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Review

The puzzle of Hox genes

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Introduction

In vertebrates, gastrulation is the crucial step that imparts the cues to the rostral-caudal patterning of the embryo's body. It is during this process that neural induction and anterior-posterior (A-P) regionalization in the neuroectoderm occur. Nieuwkoop's hypothesis suggests that these events are achieved in two steps: first, neural activation, driven by the prechordal mesoderm, converts competent ectoderm to anterior-specified neuroectoderm (i.e. "prosencephalic"). The neuroectoderm activated by the prechordal mesoderm is then converted to much posterior fates (i.e. hindbrain, spinal cord) by vertical contacts with the chordal mesoderm (Nieuwkoop, 1952). Recent findings rescue the relevance of planar signals emanating from the organizer region before the involution of mesoderm over vertical interactions originated in the underlying dorsal mesoderm, during the establishment of embryonic A-P pattern (Ruiz i Altaba 1990, 1992; Doniach *et al.*, 1992; reviewed by Guthrie, 1991 and Ruiz i Altaba, 1994). Treatments of vertebrate embryos with all trans retinoic acid (RA) during early development produce dramatic changes in the rostral-caudal axis that are correlated with profound alterations in the spatial expression of Hox-C genes, suggesting a relevant role of retinoids in rostral-caudal regionalization. Besides, genetic manipulations (overexpression and loss-of-function) of Hox-C genes provide strong support to the hypothesis that this gene family is involved in the patterning of the vertebrate A-P axis.

In this review, we attempt to provide some clues for understanding the role of retinoids and Hox-C genes in the process of rostral-caudal regionalization.

Retinoids in the patterning of A-P axis

Hox gene products, a family of nuclear transcription factors containing homeodomains, show a high degree of conservation between the vertebrate Hox clusters (Hox-C) and *Drosophila* homeotic clusters (HOM-C). In vertebrates, genes belonging to the Antp-class are located in four unlinked gene complexes (*HoxA* through *HoxD*). Within each cluster, individual genes are arranged in the same relative order as their counterparts in *Drosophila* HOM-C.

Hox-C genes are activated by RA in human embryonic carcinoma cells in a concentration-related manner from 3' to 5' direction in the four clusters (Boncinelli *et al.*, 1991). Besides, there is a spatial correlation between their clustered organization and the expression domains: 3' genes products are expressed more rostrally than 5' transcripts (Wilkinson *et al.*, 1989). In cell cultures, RA up-regulates anterior genes and down-regulates the posterior ones (Boncinelli *et al.*, 1991).

Several groups have shown that RA moves *Hox* expression boundaries rostrally within the hindbrain and cervical spinal cord and reduces the rhombomeric number with no change of the rhomboencephalic size (Table 1). Those findings include: a) the anteriorization of the expression domains of labial counterparts in *Xenopus* (Sive and Cheng, 1991) and mouse (Morriss-Kay *et al.*, 1991; Conlon and Rossant, 1992). b) The products of the zinc finger motif containing gene *Krox-20* disappeared from rhombomere 3 (r3) and expanded their r5 domain, suggesting an anterior expansion of r5 (Morriss-Kay *et al.*, 1991; Papalopulu *et al.*, 1991; Conlon and Rossant, 1992). c) Within the postotic hindbrain, both the r7 territory and the expression domain of the *Xenopus* homeoprotein *XlHbox2* were expanded in the A-P axis. The enlargement of the homeoprotein domain was due to the rostral displacement of its anterior boundary. However, no duplication of IX/X ganglionic complex was observed (López and Carrasco 1992 and unpublished results). d) In mouse embryos, RA moves rostrally the expression boundaries of *Hox*b-1, *Hox*b-2 (preotic hindbrain), *Hox*b-4 (postotic hindbrain) and *Hox*b-5 (anterior spinal cord) in the neurectoderm (Conlon and Rossant, 1992). All these findings suggest a progressive transformation of anterior hindbrain into much posterior rhombomeric identities. e) In addition, RA induces several homeotic transformations in paraxial mesoderm derivatives (Kessel and Gruss, 1991).

