may, perhaps, have been linked to evolution of appendicular structures, since such genes are coordinately expressed in the developing limbs and genital tubercle (Dollé et al., 1989, 1991a; Yokouchi et al., 1991) in which they exert an important function (Dollé et al., 1993; Small and Potter, 1993).

The coordinate expression of Hox genes may result from a successive temporal activation of these genes, in a 3′ to 5′ sequence, the *temporal colinearity* (see Dollé et al., 1989; Izpisúa-Belmonte et al., 1991; Duboule, 1992). As a consequence, genes located in 5′ positions display transcript domains that are always more distal (or posterior and distal at earlier stages) than their 3′ neighbour genes (the *spatial colinearity*). In the genital tubercle, quantitative differences are observed rather than true spatial colinear expression domains (Dollé et al., 1991a). The fact that some of these genes are functional in all axial skeleton structures has been confirmed by the experimental knock-out of *Hoxd-13*, which alters both the growth and patterning of skeletal elements arising from the limb buds and genital tubercle (Dollé et al., 1993).

Evx-1 and *Evx-2* are the two genes identified to date in mammals, which contain a homeobox homologous to that of the *even-skipped* (*eve*) gene from *Drosophila* (Bastian and Gruss, 1990). A genomic walk along the upstream (5′) part of

the human *HOXD* locus revealed that the human *EVX2* gene is located in the close vicinity of *HOXD13* (D'Esposito et al., 1991) and a similar linkage was reported in mice (Bastian et al., 1992). The human *EVX2* homeobox is found about 13 kilobases (kb) away from that of *HOXD13*. Similarly, the *EVX1* gene is linked to the HOXA complex. In this case, however, its distance from the complex is larger, as it lies approximately 45 kb upstream of the *HOXA13* gene (Faiella et al., 1992). Interestingly, both *EVX1* and *EVX2* are transcribed from the DNA strands that are opposite to those from which all *HOX* sequences are transcribed (Faiella et al., 1992; D'Esposito et al., 1991).

The developmental expression of the murine *Evx-1* gene has been studied in detail; *Evx-1* starts to be expressed at approx. 6.5 days post-coitum (dpc) in part of the embryo epiblast, and then throughout gastrulation (6.5-8.5 dpc) in a graded fashion along the primitive streak and the involuting mesodermal cells, but not anterior to the primitive streak (Dush and Martin, 1992). Later on, *Evx-1* is still expressed in the tail bud, which corresponds to the primitive streak in its histogenic potential to generate various tail structures. Starting at approx. 10.5 dpc, *Evx-1* is also expressed in discrete neural areas along the spinal cord and hindbrain (Bastian and Gruss, 1990). Finally, *Evx-1* transcripts are transiently detected in the limb buds, where they first appear in the posterior distal mesenchyme, and are subsequently maintained only in very distal (subectodermal) cells (Niswander and Martin, 1993). Thus, none of the three components of the *Evx-1* pattern during development (early, neural and limb expression) resembles the coordinate regulation of the neighbouring Hox genes, which are activated at later stages of gastrulation and in progressively more posterior areas (Yokouchi et al., 1991; Haack and Gruss, 1993).

We have analysed further the linkage of *Evx-2* to the mouse HOXD complex and show that it lies in close contact with the *Hoxd-13* gene (approx. 8 kb). Such a distance is in the range of the usual spacing between Hox genes themselves and we investigated whether *Evx-2* gene expression could, in part, be controlled by regulatory mechanisms acting on the neighbouring Hoxd genes, or instead, would be regulated independently from the complex, as seems to be the case for *Evx-1*. We report here that *Evx-2* is regulated as expected for a gene belonging to the HOXD complex, in both the limb buds and the genitalia. Interestingly, *Evx-2* expression follows temporal colinearity (i.e. shortly after the *Hoxd-13* gene), and thus does not share the early expression phase of *Evx-1* during gastrulation. However, an important aspect of *Evx-2* expression (in the CNS) makes it different from other Hoxd genes but similar to *Evx-1*. The relationships between the respective genomic locations of Evx genes and the potential control of their expression by regulatory mechanisms acting upon the HOX complexes are discussed from an evolutionary perspective.

Evx-2 IS NEAR *Hoxd-13* AND IS ACTIVATED LATE IN DEVELOPMENT

The cloning and sequence analysis of DNA fragments located upstream the *Hoxd-13* gene on genomic cosB (Dollé et al., 1991b) confirmed the linkage between the *Evx-2* sequences and the HOXD complex. Sequences were identified that correspond to the previously reported sequence of an *Evx-2* cDNA (Dush and Martin, 1992) and encode the N terminus of the *Evx-2* product. We localized the *Evx-2* ATG initiation codon to 8 kb from the *Hoxd-13* initiation codon, in an inverted orientation (Fig. 1). The spacing between *Evx-2* and *Hoxd-13* coding regions was thus found to be in the range of that observed between all five neighbouring *Abdominal-B* related Hox genes (from 5 to 10 kb; Fig. 1). This genomic organization is distinct from that of the *EVX1* locus, which was mapped, in human, at about 45 kb from *HOXA13* (Faiella et al., 1992; Fig. 1).

Dush and Martin (1992) reported the activation of *Evx-1* shortly before the onset of gastrulation; here we compared *Evx-1* and *Evx-2* expression at corresponding stages by in situ hybridization. No detectable *Evx-2* labeling was observed in 7.5 dpc embryo sections (Fig. 2A). In contrast, a graded dis-

