Initiating Hox gene expression: in the early chick neural tube differential sensitivity to FGF and RA signaling subdivides the HoxB genes in two distinct groups

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SUMMARY

Initiation of Hox genes requires interactions between numerous factors and signaling pathways in order to establish their precise domain boundaries in the developing nervous system. There are distinct differences in the expression and regulation of members of Hox genes within a complex suggesting that multiple competing mechanisms are used to initiate their expression domains in early embryogenesis. In this study, by analyzing the response of HoxB genes to both RA and FGF signaling in neural tissue during early chick embryogenesis (HH stages 7-15), we have defined two distinct groups of Hox genes based on their reciprocal sensitivity to RA or FGF during this developmental period. We found that the expression domain of 5’ members from the HoxB complex (Hoxb6-Hoxb9) can be expanded anteriorly in the chick neural tube up to the level of the otic vesicle following FGF treatment and that these same genes are refractory to RA treatment at these stages. Furthermore, we showed that the chick caudal-related genes, cdxA and cdxB, are also responsive to FGF signaling in neural tissue and that their anterior expansion is also limited to the level of the otic vesicle. Using a dominant negative form of a Xenopus Cdx gene (XcadEnR) we found that the effect of FGF treatment on 5’ HoxB genes is mediated in part through the activation and function of CDX activity. Conversely, the 3’ HoxB genes (Hoxb1 and Hoxb3-Hoxb5) are sensitive to RA but not FGF treatments at these stages. We demonstrated by in ovo electroporation of a dominant negative retinoid receptor construct (dnRAR) that retinoid signaling is required to initiate expression. Elevating CDX activity by ectopic expression of an activated form of a Xenopus Cdx gene (XcadVP16) in the hindbrain ectopically activates and anteriorly expands Hoxb4 expression. In a similar manner, when ectopic expression of XcadVP16 is combined with FGF treatment, we found that Hoxb9 expression expands anteriorly into the hindbrain region. Our findings suggest a model whereby, over the window of early development we examined, all HoxB genes are actually competent to interpret an FGF signal via a CDX-dependent pathway. However, mechanisms that axially restrict the Cdx domains of expression, serve to prevent 3’ genes from responding to FGF signaling in the hindbrain. FGF may have a dual role in both modulating the accessibility of the HoxB complex along the axis and in activating the expression of Cdx genes. The position of the shift in RA or FGF responsiveness of Hox genes may be time dependent. Hence, the specific Hox genes in each of these complementary groups may vary in later stages of development or other tissues. These results highlight the key role of Cdx genes in integrating the input of multiple signaling pathways, such as FGFs and RA, in controlling initiation of Hox expression during development and the importance of understanding regulatory events/mechanisms that modulate Cdx expression.

Key words: Hox genes, FGF signaling, Retinoids, AP patterning, Neural development, Gene regulation, Chick development, caudal, Cdx, Electroporation

INTRODUCTION

During development, neural tissues acquire anteroposterior (AP) and dorsoventral (DV) positional values by a combination of intrinsic and environmental signals. The generation of distinct segmental and region-specific identities is achieved by regulatory mechanisms that establish and maintain the spatially restricted domains of Hox gene expression along the AP axis of the embryo (Krumlauf, 1994). In mouse and chick, a total of 39 Hox genes are divided into four separate chromosomal clusters, where members from each cluster are expressed in characteristic nested or overlapping domains along the AP axis of many tissues in the developing embryo (Duboule and Dolle, 1989; Gaunt, 1988; Graham et al., 1989). The properties of spatial and temporal colinearity lead to a precise Hox code in the limbs, gut, mesoderm and neuroectoderm tissues (Dollé et
al., 1989; Izpisua-Belmonte et al., 1991a; Izpisua-Belmonte et al., 1991b; Kessel and Gruss, 1991; Wilkinson et al., 1989) and mutational analyses have shown that these tissues are important sites of normal Hox function (Favier and Dolle, 1997; Krumlauf, 1993; Maconochie et al., 1996; Trainor et al., 2000).

Understanding how the expression of homeotic genes is established and maintained is of critical importance, since experiments in many species have shown that shifts in expression boundaries can lead to transformations and alterations of segmental identity (reviewed by Maconochie et al., 1996; McGinnis and Krumlauf, 1992; Moens and Prince, 2002; Trainor et al., 2000). Regulatory analyses in transgenic and targeted mice have proved to be a useful tool in characterizing some of the upstream regulatory components of the Hox network, through the identification of local cis-acting enhancers in Hox loci. Using reporter genes, it has been possible to reconstruct patterns of expression for many of the 3' members of the Hox complexes that appear to be identical to their endogenous counterparts. With respect to the nervous system and the hindbrain in particular, the combined action of several components is required to set the precise location of anterior Hox expression boundaries (reviewed by Trainor et al., 2000). A common mechanistic theme used by several of the 3' Hox genes involves the early activation of expression through the transient action of factors like Kreisler, Krox20 or retinoid receptors, followed by the maintenance of these domains through auto- and cross-regulatory interactions mediated by the Hox genes themselves (Manzanares et al., 2001). For example, Hoxb1 and Hoxb4 are directly activated in the CNS by transiently acting retinoid-dependent enhancers, which in turn set their later segment-restricted domains of expression through triggering of separate auto/cross-regulatory elements (Gavalas et al., 2001; Gould et al., 1998; Marshall et al., 1994; Morrison et al., 1997; Morrison et al., 1995; Pöpperl et al., 1995; Studer et al., 1998; Studer et al., 1996; Whiting et al., 1991). However, to date it has been very difficult to reconstruct the proper patterns and anterior boundaries of expression for more 5' genes in Hox clusters by using local regulatory regions in transgenic approaches. No regulatory elements of the 5' genes might depend upon regulatory mechanisms that involve long-range interactions and the sharing of distal control regions, as suggested for HoxD genes in the limbs or HoxB genes in the CNS and mesoderm (Gould et al., 1997; Sharpe et al., 1998; van der Hoeven et al., 1996). Alternatively these differences might be due to the fact that they require many different components or arise as a consequence of the temporal differences in expression of 3' versus more 5' Hox genes. Hence cis-mechanisms and signaling pathways regulating 3' versus 5' Hox genes may be very different and this could be correlated with differences in patterning the head versus the trunk.