The fact that exposure of gastrulating embryos to RA induces shifts of *Hox* boundaries and transformations in the rostral-caudal axis, strongly suggested that retinoids are directly or indirectly involved in A-P patterning. The simplest explanation implied that RA acts in a graded fashion with lower levels of retinoid activity rostrally and higher levels in the caudal end (López and Carrasco, 1992; Chen *et al.*, 1994). The putative gradient could then activate Hox-C genes sequentially in the A-P axis, according to their sensitivity to retinoids (Boncinelli *et al.*, 1991). Alternatively, the involvement of other morphogens in this process cannot be ruled out. RA could be inducing a morphogen that acts in a gradual fashion in order to generate the A-P patterning. In fact, it was shown that when a RA bead is implanted into the anterior margin of the chick limb bud, a zone of polarizing activity is induced. However, RA itself was unlikely to be the morphogen mediating digit duplications in this system (Noji *et al.*, 1991; Wanek *et al.*, 1991).

Although most of the pharmacological experiments using RA suggest the existence of an A-P retinoid gradient, the actual distribution of RA in the embryo must be visualized to test this hypothesis. In this context, in the last few years, several groups gave evidence of endogenous retinoid activity during embryonic development. In mouse embryos, a transgene with three copies of the RA response element (RARE) from the RARß gene upstream of a mouse hsp68lacZ construct, was expressed following the onset of neurulation, in a restricted A-P domain in all three germ layers (Rossant *et al.*, 1991). Although there was not an obvious A-P gradient of lacZ staining, an anterior boundary was established caudal to the preotic sulcus. But the authors did not rule out the possibility of a gradient of RA responsiveness, arguing that the threshold sensitivity of the construct used might be insufficient to detect a graded retinoid activity. Other authors used an *in vitro* reporter assay to "measure" the levels of endogenous RA in embryonic neural tissue (Wagner *et al.*, 1992). The diencephalon released retinoids in levels that were 10-fold lower than those of the rat spinal cord, but they did not address the issue about different levels of RA between regions caudal to the diencephalon. But the most dramatic evidence supporting a graded distribution of retinoid activity came recently from studies in *Xenopus* embryos (Chen *et al.*, 1994). By using a reporter cell system, the authors demonstrated that retinoid levels increase 3-fold from the 2-cell stage to the neurula stage, whereas the active retinoid concentration in the

Abbreviations used in this paper: Antp, Antennapedia; A-P, anterior-posterior; CNS, central nervous system; CRABP, cellular retinoic acid binding protein; CRBP, cellular retinol binding protein; RA, *all-trans* retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid responsive element; r, rhombomere.

TABLE 1

PHENOTYPES GENERATED BY LOSS AND GAIN OF FUNCTION

Strategy	Gene	Neurectoderm and NCC derivatives		Paraxial mesoderm derivatives		References
		boundary	phenotype	boundary	phenotype	
Loss of function	Hoxa-1	r4	loss of r4, r5 and neural crest derivatives	O1	no evident alterations	Lufkin *et al.*, 1991; Chisaka *et al.*, 1992; Mark *et al.*, 1993
	Hoxa-2	r3	no evident alterations in CNS transformation of 2nd into 1st pharyngeal arch derivatives		no evident alterations	Gendron-Maguire *et al.*, 1993 Rijli *et al.*, 1993
	Hoxa-3	r5	loss of neural crest and pharyngeal arch derivatives	C1	no evident alterations	Chisaka and Capecchi, 1991
	Hoxb-4	r7	no evident alterations	C2	partial axis to atlas transformation (anteriorization)	Ramírez Solís *et al.*, 1993
	Hoxc-8	c4	no evident alterations	T5	vertebral transformations within the T7-L1 region (anteriorizations)	Le Mouellic *et al.*, 1992
	Hoxd-3	r5	no evident alterations	C1	partial transformations of atlas into basi and exoccipital bones; axis into atlas (anteriorizations)	Condie and Capecchi, 1993
	Hoxa-3/Hoxd-3		Hoxa-3 $^{-/-}$ phenotype exacerbated		complete deletion of atlas, the axis resembles a generic cervical vertebra	Condie and Capecchi, 1994
	Krox-20	r3, r5	loss of r3 and r5			Schneider-Maunoury *et al.*, 1993
Gain of function	Hoxd-4 (under promoter)	r7 (Hoxd-4)	no evident alterations	C1 (Hoxd-4)	transformation of exoccipital into cervical neural arches; basioccipital into dens axis (posteriorizations)	Lufkin *et al.*, 1992
	Hoxa-1 (under promoter)	r4 (Hoxa-1)		O1 (Hoxa-1)		
	Hoxa-7 (under ß actin promoter)	c4	abnormalities of neural crest derivatives	T3	Extra cervical vertebra (proatlas); atlas with body; transformation of axis into C3 (posteriorizations)	Balling *et al.*, 1989; Kessel *et al.*, 1990

r, rhombomeres; c, cervical level within spinal cord; C, T and O, cervical, thoracic and occipital vertebrae.