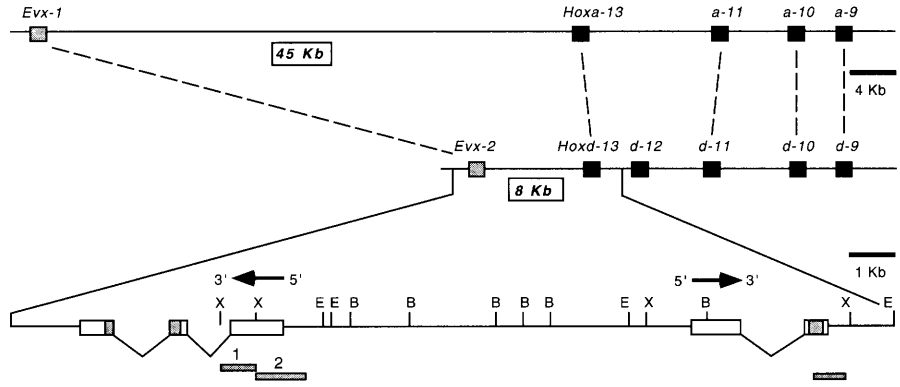

Fig. 1. Structural relationships between the HOXA/Evx-1 and HOXD/Evx-2 complexes. 12 kilobases of genomic DNA extending 5′ from the *Hoxd-13* transcription unit (Dollé et al., 1991b) were subcloned and shown, by Southern blot analysis, to contain *Evx-2* sequences (Bastian et al., 1992). Extensive sequence analysis localized the 5′ extremity of a previously reported *Evx-2* cDNA clone (Dush and Martin,1992) as well as the position of the exon containing the 5′ part of the *Evx-2* homeobox. The *Evx-2* coding sequence was found to lie on the opposite DNA strand to that of *Hoxd-13*. *Evx-2* and *Hoxd-13* ATG initiation codons were mapped and found to be separated by approx. 8 kilobases of genomic DNA. The five *Hox* subgroups related to *Abd-B* (groups 9 to 13) are shown here as black boxes. Dashed lines indicate paralogous genes on the HOXA and HOXD complexes. The HOXA complex lacks a gene member of group 12. The distances between the various genes are drawn to scale to emphasize the important difference in the spacing between *Evx-1/Hoxa-13* and *Evx-2/Hoxd-13*. The bottom line shows an enlargement of the *Evx-2/Hoxd-13* region with arrows indicating the opposite directions of transcription. The transcription units are sketched with, in dark boxes, the positions of the two homeoboxes. Stippled rectangles below the line point to the locations of the various RNA probes used for in situ hybridization experiments. X, *Xho*I; B, *Bam*HI; E, *Eco*RI (data from this work and Bastian et al., 1992; Faiella et al., 1992; D'Esposito et al., 1991; Haack and Gruss, 1993).

tribution of *Evx-1* transcripts was seen in the primitive streak, the embryonic mesoderm and the epiblast (Fig. 2A; and Dush and Martin, 1992). *Evx-2* expression was also not detected in 8.5 dpc embryos, a stage at which the *Evx-1* signal was present in caudal areas (Fig. 2B). At this stage, a restricted *Hoxd-13* signal was seen in the most posterior embryonic mesoderm and hindgut (Fig. 2B, arrow). This probably represents the time of *Hoxd-13* activation, slightly earlier than previously reported (Dollé et al., 1991b). At later stages of development, no *Evx-2* labeling was detected in the tail bud (Fig. 2C), a region equivalent to the primitive streak (Tam, 1984), even though specific signals were observed in other areas of the same embryos (see below). In contrast, both *Evx-1* and *Hoxd-13* transcripts were detected in the tail bud, although with different distributions

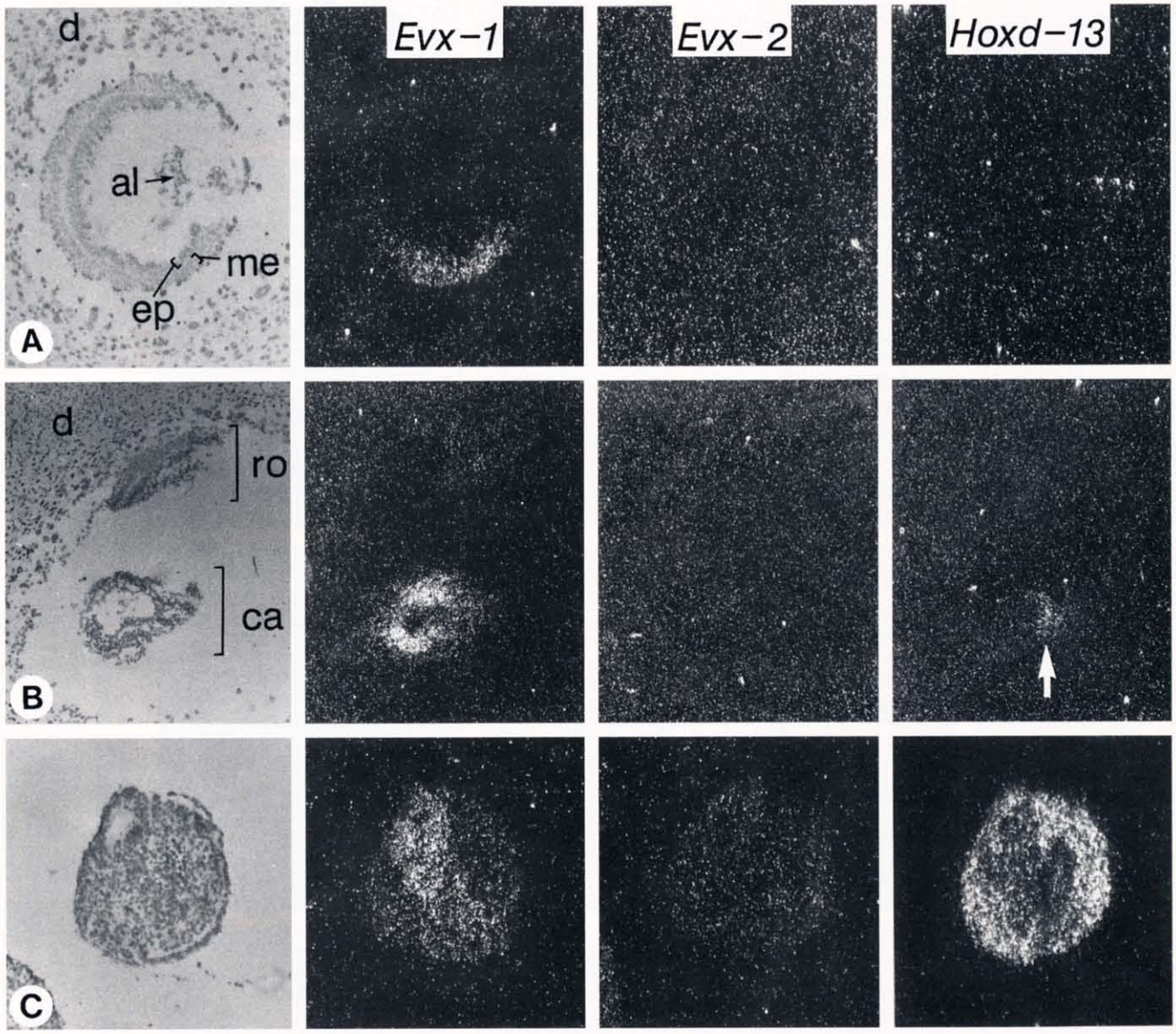

Fig. 2. Activation of *Evx-1*, *Evx-2* and *Hoxd-13*. (A) Three sections of a 7.5 dpc mouse embryo hybridized to *Evx-1*, *Evx-2* or *Hoxd-13* riboprobes. A corresponding section viewed under bright-field light is shown on the left for histological details. The caudal part of the embryo is towards the bottom of the picture, next to the allantois. (B) Sections through the caudal extremity (ca) of a 8.5 dpc embryo. In addition to the strong *Evx-1* signal, a restricted labeling is detected for *Hoxd-13* (arrow) while no *Evx-2* transcripts are observed yet. The most rostral part of the same embryo (ro) is negative for all three probes. (C) Enlargement of the tail bud area of a 10.5 dpc embryo, which shows distinct distributions of *Evx-1* and *Hoxd-13* transcripts. No *Evx-2* signal is detected even though other areas of the same embryo are positive at this stage (see below). For in situ hybridization, embryos and fetuses were collected at various stages of gestation, and the day of the vaginal plug appearance was considered as day 0.5 post-coitum (dpc). The embryos were either fixed overnight in 4% paraformaldehyde and embedded in paraffin wax, or frozen directly in OCT medium. In situ hybridization was performed both on paraffin-embedded embryo sections and frozen sections; the latter result in higher signal to noise ratio (Décimo et al., 1994) although with a somewhat poorer histology. In both cases, series of adjacent sections were collected on three sets of slides which were subsequently hybridized to *Evx-2*, *Hoxd-13* and *Evx-1* probes. ep, epiblast (embryonic ectoderm); me, mesoderm; al, allantois; d, decidual tissue; ro, rostral; ca, caudal.