In the process of neural induction, cells first take on an anterior character and then under the influence of posteriorizing signals adopt progressively more posterior fates (Slack and Tannahill, 1992). Relatively little is known about the precise signaling pathways and the balance between them that establishes Hox expression and AP patterning during development or the cis regions that integrate this information. There is emerging evidence that the compound auto- and cross-

regulatory Hox-responsive elements are also part of the mechanisms that serve to integrate some of the diverse signaling inputs that modulate Hox expression in later stages (Affolter and Mann, 2001; Grieder et al., 1997; Ryoo et al., 1999; Saleh et al., 2000). Retinoid, FGF and WNT signaling have all been experimentally linked with early posteriorizing activity. Considerable evidence has shown that in vivo, retinoic acid (RA) is an overall mediator or modulator of Hox expression (Gavalas, 2002; Gavalas and Krumlauf, 2000; Marshall et al., 1996). Excess RA causes a transformation of neural and mesodermal segments toward a posterior identity, accompanied by an anterior shift in Hox gene expression boundaries (Conlon, 1995; Conlon and Rossant, 1992; Kessel and Gruss, 1991; Marshall et al., 1992; Morrison et al., 1997; Simeone et al., 1995). The response of Hox genes to exogenous RA in embryos varies in a concentration and stage-dependent manner that correlates with the position of genes in a cluster. Inversely, retinoid deficient diets or blocking the retinoic acid pathway result in anteriorization of rhombomeres and many other AP patterning defects (Dupé et al., 1997; Dupé et al., 1999; Gale et al., 1999; Kolm et al., 1997; Niederreither et al., 1999; Niederreither et al., 2000; White et al., 2000; White et al., 1998). Functional retinoic acid response elements (RARE) have been identified in several Hox gene regulatory regions (Dupé et al., 1997; Gould et al., 1998; Huang et al., 1998; Langston et al., 1997; Langston and Gudas, 1992; Manzanares et al., 2000; Marshall et al., 1994; Packer et al., 1998; Studer et al., 1998; Studer et al., 1994; Zhang et al., 2000). Furthermore, signals from mesoderm play important roles in patterning neural tissue and retinoids have been shown to be associated with several of these signaling events (Ensini et al., 1998; Gould et al., 1998; Itasaki et al., 1996; Liu et al., 2001; Sockanathan and Jessell, 1998).

Recently, many links between the FGF pathway and regulation of Hox genes have begun to be revealed. In the chick embryo FGF signaling plays a critical role in primary and secondary neural induction and the node is an important source of FGF signals that influence the potential of neural tissue (Mathis et al., 2001; Storey et al., 1998; Streit et al., 2000; Wilson et al., 2001; Wilson et al., 2000). In FGFR1 hypomorphic mutants, the expression of Hoxd4 is shifted posteriorly by one somite and the expression domain of Hoxb9 is shifted posteriorly in the lateral mesoderm (Partanen et al., 1998), thus suggesting a rule for FGF pathways in the AP patterning of the mesoderm. The strongest evidence for the involvement of the FGF pathway for Hox gene induction in neural tissues comes from the Xenopus (Lamb and Harland, 1995; Pownall et al., 1996). FGFs in the presence of a BMP antagonist will induce posterior neural markers (Lamb and Harland, 1995). Pownall and collaborators showed that neural tissues cultured in sandwich with e-FGF-soaked beads express posterior Hox genes and that a dominant negative form of this receptor (XFD) impairs the early expression of the same genes (Pownall et al., 1996). Moreover, the same study showed that the vertebrate homologues of Drosophila caudal gene, Cdx genes, are the intermediaries of this FGF-mediated Hox induction (Isaacs et al., 1998; Pownall et al., 1996). In the mouse, Cdx genes have been shown to induce global changes in Hox expression, as illustrated by Cdx1 null mutant embryos, which show severe homeotic transformations accompanied by a change in several Hox gene boundaries in the mesoderm.
(Subramanian et al., 1995). Furthermore, a DNA motif able to bind CDX protein in vitro has been isolated in the regulatory regions of Hoxc8, Hoxb8 and Hoxa7 that are believed to be important for their regulation (Charité et al., 1998; Subramanian et al., 1995; Taylor et al., 1997). This suggests a possible mechanism for the initiation of 5′ Hox genes in chick and mouse through FGF- and CDX-dependent pathways. However Cdx genes are not exclusively involved in mediating FGF signals. RARE and LEF/TCF binding motifs have been found in a regulatory region of the Cdx1 gene (Houle et al., 2000; Prinos et al., 2001), and strong genetic synergy between Cdx1, Wnt3a and retinoid receptors has been shown in mesodermal patterning (Allan et al., 2001; Prinos et al., 2001). These results indicate that WNT and RA signaling play important roles in the early activation of Cdx1 expression. Taken together these studies suggest that Cdx genes provide a mechanism by which RA, WNT and FGF signaling may be differentially balanced and integrated, which could be important for distinct regulation of 5′ and 3′ Hox genes.

Thus, signaling and control mechanisms involved in regulation of 5′ Hox genes in the spinal cord are still poorly understood in comparison to regulation of 3′ Hox genes in the hindbrain. It is believed that RA acts in a graded manner to activate nearly all Hox genes, either directly or indirectly. The relative insensitivity of posterior 5′ Hox genes to RA could reflect a lack in their inherent ability to respond to retinoids or may be due to temporal windows of competence in the response itself. The precise contribution of Cdx genes to the regulation of posterior genes versus anterior genes in the neural tube and what signaling pathways they respond to in this process are not understood.

In this study we have systematically examined the contribution of RA, FGF and CDX pathways in the regulation of HoxB genes in the chick neural tube. We first compared the regulation of Hoxb4 and Hoxb9 in detail and then extended the analysis to the other members of the complex. In these early stages of neural development, our results surprisingly identify two distinct groups of HoxB genes based on their reciprocal abilities to respond to RA or FGF signals. This suggests that at these stages they are not regulated in a progressive colinear manner by a graded balance between FGF and RA signaling. Together these results illustrate the importance of understanding regulatory events that modulate Cdx expression to integrate the response of Hox genes to signaling pathways that establish their spatial domains of expression.