dorsal marginal zone increases 5-fold during gastrulation. Moreover, a concentration gradient of retinoid activity is evidenced in early neurula stage, with the maximum point at the posterior end of the embryo, 10-times higher than that found in the anterior end. Thus, retinoid levels are spatially and temporally regulated during *Xenopus* embryogenesis, but we do not know whether mesoderm, neurectoderm or both are the endogenous sources, how these levels are regulated and how the gradient is established.

It is possible that cellular retinoic acid binding proteins (CRABP) could be titrating endogenous RA in order to modulate free RA levels in the cell. A *Xenopus* counterpart of these proteins (xCRABP) was recently cloned. Interestingly, an A-P gradient of xCRABP transcripts was found in the hindbrain of *Xenopus* embryos, with higher levels rostrally (Ho *et al.*, 1994). Furthermore, overexpression of murine CRABP I (mCRABP I) in F9 embryonic teratocarcinoma stem cells decreases the responsiveness of these cells to exogenous RA (Boylan and Gudas, 1991). *In vitro*, the rate of RA metabolism is increased by purified mCRABP (Napoli *et al.*, 1991). These findings suggest that CRABP could be decreasing endogenous RA availability *in vivo*, thus regulating the access of RA to the nuclear receptors. Consistent with this hypothesis, the xCRABP gradient and the postulated retinoid gradient are established in opposed directions in the A-P axis.

A-P regionalization: planar vs vertical signals

Nieuwkoop postulated that in Amphibian embryos, the neuralization of the ectoderm occurs in two steps. According to this hypothesis, the first step (neural activation) is driven by a signal originated in the prechordal mesoderm, which converts competent ectoderm to anterior-specified neuroectoderm (i.e. "prosencephalic"). In the second step (neural transformation), part of the anterior specified neurectoderm is converted to much posterior fates (i.e. hindbrain, spinal cord), by vertical contacts with the chordal mesoderm (Nieuwkoop, 1952). Thus, according to this "two step" model, the response of the neurectoderm to the second interaction generates the complete A-P regionalization of the neuraxis.

In totally exogastrulated *Xenopus* embryos, the axial mesoderm does not underlie the ectoderm, but both remain connected by a small area that corresponds to the organizer region. Thus, exogastrulation provides an interesting tool for studying ectoderm development under the influence of the organizer without underlying axial mesoderm.

By using several neural markers, it has been demonstrated that neural induction occurs in exogastrulae, indicating that a planar inducing signal, probably originated in the organizer and later

TABLE 2

PHENOTYPES GENERATED BY RA TREATMENTS

Strategy	Gene	Neurectoderm and derivatives		Paraxial mesoderm derivatives		References
		boundary	phenotype	boundary	phenotype	
Retinoic acid treatments	Hoxb-1 pr / lacZ Hoxb-2 pr / lacZ Krox-20 pr/lacZ	r4 r4 r3 and r5	homeotic transformation of r2/r3 into r4/r5 (posteriorization)		N.D.	Marshall et al. 1992
	Hoxb-1	r4	Hoxb-1 expression domain shifts rostrally	O1	N.D.	Morriss-Kay *et al.*, 1991, Conlon and Rossant, 1992
	Hoxb-2	r3	Hoxb-2 expression domain shifts rostrally		N.D.	Conlon and Rossant, 1992
	Hoxb-4	r7	Hoxb-4 expression domain shifts rostrally	C2	N.D.	Conlon and Rossant, 1992
	Hoxb-5	c1	Hoxb-5 expression domain shifts rostrally	C3	N.D.	Conlon and Rossant, 1992
	Krox-20	r3 and r5	loss of r3 domain; expansion of r5 domain			Morriss-Kay *et al.*, 1991; Papalopulu *et al.*, 1991 Conlon and Rossant, 1992
	Xeb2	r7	Xeb2 expression domain and r7 shift rostrally		N.D.	López and Carrasco, 1992 and unpublished results
	Xeb1	c1	Xeb1 expression domain shifts caudally after treatment with high doses of retinoic acid		N.D.	López and Carrasco, 1992
	Hoxa-3 Hoxa-7	r5 c4	N.D. N.D.	C1 T3	Retinoic acid at 7.3 dpc: vertebral transformations (posteriorizations)	Kessel and Gruss, 1991
	Hoxc-8	c4	N.D.	T5	along the A-P axis; rostral shift of Hoxa-3, Hoxa-7 and Hoxc-8 expression domains. Retinoic acid at 8.5 dpc: vertebral transformations (anteriorizations) resticted from the mid-thoracic region to the caudal vertebra	

r, rhombomeres; c, cervical level within spinal cord; C,T and O, cervical, thoracic and occipital vertebrae; pr, promoter; dpc, days post coitum; N.D., not described.