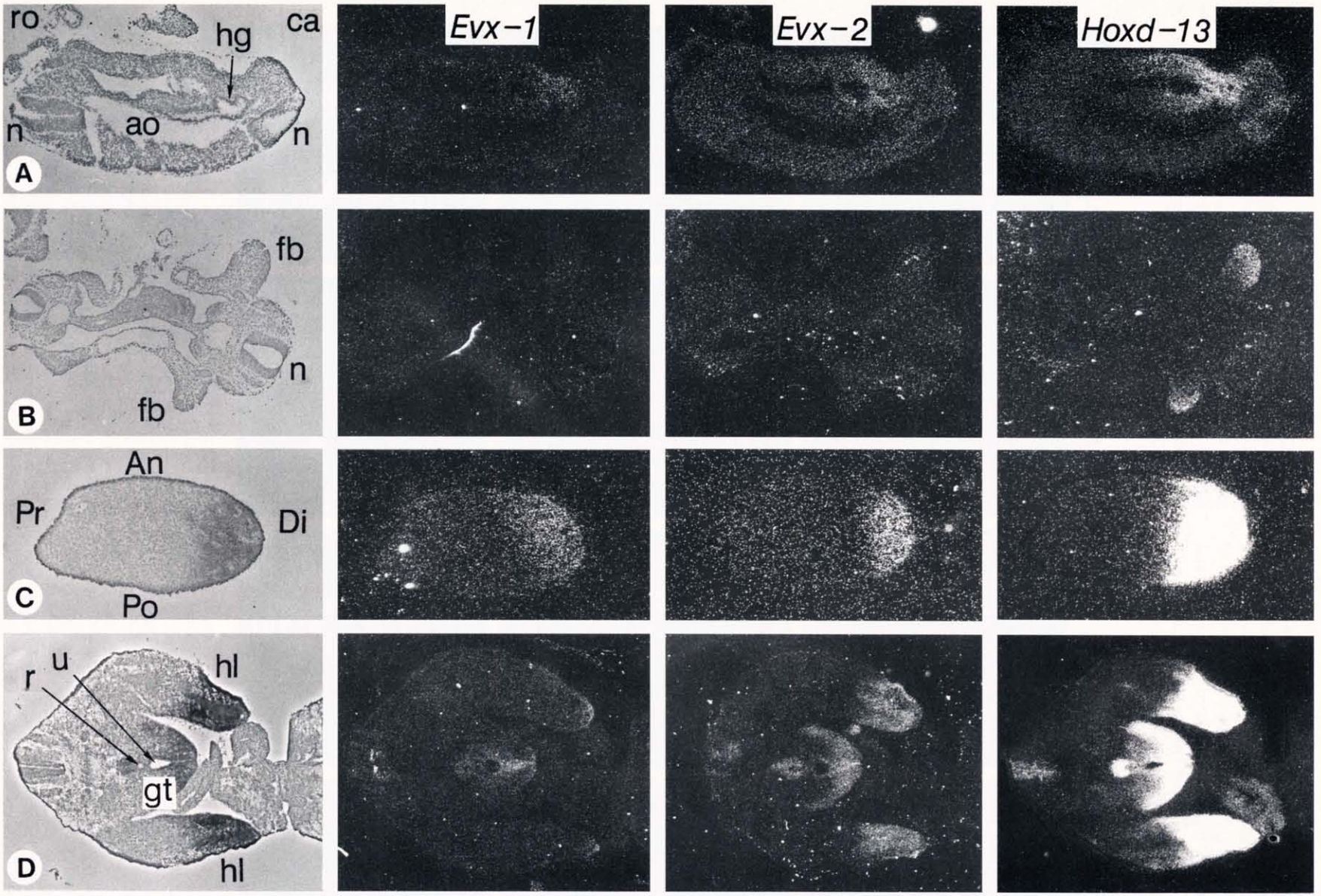

Fig. 3. Activation of *Evx-1, Evx-2* and *Hoxd-13* along the body axes. (A) Transverse section of a 9.5 dpc embryo, crossing the dorsal part of the abdominal cavity and the hindgut (cloaca). Note the similar and limited *Evx-2* and *Evx-1* transcript domains in the vicinity of the hindgut diverticulum. (B) Transverse section of a 10.0 dpc embryo, through both forelimb buds. The specimen was collected as a 10.5 dpc embryo, but was developmentally retarded. At this stage, only *Hoxd-13* transcripts are expressed in the forelimb buds. (C) Enlargement of the forelimb bud of a 10.5 dpc embryo, sectioned sagitally. (D) Transverse section through the hindlimbs and genital tubercle of a 12.5 dpc embryo. At this stage, there is a strong difference between *Evx-2* and *Evx-1* transcripts in both structures. hg, hindgut; ao, aorta; n, neural tube; fb, forelimb buds; hl, hindlimbs; gt, genital tubercle; u, urogenital sinus; r, rectum; ro, rostral; ca, caudal; Pr, proximal; Di, distal; An, anterior; Po, posterior.

(Fig. 2C). *Evx-2* expression was not merely delayed in the primitive streak and tail bud, with respect to *Evx-1*; instead, *Evx-2* was not activated in these structures.

The earliest signs of *Evx-2* transcription were seen in 9.5 dpc embryos, in the cloacal epithelium and surrounding mesoderm (Fig. 3A), from which the genital bud will arise. *Hoxd-13* was also expressed in these structures, but extending over a larger area (Fig. 3A). *Evx-1* was also expressed in this future genital region (Fig. 3A). *Evx-2* transcripts were not visible in 9.5 or 10.0 dpc limb buds while *Hoxd-13* labeling was clearly detected in posterior mesenchyme (Fig. 3B). The first detection of *Evx-2* expression in the forelimb bud was at 10.5 dpc, when it was restricted to a subset of *Hoxd-13*-expressing cells in distal and posterior mesenchyme, within an area that also expressed *Evx-1* (Fig. 3C). The co-expression of *Evx-1* and *Evx-2* in both the genital area and limb buds was transient and the two expression patterns diverged by 11.5 dpc (not shown). In 12.5 dpc fetuses, *Evx-1* was expressed in the very distal extremities of the limb mesenchyme, while *Evx-2* displayed a Hoxd-type of expression pattern, that is, across a wider distal region (Fig. 3D; see below).