MATERIALS AND METHODS

Grafting methods
Fertilized chick eggs were incubated to stages 9–16 at 37°C. Donor tissues were dissected in L15 medium. For somite transposition in the preotic region, a slit was made in stage 9 host embryos lateral to rhombomere (r)5–r2 and graft was inserted so that they made contact with the neural tube. For somite transposition in the spinal cord, somites were dissected out from the host and replaced by the donor somites. Operated host embryos were re-incubated in ovo at 37°C for 48 hours.

In vitro culture of embryos
For in vitro culture, fertilized chick eggs were incubated to stages 5–7 at 37°C. Embryos were dissected out carefully in L15 medium, with the blastoderm remaining intact. The sheet of blastoderm was folded along the body axis with the ventral surface inside, sealed in a sandwich shape and transferred to a plastic tube containing DMEM +10% FCS. Tubes were filled with 5% CO₂ and incubated on a roller culture at 37°C for 6 hours or overnight. FGF recombinant proteins (R&D systems) were applied at 200 or 400 ng/ml, Sugen 5402 (Calbiochem) at 25 μM and all-trans-retinoic acid (Sigma) at 0.7 μM.

DNA constructs and electroporation of DNA
The Xenopus dnrRARα1 was a gift from Nancy Papaolopulo (Blumberg et al., 1997) and we have previously shown that this construct blocks reporter genes under the control of retinoic response elements from Hoxb4 and Hoxd4 when electroporated into chick embryos (Itasaki et al., 1999; Gould et al., 1998). The e-FGF full-length cDNA was a gift from E. Amaya (Amaya et al., 1991). The Xenopus Xcad3, XcadVP16 and XcadEnR constructs have been examined and compared in Xenopus by Issacs, Pownall and Slack (Issacs et al., 1998; Pownall et al., 1996) who generously provided these reagents. In Xenopus XcadVP16 will phenocopy Xcad3 activity in neural assays, but it will also mimic effects of other Xcad proteins in other contexts (Issacs et al., 1998; Pownall et al., 1996). Hence, it is a useful reagent for activating general CDX targets and not just those of a particular Cdx gene, such as Xcad3. In our hands Xcad3 worked poorly in chick, while the XcadVP16 was a robust activator. The XcadEnR construct replaces the activation domains of Xcad3 and XcadVP16 with a repressor domain from engrailed, converting the construct from an activator to a repressor of CDX targets.

Different DNA constructs were electroporated unilaterally or bilaterally as described previously (Itasaki et al., 2000). Briefly, 1–5 μg/μl DNA was injected in the neural tube using a glass pipette. Since this is a closed tube DNA remains confined to neural tissue. Then, electrodos were positioned on opposite sides of the neural tube. For unilateral electroporations, five pulses of 50 mseconds at 20 volts were applied to allow the entry of the DNA into one side of the neural tube (DNA is negatively charged so only moves to the positive pole). For bilateral electroporations the position of the electrodes was reversed and the electroporation repeated.

In situ hybridization and probes
Whole-mount in situ hybridization was performed with digoxigenin-labeled probes as described previously (Henrique et al., 1995). All the following probes were hybridized at 70°C overnight. Hoxb1 cDNA (2.0 kb) (Maden et al., 1991) Hoxb4 cDNA (1.2 kb) (Yokouchi et al., 1991); Hoxb6 cDNA (300 bp) (Wedden et al., 1989), Hoxb7 cDNA (1.6 kb) (Yokouchi et al., 1991); Hoxb8 cDNA, 850 bp, a gift from K. Olberg; cdxA cDNA (2.488 kb) (Frumkin et al., 1993); cdxB cDNA (1.082 kb) (Morales et al., 1996).

RESULTS

Differences in the early dynamics of expression of Hoxb4 and Hoxb9
To gain a better picture of the process of establishing expression of Hox genes across a cluster, we selected Hoxb4 and Hoxb9 as examples of genes representing the middle and 5′ end of the HoxB cluster respectively, and examined their time-course of expression. The process of setting up Hox gene expression is dynamic and occurs at a time when the chick embryo is growing and changing extensively. In order to have a precise picture of the establishment of Hoxb4 and Hoxb9 in the neural tube, we analyzed their pattern of expression between stage 4 and stage 17. Hoxb4 expression is first detected at Hamburger and Hamilton (HH) stage 4 in the posterior half of the primitive streak (Fig. 1A). Then it expands...
Little is known concerning the regulation of Hoxb4, which is expressed more anteriorly and Hoxb9 observed for this profile is dynamic and quite different from the one and a phase of posterior regression from HH stage 11 onwards. place at HH stage 8; a phase of expansion until HH stage 9; establishment in the neural tube: an initiation phase, taking eventually reaching the level of the 20th somite at HH stage 17 regress caudally as the embryo continues to elongate, mesoderm. The boundary of shown). At this stage, it is also expressed in the posterior somites, suggesting a graded signal with a stronger influence laterally in the neural plate and segmental plate until HH stage 8+ (Fig. 1B). At stages 9-10, the expression in both the neural tube and somites 5 and 6 (Fig. 1C). The neural domain extends anteriorly, reaching the level of somites 4/5 at HH10 and the progression continues until HH stage 12, when it reaches its definitive anterior boundary at the junction between r6 and r7 (Fig. 1D,E).

Hoxb9 is first detected in the caudal neural plate at HH stage 7/8 (Fig.1F). As the embryo grows caudally, the expression becomes broader and stronger in the neural folds (Fig. 1G,H). At HH stage 10 the anterior limit of Hoxb9 in the neural tube is at the level of the prospective 9th somite (Fig. 1H) and stays fixed at the same limit until HH stage 11 (Fig. 1I and not shown). At this stage, it is also expressed in the posterior mesoderm. The boundary of Hoxb9 expression then begins to regress caudally as the embryo continues to elongate, eventually reaching the level of the 20th somite at HH stage 17 (Fig.1J). These patterns distinguish three phases for the Hoxb9 establishment in the neural tube: an initiation phase, taking place at HH stage 8; a phase of expansion until HH stage 9; and a phase of posterior regression from HH stage 11 onwards. This profile is dynamic and quite different from the one observed for Hoxb4, which is expressed more anteriorly and for which the expression in the neural tube spreads anteriorly rather than regress posteriorly during elongation of the embryo. These observations suggest a very different pattern of regulation for these two genes during development.