emanated from the notoplate, spreads through the ectodermal sac (Ruiz i Altaba, 1990, 1992). In this situation, however, certain ventral neuronal types were not differentiated and forebrain structures were absent, suggesting that they require vertical signals probably originated in the involuted axial mesoderm (head mesoderm and notochord).

Regarding the A-P character of the induced neural tissue in exogastrulae, *Xhox3*, a homeobox-containing gene related to the *Drosophila* even skipped gene (Ruiz i Altaba and Melton, 1989), is expressed in the ectodermal sac, in a region equivalent to its normal expression domain in the anterior neurectoderm (Ruiz i Altaba, 1990). The *En-2* vertebrate antigen recognized by the antibody against the *Drosophila* homeoprotein *Engrailed* is expressed as a discrete band in the midbrain. In exogastrulae, the *En-2* protein is also expressed in an equivalent region within the ectodermal sac, but the intensity of immunoreactivity and the total number of positive cells were reduced in comparison with control embryos (Ruiz i Altaba, 1992). On the other hand, the expression pattern of *wnt1* RNA is incomplete in exogastrulae, since the site of expression in the dorsal midline of the midbrain is apparently absent, and the posterior midbrain stripe seems to be located much anteriorly, near the cement gland. These results suggest that exogastrulae might lack certain forebrain and midbrain values in the A-P axis (Ruiz i Altaba, 1994).

Other authors (Doniach *et al.*, 1992) used Keller explants to study whether planar signals are sufficient for neural A-P regionalization. In these experiments, dorsal mesoderm and ectoderm are isolated as a continuous sheet from the early gastrula before mesoderm involution takes place. Explants are cultured either as a single sheet ("open face") or as two sheets with their inner faces apposed ("sandwich"). They are placed under the pressure of a glass cover slip, thus preventing mesoderm involution and vertical contact. In both "open face" and "sandwich" explants, the region-specific markers *En-2, XlHbox1, XlHbox6* and *Krox-20*, were expressed in their normal relative order along the neural A-P axis. Besides, the homeobox-containing genes were expressed in the mesoderm of Keller explants, in a pattern analogous to their normal domains outside the central nervous system (CNS).

XASH-3, a *Xenopus* homolog of the *Drosophila achaete-scute* genes, is normally expressed as characteristic transverse bands restricted to the hindbrain region in the A-P axis, and as two bilaterally symmetric longitudinal bands that are restricted in the mediolateral axis, demarcating the boundary between the presumptive dorsal and ventral halves of the neural tube. In Keller explants, the longitudinal stripes are conserved but the transverse ones are not detected. Again, planar signals do not appear to be sufficient for complete hindbrain patterning in the rostral-caudal axis (Zimmerman *et al.*, 1993).

In conclusion, planar signals are sufficient to activate some region-specific genes in their normal A-P order under the experimental conditions tested, but in the absence of vertical signals, the regionalization is incomplete.

Since in Keller explants the ectoderm and mesoderm point to opposite directions with their posterior ends in the common boundary normally defining the limit of involution, the possibility that this

region of planar contact constitutes a source of a graded signal spreading through both germ layers before mesoderm involution remains to be tested. Vertical signals might be required to keep dorsal mesoderm and neurectoderm in register after mesoderm involution and perhaps, refine and stabilize the A-P regionalization in both germ layers. However, experiments abolishing planar signals have not been yet performed to understand the relevance of planar versus vertical signals during normal development. The available data indicate that vertical signals from the axial mesoderm might be cooperating with planar signals originated in the organizer to generate a definitive A-P pattern.