EXPRESSION IN LIMB BUDS AND GENITALIA

Hoxd genes are expressed in the limb buds along spatial domains which are colinear with the ordering of the genes along the chromosome (Dollé et al., 1989). In both 11.5 dpc fore- and hindlimbs, the distribution of *Evx-2* transcripts was consistent with this gene being subject to a similar mechanism of regulation. Successive sections of hindlimb buds showed that *Hoxd-13* transcripts were distributed in slightly more extended domains than those of *Evx-2*, both more proximally, along the posterior margin of the bud (Fig. 4A), and more anteriorly, when progressing towards the tip of the bud (Fig. 4B,C). These differences were still visible in 12.5 dpc limbs (Fig. 4D). Both *Evx-2* and *Hoxd-13* transcripts were restricted to the footplates; however, the *Evx-2* domain was not as proximal as the *Hoxd-13* domain along the posterior margin and the anterior margin (Fig. 4D). The weak expression of *Evx-2* in the limbs of 13.5 dpc fetuses indicated that its transcript domain extended into all five digit primordia, up to the base of the most anterior digit I (Fig. 4E), a domain equivalent to that in which *Hoxd-13* is expressed and functional (Dollé et al., 1993). However, while transcripts of 5′-located Hoxd genes are known to accumulate at high levels in the limb extremities, with *Hoxd-13* displaying the strongest expression until at least 17.5 dpc (Dollé et al., 1991b; unpublished data), the *Evx-2* signals remained much weaker at 12.5 and 13.5 dpc (see Fig. 4D-E) and were hardly detectable at 14.5 dpc (not shown).

Hoxd genes are expressed in the genital tubercle where 5′-gene transcripts are more abundant, and maintained for longer times, than 3′-gene transcripts (Dollé et al., 1991a). Serial sections of 11.5 dpc embryos showed that *Evx-2* also had a specific expression domain in the genital bud and urogenital mesenchyme. This domain is similar to that of *Hoxd-13*, both being centred in the genital bud, and around the urogenital sinus and rectum (Fig. 5A). However, *Evx-2* transcripts were not detected in more lateral regions of the genital bud (Fig. 5B, see also Fig. 4B,C), suggesting either that *Evx-2* transcripts may be restricted to a subset of *Hoxd-13*-expressing cells in the genital bud and urogenital mesenchyme or that the transcript level is too low to be detected.

CO-EXPRESSION OF *Evx-2* AND *Evx-1* IN THE CENTRAL NERVOUS SYSTEM

Evx-2 was expressed in discrete cell layers within the embryonic central nervous system (CNS). This was first detected at 10.5 dpc and was clearly apparent until 12.5 dpc. The labeling was observed in a thin and continuous column of cells along the entire spinal cord in near mid-sagittal sections (Fig. 6A) as well as in more scattered cells of the ventral spinal cord, visible in more lateral sections (Fig. 6B). As this labeling was reminiscent of that reported for *Evx-1* (Bastian and Gruss, 1990), we compared the expression of both genes at various levels of the CNS on both transverse and coronal sections. Double-labeling experiments were performed in which the two probes were hybridized, either separately, or together on a third set of sections in order to assess possible co-expression. On transverse sections of 12.5 dpc embryo spinal cord, *Evx-2* labeling was indistinguishable from, although weaker than, that of *Evx-1* (Fig. 6C). In the cervical spinal cord, *Evx-2* signal was found in two symmetrical columns in the medial part of the ventricular zone (Fig. 6C, arrowheads; also in Fig. 6A), as well as in cells located between the ventral horns and the ventricular zone (Fig. 6C, open arrows; also in Fig. 6B) and in the most dorsal layers of the spinal cord (Fig. 6C, filled arrows). Only the former two areas were labeled in more caudal regions of the spinal cord (thoracic and lumbar; data not shown). Sections hybridized to both probes together showed no detectable extension of the signal, which indicated a probable co-expression of the two genes in the same cells (Fig. 6C; panel '1+2'). A similar co-expression was found more rostrally, at the level of the developing hindbrain (Fig. 6D). In the CNS, *Evx-1* and *Evx-2* appeared to be activated at a similar developmental stage, in 10.5 dpc embryo (data not shown).

In contrast to the similarities in the spinal cord, a striking difference was seen in the extent of *Evx-2* and *Evx-1* transcripts domains towards more rostral regions of the developing brain. While *Evx-1* transcripts were expressed up to the rhombencephalic isthmus area (the metencephalon-mesencephalon boundary, see Bastian and Gruss, 1990; Figs 6D, 7), *Evx-2* transcripts extended into the superficial layer of the entire midbrain (Fig. 7A,B). The point of divergence between *Evx-1* and *Evx-2* transcript distributions in the marginal layer was seen on coronal sections of 11.5 dpc brain (Fig. 7A), where it appeared that the *Evx-2* signal was found more rostrally than *Evx-1* in the same layer. Therefore, the expression of *Evx-2* in the superficial layer of the midbrain (Fig. 7B) corresponds to a rostral extension of domains where *Evx-2* and *Evx-1* are coexpressed.

IS *Evx-2* A *Hoxd* GENE?

Members of the vertebrate HOX gene complexes are expressed, during development, according to a set of rules that control the coordination of their functions. An interesting aspect of these rules is that they rely on the genomic organization of this gene family, that is, they rely on the respective