Differential response of Hoxb4 and Hoxb9 in the CNS to retinoids and somitic mesoderm

Little is known concerning the regulation of Hoxb9 and we wanted to investigate if signaling pathways that influence Hoxb4 also act on Hoxb9. In previous studies, we used in ovo electroporation of a dominant negative form of the retinoic receptor α1 (dnRARα1) and tissue grafting to show that retinoid signaling and somitic mesoderm are essential for the early neural expression of Hoxb4 (Gould et al., 1998; Itasaki et al., 1996) (see also Fig. 2C,D). Here, we evaluated the role of the retinoic acid pathway and somitic mesoderm in the induction of Hoxb9 in the neuroectoderm. First, HH stage 7-12 embryos were incubated in vitro with 0.7 μM RA overnight prior to analysis of Hoxb4 and Hoxb9 expression by in situ hybridization. RA treatment leads to the anteriorization of Hoxb4 expression in the neural tube (Fig. 2B) whereas it has no effect on Hoxb9 expression (Fig. 2F). This illustrates a difference in ability of the genes to respond to exogenous RA. To test if RA signaling is essential for normal Hoxb9 regulation, we electroporated the dnRARα1 construct unilaterally into the left side of the neural tube of different staged embryos and compared the expression of Hoxb9 and Hoxb4 after 24 hours of in ovo incubation. As expected from our previous work (Gould et al., 1998) expression of dnRARα1 in the electroporated side blocks the activation of Hoxb4 if compared to the non electroporated control side (Fig. 2A,C). In contrast, the expression of Hoxb9 is not affected by the presence of dnRARα1 (Fig. 2G). Hence unlike Hoxb4, Hoxb9 appears not to require RA signaling for its normal expression and lacks the ability to respond to exogenous RA treatment throughout all of the early stages we tested.

Posterior somites are able to reprogram Hoxb4 expression in the rhombomeres when grafted in the preotic region (Fig. 2D) and this inducing capacity is increased for more posterior somites, suggesting a graded signal with a stronger influence in the posterior part of the embryo (Gould et al., 1998; Itasaki et al., 1996). To assess the effect of somites in inducing Hoxb9 in the CNS, we grafted posterior somites (s23 to s25) from a stage 25 donor embryo into a more anterior region in a stage 10 host embryo, positioning them just anterior to the normal AP boundary of Hoxb9 in the neural tube at the level of somites

Fig. 1. Comparison of Hoxb4 and Hoxb9 expression in the chick neural tube. Dorsal views of different stages of embryos hybridized with Hoxb4 (A-E) or Hoxb9 (F-J). Embryos are at stage 4 (A), stage 8 (B,F), stage 9 (C,G), stage 10– (H), stage 10 (D), stage 11 (I), stage 14 (E), or stage 17 (J). Expression of Hoxb4 remains at a fixed AP boundary in the neural tube once activated, whereas that of Hoxb9 regresses posteriorly in the later stages. White arrows indicate the initial boundary of expression. HPN, Hensen’s node. Horizontal black bars mark the boundary of expression in the CNS relative to the adjacent somite (s) number or rhombomere (r).
7-9. As shown in Fig. 2H, the presence of the graft does not modify *Hoxb9* expression in the spinal cord after 24 or 48 hours of incubation. These results demonstrate differences in the ability of *Hoxb9* and *Hoxb4* to respond to posterior inducing signals such as RA and somitic mesoderm.

**Hoxb4 and Hoxb9 display different sensitivities to FGF treatment**

We next investigated the potential role of the FGF pathway in *Hoxb4* and *Hoxb9* induction in the avian neural tube. HH stage 7-9 embryos were cultured overnight *in vitro* in the presence of 200 ng/ml of FGF2 (Fig. 3) or FGF4 (not shown) recombinant proteins. Analyses by in situ hybridization showed that *Hoxb9* expression is shifted anteriorly in the neural tube up to the level of the otic vesicle (Fig. 3E). This shift is specific to the neural tube, as the somitic boundary is not affected. However, this effect could be mediated through a cascade of events initiated in the mesoderm. To exclude this possibility, we over-expressed the *Xenopus* homologue of FGF4, e-FGF, specifically in the neural tube, by in ovo electroporation of a DNA construct. In this case, *Hoxb9* expression is also induced and extends anteriorly in the electroporated side of the neural tube (Fig. 3F). This confirms that the FGF effect can be mediated or initiated specifically in the neural tube. For comparison, we also examined the sensitivity of *Hoxb4* to FGF2, FGF4 and e-FGF treatments under the same experimental conditions. The *Hoxb4* expression domain remains unaffected by both *in vitro* FGF treatment (Fig. 3B) and e-FGF electroporation (Fig. 3C). We also tested different FGF members to see if this effect on *Hoxb9* was specific to FGF2, FGF4 or e-FGF and if the insensitivity of *Hoxb4* was due to the use of these particular FGF ligands. Using FGF8 or FGF10 there was no shift in *Hoxb9* or *Hoxb4* expression (not shown), suggesting that this effect on *Hoxb9* specifically involves signaling through FGF2 and/or FGF4 and their receptors and that the insensitivity of *Hoxb4* to FGF treatment is a general property of its responsiveness.

**Cdx genes are targets of FGF signaling**

*Cdx* genes appear to play key roles in the response to axial signaling. It has been shown in *Xenopus* that *Cdx* genes are the mediators of FGF signaling to initiate Hox gene expression (Pownall et al., 1996). To assess if the *Cdx* genes could be the targets of FGF signaling in the avian embryo, we treated HH stage 5 to 7 embryos with FGF2/4 in *vitro* under conditions that induce *Hoxb9* and monitored expression of *cdxA* and *cdxB*. Both gene expression domains were anteriorized in the neural tube upon FGF treatment (Fig. 4B,D). Moreover, the change in *cdxA* expression was detectable after 6 hours, showing that *Cdx* genes are early targets of FGF signaling. This induction is temporally dynamic, as HH stage 7 embryos treated overnight showed no persistent *cdxA* expression as observed for untreated embryos (not shown). This shows that FGF has the ability to induce *Cdx* expression in early stages but this effect is stage dependent and expanded *Cdx* expression in neural tissue is not maintained in the later stages.