In this context, it is important to discuss the role of RA as a putative A-P regionalizing signal. RA can modify mesodermal patterning in early *Xenopus* embryos (Ruiz i Altaba and Jessell, 1991a; Sive and Cheng, 1991), but other authors reported direct effects on the neurectoderm (Durston *et al.* 1989; Sive *et al.*, 1990). The actual source of endogenous RA is unknown. However, the notochord has exhibited polarizing activity when grafted on the chick wing bud (Hornbruch and Wolpert, 1986), and cellular retinol binding protein (CRBP) is expressed there (Maden *et al.*, 1990). CRBP is also present in the floor plate (Maden *et al.*, 1990), a structure with polarizing activity that can convert retinol to RA (Wagner *et al.*, 1990). These data suggest that two midline structures, the floor plate and the notochord, may be sources of endogenous retinoids.

Although the gene reporter experiments performed in transgenic mouse embryos show that, following the onset of neurulation, endogenous retinoid activity is present in a restricted A-P domain in all three germ layers (Rossant *et al.*, 1991), they do not answer where the source of retinoids is located in the developing embryo. Thus, it will be important to discern the structures endowed with the enzymatic machinery that synthesizes morphogenic retinoids.

According to the hypothesis of Nieuwkoop, a gradient model in the chordal mesoderm has been proposed in order to explain the alterations produced by RA treatments at gastrulation in *Xenopus* (López and Carrasco, 1992). If a morphogen gradient is organized in the chordal mesoderm during gastrulation, it could be interpreted by the overlying activated neurectoderm cells to acquire their positional values through the expression of Hox genes. In this context, the putative morphogen gradient could be acting as Nieuwkoop's "transforming wave", where RA might be involved (Durston *et al.*, 1989). Alternatively, and according to the planar model, RA could be participating in the putative graded signal that might be radially emanating from the interphase between non-involuting marginal zone and involuting marginal zone, simultaneously imparting positional values to neurectoderm and dorsal mesoderm before the latter involutes.

In addition, the floor plate could be a source of retinoids at later stages (Wagner *et al.*, 1990). This could explain why RA applications can alter neural patterning during later events, inducing ectopic anterior expression of serotonin and *Xhox3* (Ruiz i Altaba and Jessell, 1991b) and a rostral shift of the *Xenopus* homeoprotein *XlHbox2* (Paez Pereda and Carrasco, unpublished results). Interestingly, there are nearly no differences in retinoid activities between floor plate and dorsal spinal cord. However floor plate has polarizing activity whereas dorsal spinal cord has not (Wagner *et al.*, 1990, 1992). Thus, it is possible that a non-retinoid signal could account for the polarizing activity of the floor plate, and RA could be the inducer of the polarizing signal, as was suggested for the chick limb bud (Noji *et al.*, 1991; Wanek *et al.*, 1991). This is an important point which remains to be tested.

Programming the rostral-caudal axis: the role of Hox-C genes

The phenotypes resulting from loss of function and overexpression of Hox-C genes (Table 2) and treatments with RA (Table 1) suggest that, in molecular terms, the vertebrate CNS and the paraxial mesoderm reveal different levels of complexity along the developing A-P axis.

Forebrain and midbrain, which are the latest phylogenetic acquisitions of vertebrates (Gans and Northcutt, 1983; Northcutt and Gans, 1983), are induced by the prechordal mesoderm (Nieuwkoop, 1952). The expression of Hox-C genes has not been described so far as trespassing the hindbrain. In addition, the most cephalic structures are progressively reduced after exposure of *Xenopus* embryos to increasing concentrations of RA at blastula stages (Durston *et al.*, 1989; Papalopulu *et al.*, 1991), during gastrulation (Durston *et al.*, 1989; López and Carrasco, 1992) or at neurula stages (Ruiz i Altaba and Jessell, 1991a). Although treatments of *Xenopus* neurulas with high concentrations of RA lead to a reduction of the cephalic structures, they do not completely delete eyes or cement glands as happens with treatments at earlier stages. Besides, treatments of *Xenopus* embryos with RA at the late gastrula to the early neurula stage changed the cell fate of the A1 blastomere lineage, which is confined to the dorsoanterior part of the tailbud stage embryo, from a mostly neuronal phenotype to an epidermal one (Agarwal and Sato, 1993). The inhibition of goosecoid expression (Cho *et al.*, 1991) and the ectopic activation of the *Xenopus* homeoprotein *XlHbox1* in the more rostral region of the CNS after exposure to high doses of RA (López and Carrasco, 1992) further suggest that RA should not normally act at this level. Moreover, the gene reporter experiments detecting endogenous RA activity (Rossant *et al.*, 1991; Wagner *et al.*, 1992; Chen *et al.*, 1994) strongly support this hypothesis. In addition, the idea of a distinct genetic program for forehead morphogenesis is favored by the expression of homeobox-containing genes that do not belong to the Antp-class (Price *et al.*, 1991; Simeone *et al.*, 1992).These observations could imply that the forehead relies on a genetic program, different from hindbrain and spinal cord, which does not depend on RA modulations, at least during early events.