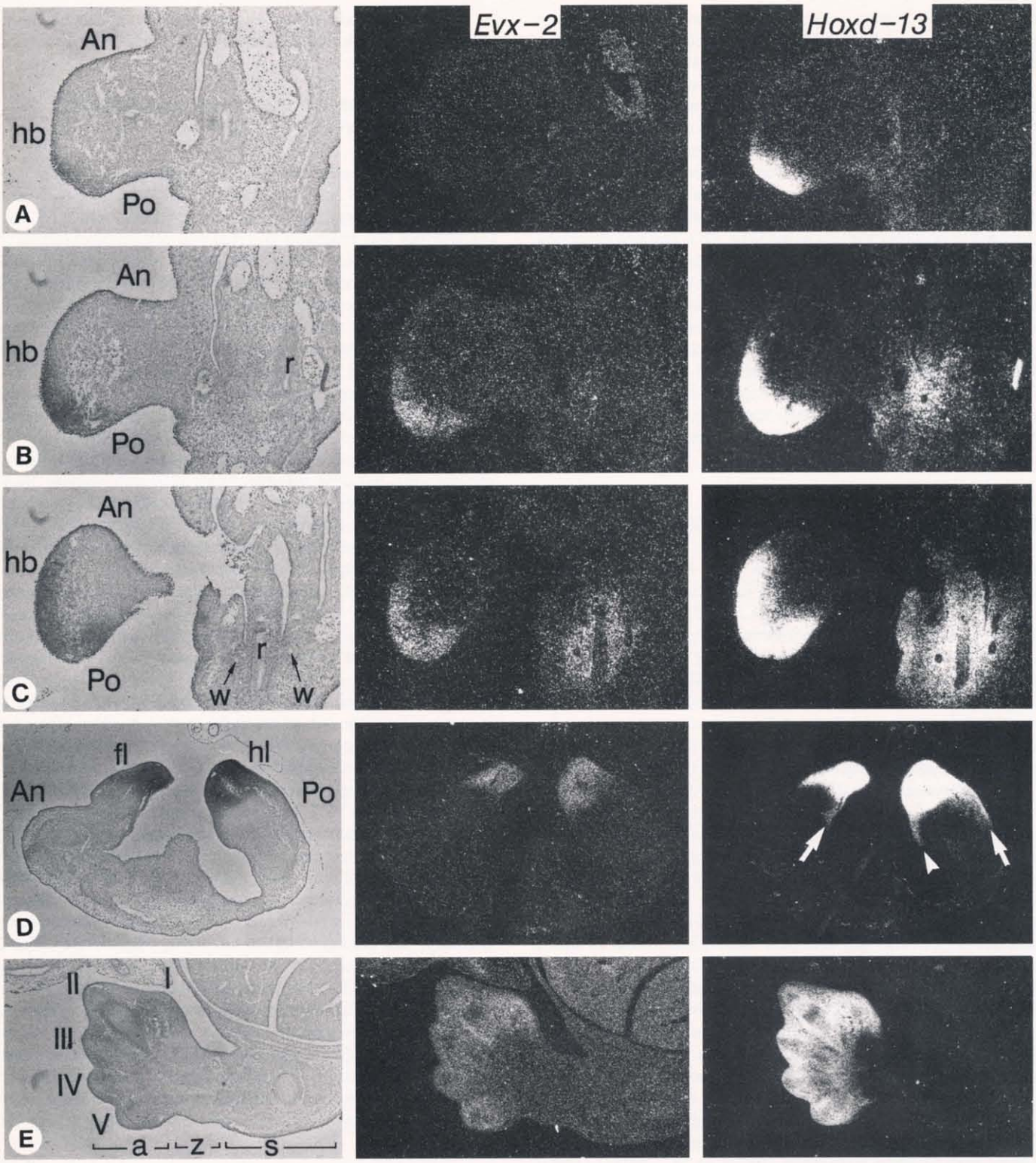

Fig. 4. *Evx-2* expression in developing limbs. (A-C) Three serial sections of an 11.5 dpc hindlimb bud. The bud is sectioned along its A-P axis, and from A to C, the sectioning progresses towards the tip of the bud (i.e. the sections progress from the dorsal side towards the ventral side of the embryo). In addition to the distinct domains of *Evx-2* and *Hoxd-13* transcripts in the hindlimb bud, compare also the labeling patterns in the trunk (urogenital area). (D) Sagittal section of a 12.5 dpc fetus, crossing both the forelimb and hindlimb. (E). Sagittal section of a 13.5 dpc fetus through the footplate of the hindlimb (crossing all five digit anlagen). hb, hindlimb bud; An, anterior; Po, posterior; r, rectum; w, wolffian ducts; fl, forelimb; hl, hindlimb; I, II, III, IV, V, digit primordia; a, autopod (footplate); z, zeugopod; s, stylopod.

position of each gene within its complex. Thus, a particular position will coincide with both the time of activation (temporal colinearity, Dollé et al., 1989; Izpisúa-Belmonte et al., 1991) and the craniocaudal position of the expression domain (spatial colinearity, Gaunt et al., 1988). Though the mechanisms underlying these rules have yet to be discovered, it has been hypothesized that spatial and temporal colinearities were major contributory factors to the conservation of this clustered organization during evolution (Duboule, 1992, 1994).

The two vertebrate genes *Evx-1* and *Evx-2* are related to the *Drosophila even-skipped* gene and are closely linked to the 5′ extremities of the HOXA (*Evx-1*) and HOXD (*Evx-2*) complexes (D'Esposito et al., 1991; Faiella et al., 1992; Bastian et al., 1992). Interestingly, while the distances between paralogous Hox genes have been relatively well conserved among the various HOX complexes (an average of approx. 10 kb), the distances between the Evx genes and the HOXA and D complexes have not been equally conserved; *EVX1* is separated from *HOXA13* by 45 kb, whereas *EVX2* was found in the close vicinity of *HOXD13* (D'Esposito et al., 1991; Faiella et al., 1992). We have further analysed the mouse *Evx-2* locus and found that this gene lies 8 kb from the *Hoxd-13* gene, in an inverted orientation. Thus, while the distance between *EVX1* and *HOXA13* is well above those usually found between vertebrate Hox genes, the spacing between *Evx-2* and *Hoxd-13* is in the range of the distances separating Hoxd genes themselves.

The expression profile of *Evx-1* during development (Bastian and Gruss, 1990, Dush and Martin, 1992, this work) suggests that its physical linkage to the HOXA complex is not associated with a 'Hox type' of regulation. Its early expression, at the onset of gastrulation, does not follow temporal colinearity (it is expressed well before *Hoxa-13*) and, subsequently, no restriction to posterior regions is observed in the spinal cord expression (as is the case for *Hoxa-13*). Even though *Evx-1* transcripts are found in both limb buds and genital tubercle, their distribution in time is distinct from that of *Hox* transcripts in these structures. As the *Evx-2* gene was found to be physically closer to the HOXD complex, we analysed whether its