When we compared the kinetics of the response to FGF between *Hoxb9* and *cdx* genes, it appeared that *cdxA* and *cdxB* respond earlier/faster than *Hoxb9*. This observation raised the possibility that CDX proteins could act downstream of FGF signaling to activate the avian *Hoxb9* gene. To assess this possibility, we utilized a construct (*XcadVP16*) encoding a fusion protein between the *Xenopus* caudal 3 (*Xcad3*) and the VP16 activation domain. *Xcad3* is a homologue of the avian *cdxB* gene and *XcadVP16* has been shown in *Xenopus* to strongly transactivate targets in a manner similar to *Xcad3* (Isaacs et al., 1998; Pownall et al., 1996), and also acts as a general activator of CDX target genes in other contexts. Using in ovo electroporation, we over-expressed *XcadVP16* unilaterally in the left side of the neural tube and assayed for its effects on *Hoxb9* expression after a further 20 hours of in

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**Fig. 2.** Effect of RA treatment and somite grafting on *Hoxb4* and *Hoxb9* expression in the neural tube. Dorsal views of stage 15 (A-C,G) stage 19 (D,H) and stage 14 (E,F) embryos hybridized with *Hoxb4* (A-D) or *Hoxb9* (E-H). (A,E) Untreated embryos. (B,F) Retinoic acid treated embryos. Exogenous application of retinoic acid causes anterior shift of the expression domain and creates a new anterior limit of *Hoxb4* expression (black arrow in B) while *Hoxb9* does not show any anterior shift (F). (C,G) Embryos electroporated with a dnRAR expressing construct unilaterally on the left side of the neural tube. dnRAR causes down-regulation of endogenous *Hoxb4* expression (white arrowheads in C) while *Hoxb9* expression is not affected (G). (D,H) *Hoxb4* and *Hoxb9* expression in grafted embryos, whereby posterior somites 23-25 of a stage 15 donor embryo were transposed into an anterior region of a stage 15 host embryo at the level of somite 7-9 and cultured for 36hrs. The grafted somites induce upregulation of *Hoxb4* (*", D") while there is no change in the pattern of *Hoxb9* expression (*", H"). Black arrowheads in H show position of graft. OV, otic vesicle; SM, somite grafts.
ovo incubation. Compared with the control side or non-electroporated embryos, we detected an upregulation of *Hoxb9* anterior to its normal domains of expression in patchy groups of cells only in the electroporated side (Fig. 5B,E). This demonstrates that increasing CDX activity by ectopic expression of *XcadVP16* is sufficient to induce neural expression of *Hoxb9*.

To confirm that the FGF pathway is acting though *cdx* genes to induce *Hoxb9* expression, we overexpressed a dominant negative form of *Xcad3* (*XcadEnR*) by electroporation in one side of the neural tube and cultured the whole embryos overnight in the presence of FGF2. This construct encodes a fusion protein between *Xcad3* and the transcriptional repression domain of Engrailed and has been shown in *Xenopus* to act as a dominant negative form of *Xcad3* and CDX activity (Isaacs et al., 1998; Pownall et al., 1996). The expression of this construct in the dorsal/left side of the neural tube impairs the upregulation of *Hoxb9* dependent upon FGF2 treatment (Fig. 5C,F). We noted that endogenous *Hoxb9* expression was not affected using *XcadEnR*, however this expression is initiated at earlier stages. In attempts to block this endogenous domain by performing electroporations at earlier stages (HH 5-7), the survival rate of embryos following the manipulations is poor and precludes analysis. Hence activation (*XcadVP16*) and inhibition (*XcadEnR*) of CDX activity have reciprocal effects upon *Hoxb9* expression. These results strongly suggest that FGF2-induced ectopic expression of *Hoxb9* in the neural tube is *cdx* dependent.

**Sensitivity to RA or FGF defines two groups of *Hoxb* genes related to their position in the cluster**

The above results suggest that posterior versus anterior or midcomplex *HoxB* genes respond in distinct manners to different signaling pathways. This ‘opposed’ or reciprocal sensitivity is rather unexpected because *Hox* genes are clustered and believed to be coordinately regulated along the complex in a sequential manner, which is the basis of temporal and spatial colinearity. There could be a progressive shift in the balance between RA and FGF regulation across the complex or specific groups of genes may exclusively be able to respond to one or both of these pathways in the neural tube to regulate *Hox* expression. Therefore, we examined the level of sensitivity of *Hox* expression to RA and FGF signals across the *HoxB* complex. Fig. 6 shows in situ hybridization of chick embryos with several *HoxB* genes in control, RA-treated and FGF2-treated embryos. We found that *Hoxb1*, *Hoxb3* and
Hoxb5 react as Hoxb4 and are sensitive to retinoic acid treatment and insensitive to FGF2 treatments (Fig. 6). In chick, Hoxb2 is not normally expressed at significant levels in the neural tube, unlike in the mouse (Vesque et al., 1996). The other genes we tested, Hoxb6, Hoxb7 and Hoxb8 behave as Hoxb9 and are rapidly anteriorized upon FGF2 treatment (Fig. 6). They are also refractory to retinoic acid treatment (Fig. 6) and their expression is unaffected by the presence of the dnrARa1 construct (not shown). These results divide the HoxB complex into two groups of genes based on their differential sensitivity to RA or FGF at these stages. Surprisingly despite varying concentrations, timing and stage of analysis none of the genes simultaneously showed sensitivity to both RA and FGF treatments. This suggests that the change in the regulation or shift in responsiveness is not progressive along the complex, but undergoes a distinct switch. The position of the shift in RA or FGF responsiveness of Hox genes may be time-dependent. Hence, the specific Hox genes in each of these complementary groups may vary in later stages of development or other tissues. In some contexts Hox genes may simultaneously respond to both signals.

### The role of cdx in altering the Hox response to FGF and RA

In evaluating the potential basis for this sharp change in response, it is striking to note that the anterior limits of expression of the Hoxb genes (Hoxb6-Hoxb9) before and after FGF treatment never pass through the hindbrain/spinal cord boundary (Fig. 6). Our experiments with Hoxb9 and XcadEnR showed that CDX activity is important in mediating the response to FGF and we noted that the normal and FGF-induced domains of cdxA and cdxB expression similarly never extend anteriorly into the hindbrain (Figs 4, 5). The Hox genes not responsive to FGF (Hoxb1-Hoxb5) have normal limits of expression in the hindbrain, where CdX is not expressed. Hence, there is a good correlation between Hox genes that are expressed with anterior limits in the spinal cord, responsiveness to FGF and domains of CdX expression. The apparent insensitivity of 3’ Hoxb genes to FGF treatment may not arise through lack of ligands or receptors, but could instead reflect a loss in the competence of cells in the preotic region to activate Hox genes upon exposure to FGF. It is possible that restriction or absence of CdX expression in more anterior regions could be a limiting factor that modulates the competence to FGF response. This is consistent with our results using the XcadEnR construct to block CDX activity, and shows that CDX-dependent activity can act downstream of FGF signaling to modulate 5’ Hox genes in chick neural tube.