The hindbrain constitutes a very complex structure composed of discrete territories named rhombomeres (Lumsden, 1990). The rostral boundaries of expression of the clustered *Hox* genes coincide with the morphological boundaries of the odd rhombomeres (r3, r5, r7) (Wilkinson and Krumlauf, 1990). The discrete streams of rhomboencephalic neural crest cells have recently been identified (Lumsden *et al.*, 1991). Their migratory pathway explains the metameric relationship between hindbrain and branchial arches that correlates with the localization of the anterior boundaries of *Hox* expression domains (Hunt *et al.*, 1991).

The first, second and third branchial arches receive neural crest cells from rhombomeres r2, r4 and r6 respectively; r3 and r5 are spacers between territories. This spacing seems to be related to the expression of *msx* genes in cells programmed to die (apoptosis) within the odd rhombomeres, probably influenced by signals provided by the even rhombomeres (Graham *et al.*, 1993).

The phenotypes arising from null mutations of *Hox*a-1 and *Hox*a-3 or from overexpressing *Hox* genes by treatments with RA indicate that the preotic hindbrain responds distinctly. For instance, the "knock-out" of *Hox*a-1 (Lufkin *et al.*, 1991; Chisaka *et al.*, 1992; Mark *et al.*, 1993), *Hox*a-2 (Gendron-Maguire *et al.*, 1993; Rijli *et al.*, 1993) and *Hox*a-3 (Chisaka and Capecchi, 1991) does not

apparently induce homeosis in the CNS (see Table 1). Some neural crest and pharyngeal arch derivatives seem to be lost in *Hox*a-1 and *Hox*a-3 mutants, and an apparent homeotic transformation of second into first pharyngeal arch derivatives occurs in *Hox*a-2 mutant mice. In addition, in *Hox*a-1 mutants, r4 is markedly reduced and r5 is almost absent (Mark *et al.*, 1993). Surprisingly, the only homeotic duplication in the vertebrate CNS reported so far involved the transformation of r2/r3 into r4/r5 as a result of RA treatment of gastrulating mouse embryos at gastrulation (Marshall *et al.*, 1992; Table 2). Thus, it is tempting to speculate that complete homeosis, at least for this particular region of the neuroectoderm, needs the ectopic overexpression of all paralogues of the first and second groups in the prospective r2/r3 territories to transform their identity, as may happen in RA treatments.

In spite of the above-mentioned homeotic transformation in the CNS after exposure to exogenous RA, other authors, following similar treatments, have not reported such findings (Morriss-Kay *et al.*, 1991; Sive and Cheng, 1991; Conlon and Rossant, 1992; López and Carrasco, 1992 and unpublished results). They merely describe a general rostral shift of homeoprotein boundaries without any evidence of homeotic duplications, and report that the number of rhombomeres was reduced and the posterior rhombomeres were expanded. The explanation for this contradiction assumes that the timing and the concentration of RA must be critical in those experiments. This special kind of transformation, which does not imply actual duplication, seems to be a dose-dependent phenomenon. For instance, *Xenopus* embryos treated with 10^{-5} M RA at gastrulation lost their otocyst. The *XlHbox2* expression domain, whose anterior boundary is in r7 (López and Carrasco, unpublished results), was enlarged, and it reached the anterior end of the CNS (López and Carrasco, 1992). We reinterpret those findings as a loss of preotic hindbrain at the expense of the extreme enlargement of r7.

Nevertheless, it remains to be analyzed whether the transformation reported by Marshall is truly homeotic. The authors interpret that the *Krox-20* domain in r3 was transformed into the r5 domain. However, the activation of the anterior stripe of *Krox-20* in RA-treated mice did not follow the same temporal pattern as the activation of *Krox-20* in r5, and this is not what we expect from a genuine transformation of r3 into r5. Conversely, the anatomical transformation of the trigeminal motor nerve to a facial identity strongly favors the idea of a homeotic duplication, but functional data from the transformed ganglia are not available.