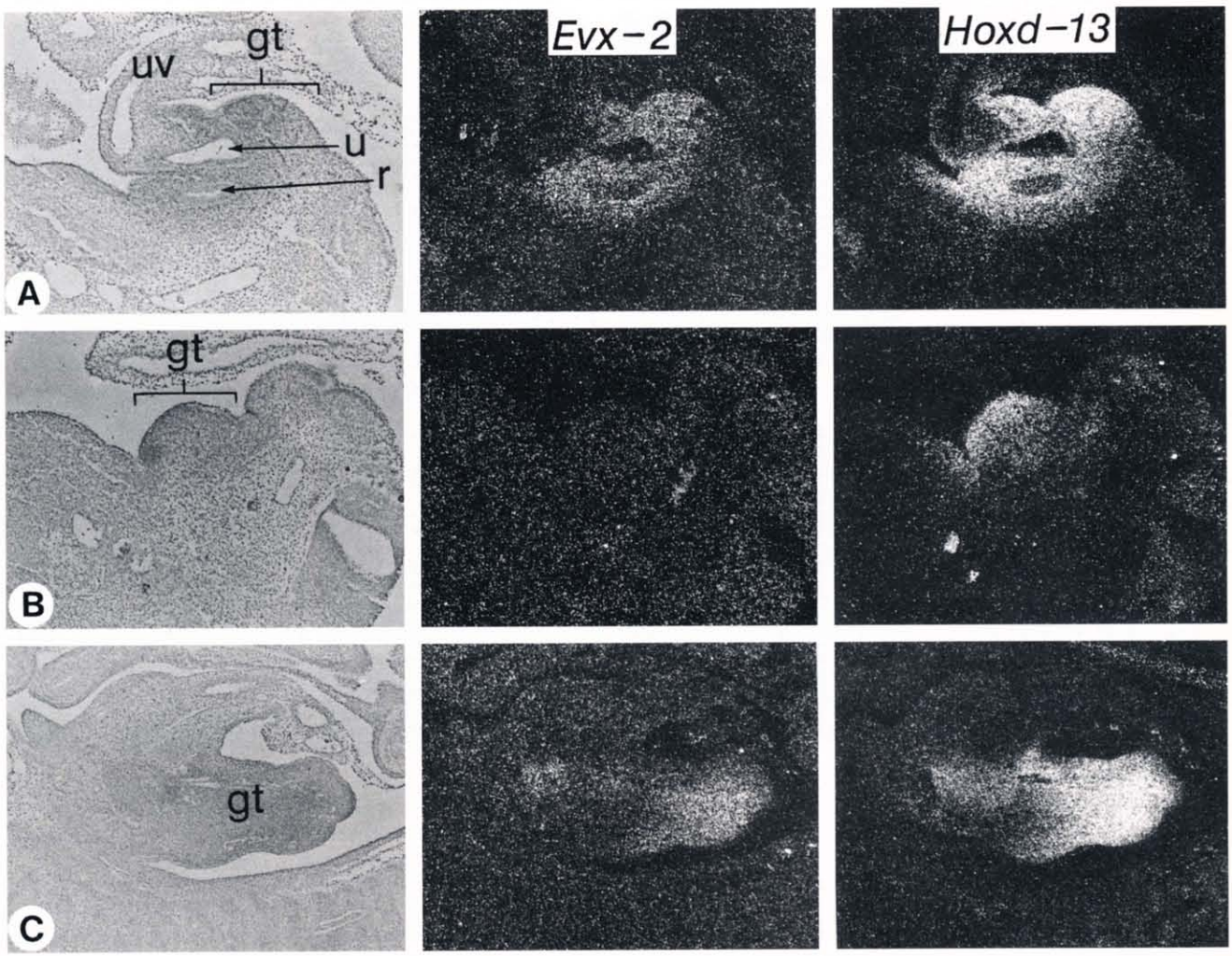

Fig. 5. *Evx-2* expression in the genital area. (A,B) Two serial sagittal sections through the genital bud of a 11.5 dpc embryo. Due to the curvature of the embryo, the sectioning becomes more lateral towards the caudal part of the embryo (right side of the pictures). Section (A) goes through medial parts of the genital region and crosses the umbilical vessels, rectum and urogenital sinus, while (B) covers more lateral regions. (C) Sagittal section through the genital tubercle of a 13.5 dpc fetus. gt, genital tubercle; u, urogenital sinus; r, rectum; uv: umbilical vessels.

expression could be subject to regulatory influences of the nearby HOXD complex. Our results show that the expression of *Evx-2* corresponds, in many respects, to that of a genuine *Hoxd* gene. However, an important aspect of regulation conserved between *Evx-1* and *Evx-2* relates to expression in the central nervous system.

Evx-2 expression seems to comply with temporal colinearity of the HOXD complex (Dollé et al., 1989; Izpisúa-

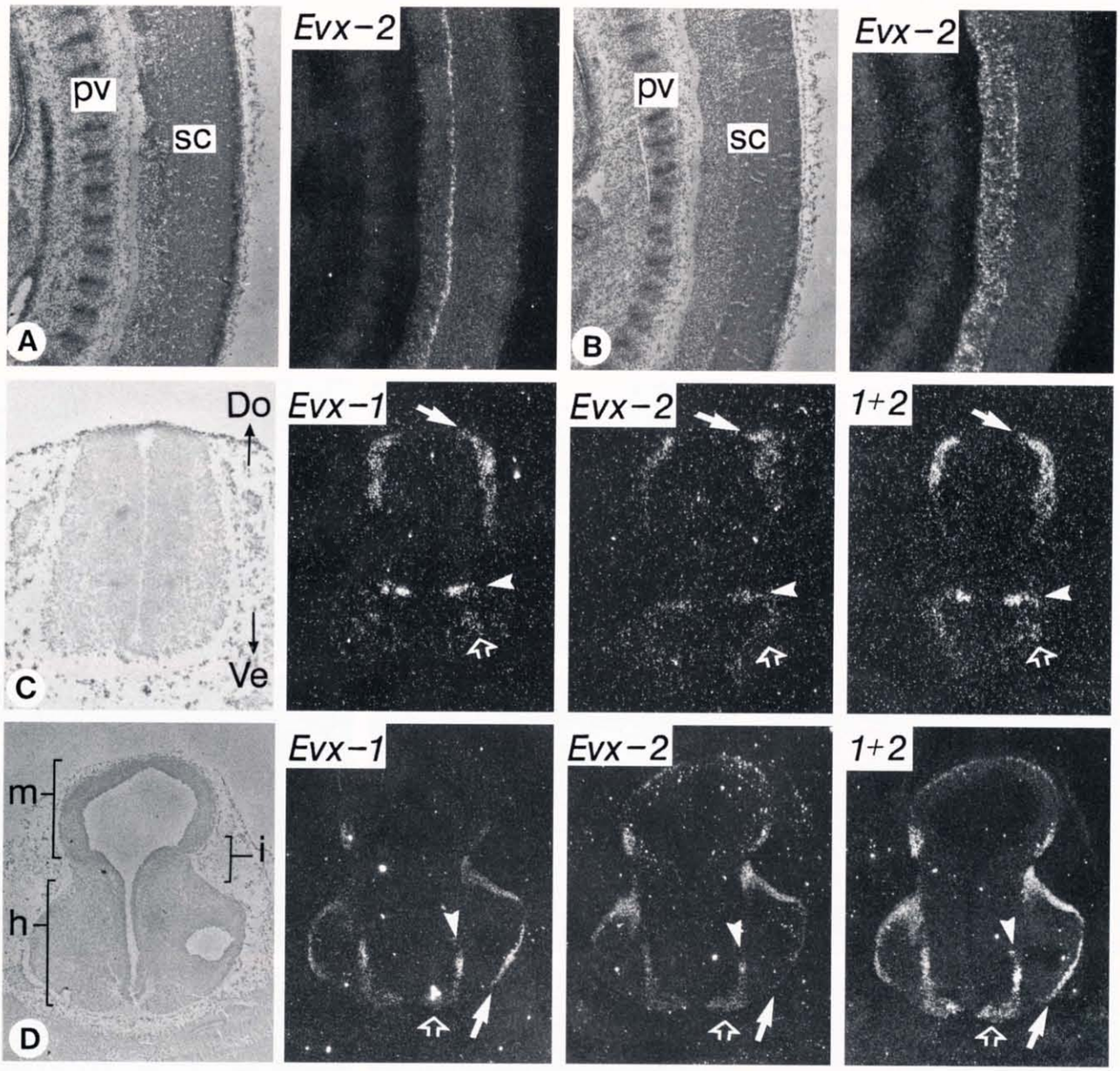

Fig. 6. *Evx-2* expression in the hindbrain and spinal cord. (A,B) Two serial sagittal sections through the thoracic spinal cord of the same 12.5 dpc fetus, showing two distinct components of *Evx-2* expression. Section A, which is rather medial, is essentially through the ventricular zone and shows the thin column of *Evx-2*-expressing cells in this area (also visible in C, arrowhead). Section B is slightly more lateral and crosses the ventral part of the mantle layer. A few scattered cells express *Evx-2* all along this ventral area (visible in C, open arrow). (C). Three adjacent transverse sections of the cervical spinal cord from a 12.5 dpc fetus were hybridized to *Evx-2* (middle panel), *Evx-1* (left panel) and to a mixture of both probes (right panel). This transverse view shows the two populations of labeled cells shown in A and B; the medial column (arrowheads) and the ventral cells (open arrows). In addition, the dorsal-most layers are also labeled at the cervical level (filled arrows). Note the similarity of the labeling patterns for both probes, as well as in the double labeling. (D) Coronal sections through the hindbrain of a 12.5 dpc fetus. The same three components of *Evx-1* and *Evx-2* expression are found along the hindbrain; the thin column of cells (arrowheads), groups of ventral cells (open arrows) and the most superficial layer (filled arrows). The labeling patterns obtained with each probe, and with the mixture of both probes, are indistinguishable in the hindbrain. Note, however, the sharp boundary of *Evx-1* transcripts close to the rhombencephalic isthmus, whereas *Evx-2* transcripts extend in the superficial cells of the midbrain (also observed in the double labeling experiment). sc, spinal cord; pv, prevertebral column; do, dorsal; ve, ventral; h, hindbrain; m, midbrain; i, isthmus.