In order to investigate these potential links between cdx expression and competence, we examined the effects of elevating CDX activity upon Hoxb4 expression. Experiments in Xenopus have demonstrated that a fusion between Xcad3 and the VP16 activation domain (XcadVP16) will phenocopy Xcad3 activity in neural assays, and also mimic effects of other Xcad proteins in other contexts (Isaacs et al., 1998; Pownall et al., 1996). Hence, it is a useful reagent for increasing CDX activity and activating general CDX targets. As shown in Fig. 7A, electroporation of the activated XcadVP16 fusion construct into the left side of the neural tube leads to an anterior induction and extension of the Hoxb4 expression domain into the hindbrain and midbrain territories. This shows that Hoxb4 is capable of being induced by CDX activity, and suggests that mechanisms limiting CDX expression to more posterior regions of the neural tube prevent posterior Hox genes from responding to FGF signaling. Cdx genes are not only involved in mediating FGF signals as RARE and LEF/TCF regulatory motifs have been found in the Cdx1 gene (Houle et al., 2000; Prinos et al., 2001), and there is genetic synergy between Cdx1, Wnt3a and retinoid receptors in mesodermal patterning (Allan et al., 2001; Prinos et al., 2001). Therefore we wanted to exclude the possibility that the induction of Hoxb4 expression by the activated XcadVP16 construct reflected a role for Cdx genes in mediating a retinoid response of Hoxb4. Towards this end we electroporated embryos with a XcadEnR vector, incubated them in RA overnight and assayed for Hoxb4 expression. The pattern of RA-induced anteriorization of Hoxb4 expression we previously observed in the hindbrain (Fig. 2B) was unaltered by the presence of XcadEnR (data not shown). This shows that in the context of this experiment, the response of Hoxb4 to RA is not going through a CDX-dependent pathway in the hindbrain.
XcadVP16 can activate Hoxb9 in the hindbrain in association with FGF

While we observed that FGF did not induce an anterior shift of Hoxb6-Hoxb9 that extended into the hindbrain, the results above open the possibility that all HoxB genes can potentially be activated by FGF in the hindbrain if CDX activity is provided. We first tested the potential of XcadVP16 alone to activate Hoxb9 in the preotic region and found that, unlike Hoxb4 (Fig. 7A) it had no effect (not shown). However, we tested the combined effect of FGF treatment and XcadVP16 expression to see if we could bypass the restriction of induction in the hindbrain. HH stage 8+ embryos were electroporated with XcadVP16 in the left side of the neural tube and simultaneously treated in culture with FGF overnight. Under these conditions, the neural domain of Hoxb9 now extends to the most anterior rhombomeres in the hindbrain, as compared to the non-electroporated but FGF-treated control side on the right (Fig. 7B). This result suggests that the expression of Hoxb9 in the hindbrain requires not only the presence of CDX activity, but other events controlled by FGF. As Cdx genes have been shown to possess autoregulatory feedback loops that maintain their expression following early activation (Prinos et al., 2001), it is possible that the addition of FGF is required to reinforce XcadVP16 activity and stimulate such a feedback circuit to induce Hoxb9. Together these results illustrate the importance of regulatory events that modulate CDX expression to integrate the response to signaling pathways and control the ability and spatial extent of the Hox response.

DISCUSSION

The establishment of Hox expression boundaries requires a complex balance of interactions between several signaling pathways. In this study, by analyzing the response of HoxB genes to both RA and FGF signaling in neural tissue during chick embryogenesis, we have defined two distinct groups of Hox genes, based on their reciprocal sensitivity to RA or FGF. We showed that the most 5′ members of the HoxB complex (Hoxb6-Hoxb9) can be activated or induced following FGF treatment in regions where they are not normally expressed and that these same genes are refractory to RA treatment. Furthermore through analysis of Hoxb9 we have demonstrated that this FGF effect is mediated in part through the activation and function of Cdx gene activity. Conversely, the 3′ HoxB genes (Hoxb1 and Hoxb3-Hoxb5) respond to RA but not FGF treatments and analysis of Hoxb4 revealed that it is capable of responding to Cdx expression. Our findings suggest a model
domain of expression prevent 3

Effect of expression and FGF treatment in the XcadVP16

Fig. 7. Effect of XcadVP16 expression and FGF treatment in the hindbrain. Dorsal views of stage 15 embryos hybridized with Hoxb4 (A) or Hoxb9 (B), after electroporation with the XcadVP16 expressing construct unilaterally on the left side of the hindbrain. The embryos were cultured overnight (A) in the absence of or (B) in the presence of an exogenous FGF. In both cases (A,B), upregulation and anterior expansion of each Hox gene is observed in the anterior hindbrain region (area between arrowheads) in response to FGF treatment (see also Fig. 5A for comparison). The asterisk (*) in A shows that the anterior limit of expression on the non-electroporated side is at the level of otic vesicle. The asterisk in B is just posterior to the OV.

whereby all HoxB genes are actually competent to interpret the FGF signal via a CDX-dependent pathway, but mechanisms that axially restrict the Cdx domain of expression prevent 3' genes from responding to FGF signaling in the hindbrain. The presence of retinoid response elements adjacent to Cdx1 suggest it is possible that retinoid signaling itself plays a role in restricting the Cdx response to FGF. These results raise several interesting issues with respect to the control or transcriptional readout of signaling events that initiate Hox expression in development.