Strikingly, although homeotic duplications were not observed in the CNS, several examples of homeotic transformations were reported for paraxial mesoderm derivatives (Tables 1 and 2). The overexpression of *Hox*d-4 driven by the *Hox*a-1 promoter induces a posteriorization in sclerotome derivatives, where the exoccipital bone is transformed into ectopic neural arches and the basioccipital acquires a dens axis-like identity (Lufkin *et al.*, 1992).

Another example is shown by the "knock out" of *Hox*b-4, where the second cervical vertebra is transformed into an atlas (Ramírez-Solís *et al.*, 1993). This homeotic transformation is incomplete since the axis conserves the dens and its vertebral body but acquires the characteristic neural arch and ventral tubercule of the first vertebra. Feasibly, the *Hox*b-4 paralogues are active and specify other aspects of the vertebral identity conserved in these mutant mice (Ramírez-Solís *et al.*, 1993). In fact, the null inactivation of *Hox*d-3 also induces a partial axis to atlas anteriorization, but the transformed vertebra loses the dens axis, as opposed to the phenotype obtained by *Hox*b-4 loss of function (Condie and Capecchi, 1993). Therefore, each vertebra may be considered as a mosaic, and the whole identity would result from the cooperative action of all paralogues and/or more than one set of paralogues.

The null mutation of *Hox*a-3 deletes several neural crest and pharyngeal arch derivatives but does not affect the craniocervical joint. On the other hand, the inactivation of its paralogue *Hox*d-3 induces anteriorizations of the first and second cervical vertebrae, but the neuroectoderm derivatives appear to be normal (Condie and Capecchi, 1993). These findings and the information available from null phenotypes of Hox-C genes reported so far prompt the speculation about a tissue-specific function for each paralogue. For instance, the knock-out of *Hox* genes that belong to the A cluster (*Hox*a-1, *Hox*a-2 and *Hox*a-3) results in alterations of neurectoderm and neural crest derivatives, whereas examples of inactivation of genes belonging to the three remaining clusters (*Hox*b-4, *Hox*c-8 and *Hox*d-3) show transformations of paraxial mesoderm derivatives (Table 1). Nevertheless, a cooperation between Hox paralogues might exist through both germ layers, despite this apparent tissue specificity. For example, although the null inactivation of *Hox*a-3 (Chisaka and Capecchi, 1991) and *Hox*d-3 (Condie and Capecchi, 1993) have no overlapping abnormalities, the double mutant mice for both paralogous genes have more severe abnormalities than predicted from the sum of the individual mutant phenotypes (Condie and Capecchi, 1994). The atlas, which was partially transformed into basi and exoccipital bones in *Hox*d-3 nulls, was instead completely deleted in double mutants. On the other hand, the phenotype corresponding to the *Hox*a-3 inactivation was also exacerbated by the double mutation. In both phenotypes, the exacerbations depend on gene dosage, since their severity increases as the number of wild type copies of both paralogous genes decreases. Thus, strong synergistic interactions may exist between *Hox*a-3 and *Hox*d-3.

In the vertebral column, treatment of pregnant mice with RA at 7.3 d.p.c. induces posteriorizations that correlate with the anterior shift of *Hox*a-7 and *Hox*c-8 domains (Kessel and Gruss, 1991). When pregnant females are treated with RA at 8.5 d.p.c., the pattern changes dramatically: several vertebral anteriorizations occur, but they are restricted to a region extending from the mid-thoracic region to the caudal vertebra (Kessel and Gruss, 1991). These authors interpret that the posterior genes responded to RA with a loss of function, and the explanation for the anteriorizations supposes that the expression domains of the anterior *Hox* genes were caudally shifted after RA exposure on day 8.5. Although they did not show *in situ* hybridizations for *Hox* gene products in this situation, another study performed on *Xenopus laevis* favors this idea (López and Carrasco, 1992). The results show that the expression domain of the homeoprotein *XlHbox1*, which is normally restricted to the cervical spinal cord, reaches the caudal end of the embryo after treatment of *Xenopus* gastrulae with high concentrations of RA. However, the anterior boundary of *XlHbox1* remains unaffected. Thus, the caudal expansion of *XlHbox1* domain may be the result of the loss of function of much posterior genes rather than a direct up-regulation of *XlHbox1* by RA. This would imply that a "cross talk" may be established between neighboring Hox genes. Likely, the thoracic region marks the transition where the behavior of Hox-C genes changes in response to RA, in agreement with the results of cell culture experiments (Boncinelli *et al.*, 1991).