Belmonte et al., 1991), as there was no area in the embryo where *Evx-2* transcripts could be detected prior to those of *Hoxd-13*. *Evx-2* transcripts were detected earliest in 9.5 dpc embryos, in a region of the genital anlage corresponding to a subset of the *Hoxd-13* domain. This suggests that *Evx-2* is activated slightly after *Hoxd-13* in the cell population that will eventually give rise to the genitalia. This delay was even clearer in limb buds, where *Evx-2* expression was detectable only by day 10.5 in posterior forelimb mesenchyme, a region that expressed *Hoxd-13* at 9.5-10.0 dpc. Possibly as a result of this temporal progression, the *Evx-2* transcript domain in developing limbs appears to be more restricted than that of *Hoxd-13*. This is in good agreement with the spatial colinearity observed amongst Hoxd genes in limbs (Dollé et al., 1989).

Temporal colinearity may be respected in the activation of *Evx-2* in the central nervous system as well, even though this is a structure where the *Evx-2* expression pattern is clearly distinct from that of all the neighbouring Hoxd genes. Therefore, there is a correlation between the close association of *Evx-2* to the HOXD complex and the fact that its developmental expression follows the rules of temporal and spatial colinearities acting upon the adjacent complex. In this respect, and in contrast to *Evx-1*, *Evx-2* could be considered as an additional *Hoxd* gene. Such an assimilation is based on the location and regulation of *Evx-2* and does not necessarily imply similar functions of the gene products. Furthermore, the following reservations are noteworthy; first, the *Evx-2* signal was always much weaker than that of *Hox* genes, making it possible that the distributions of transcripts, in both space and time, were underestimated due to technical problems with detection of gene activity. However, results obtained with other mouse *Evx-2* probes confirm that the gene is expressed at a low level, as is also suggested by comparison of *Evx-2* versus *Hoxd-13* expression in zebrafish (P. Sordino and D. D., unpublished data). Second, the expression of *Evx-2* in the CNS clearly contradicts spatial colinearity as *Hoxd-13* is not expressed in the CNS anterior to the sacral region. In addition, it should be noted that *Evx* and *Hoxd* genes are not expressed in the same cellular subsets in the spinal cord.

IS *Evx-2* AN *Evx* GENE?

Previous analyses of vertebrate and invertebrate genes from the *even-skipped* family revealed two features of expression in common. First, there are similarities in the early expression pattern, which is initiated prior to gastrulation and subsequently restricted to posterior parts of the embryo. This is exemplified by the expression,during gastrulation, of mouse *Evx-1* (Dush and Martin, 1992) or zebrafish *eve-1* (Joly et al., 1993), and by grasshopper and beetle *eve* genes until caudal extension (Patel et al., 1992, 1994). In vertebrates, this early expression is maintained in the tail bud. Second, these genes have specific expression patterns in the CNS, for example, *eve* in *Drosophila* (Doe et al., 1988). *Evx-2* is the first example of an *eve*-related gene that does not show either the early-posterior expression, or its persistence in the tail bud. As such, this gene is rather atypical amongst the *eve* gene family. A posterior expression is however resumed later in development, at a position similar to *Hoxd* gene expression in the genital

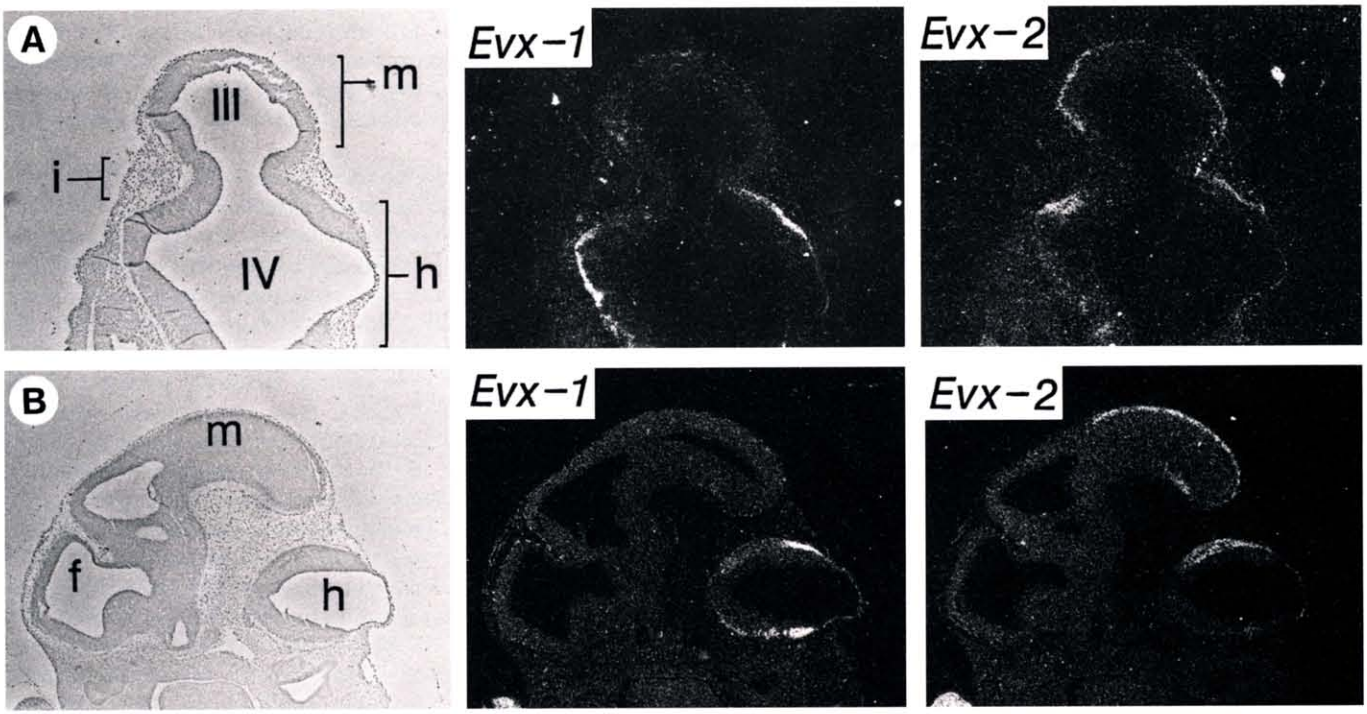

Fig. 7. *Evx-2* specific neural expression in the midbrain. (A) Coronal section through the head of a 11.5 dpc embryo. Both *Evx-1* and *Evx-2* transcripts are expressed in superficial marginal cells of the hindbrain. In addition, *Evx-2* transcripts are found in superficial marginal cells of the midbrain. (B) Sagittal section of the head of a 11.5 dpc fetus. *Evx-2* and *Evx-1* are co-expressed within the hindbrain but only *Evx-2* labelling is seen in superficial cells of the midbrain. m, midbrain; h, hindbrain; f, forebrain; i, isthmus; III, third ventricle; IV, fourth ventricle.

anlage and following the activation of *Hoxd-13*. In limbs and genitalia, *Evx-1* and *Evx-2* expression were similar at first, but soon diverged, essentially through a decrease of *Evx-1* expression. Such differences in the dynamics of the expression domains suggest that the systems of expression maintainance may have differentially evolved between the two genes.