Differences in the dynamic nature of Hoxb4 versus Hoxb9 expression

In the neural tube, the Hoxb4 and Hoxb9 expression domains are established following a very different sequences of events. Typical of most other 3' Hox genes (Maconochie et al., 1996), Hoxb4 expression is first initiated in the posterior part of the neural tube and this domain spreads forward over time, eventually reaching a distinct anterior boundary that is maintained in later stages (Fig. 1). This progressive process does not reflect the output or response of a single control region but is mediated by the combined activities of a series of neural regulatory regions (Gould et al., 1998; Gould et al., 1997; Sharpe et al., 1998; Whiting et al., 1991). In contrast, Hoxb9 is activated directly at an axial level that constitutes its most anterior limit of expression and then its neural AP boundary regresses caudally during the later stages of development (Fig. 1). This posterior regression suggests that the factors activating Hoxb9 are continually changing their spatial distribution or activity and indicates the absence of mechanisms that maintain a sharp and distinct fixed boundary. This pattern for Hoxb9 is also consistent with the posterior shift in Cdx expression in the developing chick neural tube.

Activation of early Hox expression by FGF is mediated via CDX activity

We demonstrated that FGF treatment leads to an anteriorization of the expression domain of several HoxB (Hoxb6-Hoxb9) genes in the chick neural tube. In addition, we showed that cdx gene activity is required to transduce this FGF signal by using a dominant negative form of Xcad (XcadEnR) (Fig. 5). Furthermore ectopic expression of XcadVP16 can induce 3' HoxB genes and in combination with FGF induce 5' HoxB members in the hindbrain. These results are consistent with the Hox expression data obtained in Xenopus neural tissue following modulation of CDX activity (Isaacs et al., 1998; Pownall et al., 1996) and also with ectopic expression of chick cdxB in cardiac tissue, which induces a posterior program of Hox expression (Ehrman and Yutzey, 2001). Such a relationship was also suggested by the presence of CDX recognition boxes in the vicinity of mouse Hox regulatory regions (Charité et al., 1998; Subramanian et al., 1995; Taylor et al., 1997). However, null mutations of Cdx1 in mouse lead to the mis-regulation of anterior Hox genes only in the mesoderm, not in neural tissue (Subramanian et al., 1995). This could be due to a difference in the function of Cdx genes between mouse and chick. FGFRI mutants display changes in Hox expression exclusively in the mesoderm and expression of Cdx genes is not affected (Partanen et al., 1998). This is a hypomorphic allele but could also reflect the fact that the FGF effect we observed is not mediated thought FGFR1. When we tested different FGF ligands, only FGF4 and FGF2 (not FGF8 or FGF10) had an effect on Hox expression. These two members of the FGF family can use FGFR1, FGFR3 or FGFR4 to transduce their signal (Szebenyi and Fallon, 1999). All three receptors are present in the chick neural tube (Walsh and Mason, 2000) and it is thus possible that FGFR3 or FGFR4 are preferentially used or can compensate for FGFR1 in the neural context of our experiments.

Sensitivity to early RA or FGF treatment in the neural tube defines two distinct groups in the HoxB complex

When we compared the RA and FGF sensitivity for all of the HoxB genes in early chick embryos, we did not find any genes for which the anterior boundary was anteriorized or induced by both treatments at the stages examined. Rather, the responsiveness of members of the HoxB complex to the two signaling pathways seemed to be mutually exclusive during the stages examined. The sharp reciprocal transition from RA to FGF responsiveness in moving from the 3' (Hoxb1 to Hoxb5) to the 5' (Hoxb6-Hoxb9) Hox genes is surprising (Fig. 6). In mouse the 3' Hox genes do not respond uniformly to RA treatment, as there is a progressive temporal shift in their competence or ability to respond to RA during gastrulation, such that successively more 5' genes respond in later time windows (Bel-Vialar et al., 2000; Conlon, 1995; Conlon and Rossant, 1992; Marshall et al., 1992; Morrison et al., 1997). Hence, it had been suggested that the most posterior 5' Hox genes might also be progressively sensitive to RA in later stages at the end of or after gastrulation. While our experiments demonstrated a clear drop-off in RA responsiveness, they
focused on the early stages of expression, during the phase when initial Hox patterns are being established. Therefore it is possible there may be further changes or shifts in the RA sensitivity of 5’ Hox genes at later stages. However, later RA responsiveness might be complicated because it occurs during a maintenance or a refinement phase of Hox expression, as has been proposed for retinoic acid function in mouse mesoderm and skeletal structures (Kessel, 1992; Kessel and Gruss, 1991).

**HoxB genes and the competence to respond to the FGF signaling pathway**

Interestingly, upon FGF treatment the expression of the FGF responsive 5’ Hox genes reach the same anterior level just posterior to the otic vesicle, which corresponds to the limit between the hindbrain and the spinal cord. Hence, the inability of the 3’ Hox genes to respond to early FGF signaling may be directly or indirectly related to a lack of competence of the hindbrain itself in response to FGF treatment. Like the sharp transition in RA response, this too is surprising, as in later stages, FGF is expressed in the region and FGF treatment of the chick neural tube leads to ectopic expression Krox20 and creistler in the hindbrain while inhibition of FGF signaling downregulates their hindbrain domains (Marin and Charnay, 2000). Hoxa2 expression is also modulated in the anterior chick hindbrain in response to FGF8 signals generated at the mid/hindbrain isthmus (Irving and Mason, 2000; Trainor et al., 2002). Hence, FGFs are expressed in the hindbrain region and the hindbrain is capable of responding to FGF signaling in some contexts or stages.

Our results show that the FGF responsiveness of 5’ Hox genes is dependent upon CDX activity and that the response of Cdx expression is itself limited to the spinal cord region. This raises the possibility that the lack of competence in FGF response of 3’ Hox genes in the hindbrain is a consequence of the restriction in Cdx activation in the hindbrain following FGF treatment. Supporting this idea we showed that Hoxb4 is expanded in the hindbrain upon XcadVP16 ectopic expression and that the combination of Cdx gene activity and FGF are able to induce Hoxb9 in the hindbrain. These findings are consistent with ectopic expression experiments using adenoviral vectors in chick cardiac tissues, where anterior expression of cdxB is capable of inducing posterior genetic programs, such as expression of Hoxa6, Hoxc6 and Hoxc8 (Ehrman and Yutzey, 2001). We also found that the dominant negative XcadEnR construct does not antagonize the RA-induced expansion of Hoxb4 expression in the hindbrain, indicating that the RA response is not being directed through CDX activity at this stage.