Another example of homeotic transformations affecting the paraxial mesoderm is given by the null mutation of *Hox*c-8 (Le

Mouellic *et al.*, 1992). The anterior boundary of this gene reaches the cervical region of the spinal cord and extends from the fifth thoracic to the first lumbar vertebra in the paraxial mesoderm. The phenotype shows at least four homeotic transformations from the 7th thoracic to the 1st lumbar vertebrae but again, no malformations in the CNS were observed.

Finally, the deletion of structures instead of homeotic transformations observed as a result of the targeted disruption of *Hox*a-1, *Hox*a-2 and the double mutation *Hox*a-3/*Hox*d-3, made Condie and Capecchi postulate that Hox genes might be regulating the proliferation rate of precursor cells, instead of specifying cell identity (Condie and Capecchi, 1994). However, we think that there is no contradiction between the two roles. What makes two vertebrae different may be distinct proliferation programs regulated in space and time by different *Hox* codes.

Metamerism: CNS vs somitic mesoderm

In *Drosophila melanogaster*, the combined actions of pair rule and segment polarity genes divide the embryo into parasegments (Ingham, 1988). As a result of this segmentation prepattern, mutations affecting homeotic selector genes duplicate the identity of segmented territories (homeosis). At present, the only example of homeotic duplication in the vertebrate CNS was reported by Marshall *et al.* (1992), involving the transformation of r2/r3 into r4/r5 after RA treatment. Besides, the inactivation of *Krox-20* reduces or eliminates r3 and r5, behaving as the *Drosophila* segmentation pair-rule genes (Schneider-Maunoury *et al.*, 1993). The lack of homeotic duplications in the CNS caudal to the otic vesicle can be explained if each rhombomere with its own boundaries is specified by a distinct *Hox*-code instead of depending on a segmentation prepattern, as might occur in the preotic hindbrain or in somitic derivatives where genuine homeotic transformations occur.

Evolutive implications

It is pertinent to note the idea that HOM/HOX genes were "recycled" during evolution to model the rostral-caudal axis in both Protostomes and Deuterostomes. Their organization in clusters appear to have been used as a strategy for ordering the sequence of activation. In this context, the acquisition of vertebral character by the basi and exoccipital bones, as a result of ectopic overexpression of *Hox*d-4 driven by *Hox*a-1 promoter in mice, resembles the Cyclostomes paleocranium (Lufkin *et al.*, 1992). This suggests that evolution might be introducing discontinuous morphogenetic modifications — "punctuated equilibrium" according to Gould and Eldredge (1993) — within *Hox* regulatory regions to change their expression domains in order to obey phylogenetic requirements. For instance, altering the sensitivity of *Hox* genes to retinoids is a feasible manner of specifically switching the anterior expression boundaries to a new position in order to create new structures. In the future, unraveling the hierarchy of genes regulated by homeoproteins will hopefully illuminate the roles of cell proliferation programs in the modeling of anatomical structures.

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Summary

During the last few years, the regionalization of the rostral-caudal axis has been extensively studied through treatments with RA and genetic manipulations of Hox-C genes. RA shifts several *Hox* expression boundaries rostrally, deletes anterior rhombomeres but expands the caudal ones, and induces homeotic transformations in the vertebral column. These phenotypes indicate that retinoids may act in a graded fashion in the A-P axis, with maximum activity caudally. This excludes forebrain and midbrain, which apparently depend on neither Hox-C genes nor RA modulations, at least during early development.

The phenotypes resulting from ectopic overexpression and loss of function of *Hox* genes described so far show homeotic transformations in paraxial mesoderm derivatives but not in the neurectoderm. An explanation for this discrepancy implies that the paraxial mesoderm may be already segmented in molecular terms at the time of *Hox* activation. Conversely, the activation of a distinct *Hox*-code without a previous "molecular segmentation" may specify rhombomeres with their own boundaries. This would explain why RA expands but does not duplicate the postotic rhombomeres.

Finally, the atavistic transformations obtained by overexpressing *Hox* genes in the wrong place suggest that evolution might be introducing modifications within *Hox* regulatory regions. Thus, changes in their expression domains could sustain phylogenetic requirements.

KEY WORDS: *Hox-C genes, retinoic acid, regionalization, homeotic transformation*

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