In contrast, *Evx-2* expression in the CNS was almost identical to that of *Evx-1*, both in timing and distribution. Both genes were activated around day 10 of development and *Evx-2* transcripts showed the same cell-type specificity, mostly in post-mitotic cells (early differentiating neurons). These highly restricted expression domains were located at presumptive areas for interneurons (Bastian and Gruss, 1990). It is therefore probable that the function of both *Evx-2* and *Evx-1* in the CNS, is to identify particular types of neural cells; a role with parallels to the *Drosophila eve* gene (Doe et al., 1988). This aspect of *Evx-2* regulation, clearly shared by *Evx-1*, is likely confered by some CNS-specific regulatory sequences which, were conserved after large-scale duplication of an original HOM/Evx complex.

Although both genes are probably co-expressed along the spinal cord and hindbrain, the cranial boundaries of the *Evx-1* and *Evx-2* expression domains in the CNS are different. While the rostral limit of *Evx-1* expression is found at the rhombencephalic isthmus (the hindbrain-midbrain transition), *Evx-2* transcripts extend more rostrally into the midbrain, up to the midbrain-forebrain junction. In the midbrain, the unique expression of *Evx-2* is observed in cell layers similar to those expressing both *Evx* genes at more caudal levels of the CNS. It is thus conceivable that this additional *Evx-2* rostral domain reflects an anterior extension of the original *Evx* function. This functional extension may have followed the duplication of an original HOX/Evx complex, in prechordates or early in the vertebrate lineage (see below), in parallel to the acquisition of a complex anterior nervous system. It is noteworthy that both *Evx-1* and *Evx-2* rostral limits of expression coincide with two important morphological sub-divisions of the embryonic brain, between the mesencephalic-metencephalic and metencephalic-diencepalic vesicles, respectively.

EVOLUTION OF THE *Evx/Hox* FUNCTIONAL RELATIONSHIPS

These results, together with previously published data, suggest the following scheme for the history of the functional relationships between *eve/HOM* related genes. The conservation of an original linkage between an ancestral *eve* gene and an ancestral HOM complex (presumably formed originally by horizontal gene duplication) may have been favoured by a primordial function of *eve* in the early specification of the 'posterior' in development. This assumption is supported by the presence of an *eve*-like gene linked to a potential HOM complex in a Cnidarian (Miller and Miles, 1993). Before the separation between protostomes and deuterostomes, this ancestral gene was secondarily recruited for neuronal specification, probably in association with the evolution of different cell types in an ancient CNS. At this point, temporal colinearity must have been imposed on the HOM complex (if one assumes that this mechanism is used in some present day arthropods or annelids; Duboule, 1992). However, because of its early function, it is probable that the ancestral *eve* gene was not subject to this additional regulation. It nevertheless remained at the vicinity of the complex, perhaps because of the regulatory control determining posterior specificity. In many arthropods, a similar configuration could still exist nowadays, where an *eve*-like gene would be next to the HOM complex (on the 'posterior' side), playing an important function during early posterior development (Patel et al., 1992). In long germ band insects, a novel function (in addition to neural and posterior functions) was developed for *eve,* as reflected by the pair rule expression pattern observed in Diptera *eve* (see Patel, this volume). In fact, a reminiscence of the ancestral posterior pattern may be seen in flies, with the expression of *eve* in the primordial proctodeum at the end of germ band extension (Goto et al., 1989).

In early deuterostomes, a primitive *Evx* gene was thus linked to an ancestral HOX complex and probably had functions during both gastrulation and neuronal specification. The present configuration of the vertebrate HOXA/Evx-1 complex may reflect this ancestral situation, with the *Evx* gene relatively independent from *HOX* regulation. In cephalochordates, a situation quite similar to the ancestral one may be observed, since these close relatives of the vertebrates still retain a single HOX complex (see Holland et al., this volume). In the lineage leading to vertebrates, either in parallel with, or soon after, the large scale complex duplications, a rearrangement might have taken place in the upstream part of the HOXD complex resulting in a reduction in the distance separating *Hoxd-13* from *Evx-2*. (The alternative, that the *Evx-1* gene could have been secondarily removed from the immediate proximity of *Hoxa-13* would imply that an ancestral *Evx* gene, before duplication, had no early posterior function. We consider this unlikely, as discussed above.) Since the large scale HOX complex duplications probably occurred in parrallel to the design of a complex central nervous system, it is possible that at this time one of the duplicated Evx genes (*Evx-2*) was also recruited to extend its original function up to a more anterior region.

The close vicinity of the *Evx-2* gene to the HOXD complex correlates with the two essential differences between *Evx-2* and other members of the *eve* gene family. On the one hand, the aquisition by *Evx-2* of expression features that are characteristic of Hox genes; on the other hand, the suppression of a major trait of the *eve* family, the early phase of expression. We suggest that these two novel components of *Evx-2* expression pattern arose by this gene coming under the influence of the neighbouring HOXD complex. As a consequence, while the *Evx-2* gene became expressed in limbs and genitalia through the control of some of the *Hoxd-13* regulatory (enhancer) elements, its early phase of expression was suppressed by temporal colinearity. Indeed this latter mechanism would not allow the *Evx-2* gene to be transcribed earlier than *Hoxd-13*, that is not before 8.5-9 days of gestation. The molecular mechanisms involved in temporal colinearity are not known. One potential explanation is the sequential opening (accessibility) of the HOX complexes, from 3′ to 5′, to the transcription machinery during gastrulation (Dollé et al., 1989; Duboule, 1992). In this context, the inactivation, in the course of evolution, of the early phase of *Evx-2* expression by its translocation next to the 5′ extremity of the HOXD complex (i.e. near an early 'inactive configuration') may be mechanistically

similar to position effect variegation in *Drosophila* (Reuter and Spierer, 1992). If this were the case, however, one might not expect *Evx-2* to be expressed anterior to *Hoxd-13,* even in different cell types. Analysis of the location and structure of the regulatory element(s) conferring CNS specificity on *Evx-2* may, therefore, give insight into the mechanism involved not only in *Evx-2* regulation, but also in temporal colinearity of HOX complexes.

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