These results suggest that in early stages all Hox genes are competent to respond to the FGF signal if CDX proteins are present. During gastrulation, Cdx expression domains are very dynamic and move posteriorly during regression of the node (Frumkin et al., 1993; Marom et al., 1997; Morales et al., 1996). Since the node is a source of FGF (Mathis et al., 2001), it is possible that the neural plate in the pre-otic region is transiently exposed to FGF signals during regression of the node. This early FGF input together with transient Cdx expression could be important in some aspect of activating expression of anterior Hox genes. The progressive posterior regression of Cdx expression in the chick might account for a sliding scale or morphogenetic gradient that sets different AP identities (Frumkin et al., 1993). Our experiments are consistent with this view and we propose that over time, as each Hox gene gets activated, it sees a more posterior domain of Cdx expression and consequently has a more posterior limit of expression. This might help to explain the posterior shift in

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**Fig. 8.** Models of Hox response to FGF based on different effects of FGF on Cdx expression and Hox accessibility. (A) In normal development, Hox loci (colored boxes) are progressively opened over time in an anterior to posterior direction. During this period, Cdx expression domains (grey shaded areas) are gradually regressing toward the caudal end of the neural tube. As each Hox gene in a complex is believed to be accessible at a slightly different time, when it becomes accessible it is exposed to a different pattern or level of Cdx expression, to which it can respond. Hence the final boundary of a given Hox gene is determined by two parameters: the time when the Hox locus is accessible and the position of the Cdx anterior boundary at this particular time. As the embryo develops, Hox loci become accessible in a domain where Cdx is not expressed so they are not capable of being induced. This sets up the nested patterns of expression shown at the right. (B) The addition of FGF leads to an anterior expansion and maintenance of Cdx domains of expression over time and leads to an extended accessibility of Hox complexes along the entire AP axis. This dual effect induces an anteriorization of Hox domains in the neural tissue.
Hoxb9 expression we detected by in situ analysis. As there are multiple cdx products differentially expressed over time, specificity of recognition in any individual member could add a further degree of complexity in regulating of Hox expression or integrating separate signaling pathways.

**FGF and models for the accessibility state of Hox loci**

Models that attempt to explain the colinear properties of Hox complexes frequently incorporate global regulation of graded or differential accessibility of a complex, with variations in the availability of upstream factors needed to activate local control elements (Bel et al., 1998; Kmita et al., 2000; Sharpe et al., 1998). The fact that even the 5’ posterior gene Hoxb9 can be activated by CDX activity in the hindbrain, implies that at the stage we did our experiments, all members of the HoxB complex might be accessible in the CNS.

This situation with respect to Hox expression and FGF signaling could be explained in several ways. The first would be that Hox loci are all equally accessible to FGF signaling along the entire AP axis at the time of our experiments and differential modulation is dependent upon variations in the transcription factors (CDX) needed to potentiate respective Hox expression. In this model the anterior boundaries of the different Hox genes in the CNS would not reflect a graded accessibility state of their complex, but instead would be set by FGF though modulation of the timing and the extent of the Cdx expression domain along the AP axis.

Another model to explain the overall accessibility state is that applying FGF provokes the opening of the Hox complexes in a more anterior position than normal. This renders them more accessible to transcription factors (CDX proteins) along the entire AP axis, inducing anterior shifts in expression. It has been proposed that for a defined AP position, Hox complexes become progressively more accessible over time (Gaunt, 2000). In this model, all Hox loci become accessible along the entire axis at a certain time only after an FGF-dependent internal clock controlling the relative accessibility of each gene had fully opened a complex (Fig. 8A). There is evidence of a segmental clock in the mesoderm and it has been recently suggested that the activation of Hox genes is in phase with the segmentation clock and that FGF is involved in the regulation of the rhythm of this clock (Dubrulle et al., 2001; Zakany et al., 2001). However to date, such a clock has not been described for the neural tube and it is possible that these two tissues use different strategies to define AP values. Alternatively events or a clock patterning mesoderm may indirectly regulate events in the neural tube through tissue interactions.

Our results argue in favor of a model that includes the dynamic nature of factors like CDX and modulation of accessibility of the complex. It is clear that Cdx expression in the chick neural tube is dynamic and dependent upon FGF signaling and that FGF-mediated stimulation of Hox genes requires CDX. This highlights the importance of the regulatory interactions between FGF and Cdx genes, in accord with both models. However, while robust upregulation of Hoxb4 in the hindbrain can be mediated Xcad3VP16 alone, induction of Hoxb9 is more patchy or limited and does not extend as far anteriorly in the hindbrain (Fig. 7). This suggests a difference in the competence or accessibility state of Hoxb9 versus Hoxb4 in the hindbrain. When we overexpressed XcadVP16 in combination with FGF treatment, Hoxb9 was also strongly upregulated in the hindbrain, whereas neither of these treatments alone was sufficient to induce Hoxb9 expression in this domain. This suggests that in addition to the input from CDX, FGF treatment has in some way rendered the Hoxb9 locus more accessible, thus allowing the activation of Hoxb9 transcription. In this combined model (Fig. 8), FGF signaling would have a dual role in modulating the accessibility of the Hox complex along the axis and in activating the expression of Cdx. However, we cannot exclude the possibility that the combination of ectopic Cdx and FGF treatment is more effective at inducing Hoxb9, as a result of the action or induction of other factors/co-factors required to transduce the CDX-mediated signal and trans-activate the 5’ Hox genes in the hindbrain. It has been suggested that the retinoic acid pathway could also be involved in the accessibility state of the complexes (Bel-Vialar et al., 2000; Kmita et al., 2000) and retinoid nuclear receptors are known to be part of complexes containing HAT and HDAC chromatin remodeling enzymes (Featherstone, 2002). Therefore any progressive opening of chromatin and accessibility of Hox complexes could be controlled by a balance between the influence of retinoid and FGF signaling pathways.

Finally, recent results show that in the neural plate cells adopt successively different mature fates as they move or are forced out of the node region toward more anterior regions (Mathis et al., 2001). These same authors also present data indicating that FGF signals in a node stem zone are used to maintain a consistent pool of immature neural precursors during elongation of the tube. This data opens the alternative possibility that FGF acts as a caudalizing factor for the neural tube because it prolongs the window of time during which cells are exposed to additional caudalizing factors, FGFs, WNTs or retinoids (Vasiliauskas and Stern, 2001). Our results lend strength to the idea that cdx genes appear to integrate signaling from multiple signaling pathways and it is tempting to suggest that CDX is a pivotal general caudalizing factor.

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