Int. J. Dev. Biol. 43: 665-674 (1999)

Novel interactions between vertebrate *Hox* genes

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ABSTRACT Understanding why metazoan *Hox/HOM-C* genes are expressed in spatiotemporal sequences showing colinearity with their genomic sequence is a central challenge in developmental biology. Here, we studied the consequences of ectopically expressing *Hox* genes to investigate whether *Hox-Hox* interactions might help to order gene expression during very early vertebrate embryogenesis. Our study revealed conserved autoregulatory loops for the *Hox*4 and *Hox*7 paralogue groups, detected following ectopic expression *Hoxb-4* or *HOXD4,* and *Hoxa-7,* respectively. We also detected specific induction of 5' posterior *Hox* genes; *Hoxb-5* to *Hoxb-9,* following ectopic expression of *Hoxb-4/HOXD4*; *Hoxb-8* and *Hoxb-9* following ectopic expression of *Hoxa-7*. Additionally, we observed specific repression of 3' anterior genes, following ectopic expression of *Hox*4 and *Hox*7 paralogues. We found that induction of *Hoxb-4* and *Hoxb-5* by *Hoxb-4* can be direct, whereas induction of *Hoxb-7* is indirect, suggesting the possibility of an activating cascade. Finally, we found that activation of *Hoxb-4* itself and of posterior *Hox* genes by *Hoxb-4* can be both non-cell-autonomous, as well as direct. We believe that our findings could be important for understanding how a highly ordered *Hox* expression sequence is set up in the early vertebrate embryo.

KEY WORDS: *Hox genes, autoregulation, cross-regulation, establishment, Xenopus laevis*

Introduction

Hox/HOM-C genes are an evolutionarily conserved, chromosomally clustered family of genes encoding transcription factors which specify positional identities along the anteroposterior axis in vertebrates and other metazoans (Duboule and Morata, 1994; McGinnis, 1994; Ruddle *et al.*, 1994; Carroll, 1995; Capecchi, 1997; Sharkey *et al.*, 1997; Lewis, 1998). In most vertebrates, they are organised in four chromosomal complexes, each containing up to 11 genes. The expression of *Hox/HOM-C* genes is characterised by spatial colinearity: the anterior expression boundaries of these genes occur in a sequence which matches their chromosomal order. Most metazoan embryos also show temporal colinearity; *Hox* genes are expressed in a temporal sequence which reflects their chromosomal order. Most available data about the regulation of vertebrate *Hox* gene expression concerns maintenance of the established pattern: less is known about the mechanisms that initially lead to *Hox* expression at appropriate levels along the embryonic axis. This contrasts with the situation in *Drosophila*, where much more is known about both phases. It now seems likely that only some elements of the mechanism mediating maintenance are conserved between *Drosophila* and vertebrates. This may relate to the fact that *Drosophila*, in contrast to most other animals, shows little sign of temporal colinearity (Duboule and Morata, 1994), a characteristic of the establishment phase in vertebrates. Nevertheless, we are still far from understanding all of the regulatory interactions involved in generating the proper spatial and temporal patterns of vertebrate *Hox* gene expression. This is a central challenge in today's developmental biology.

There are strong indications that interactions among *Hox/HOM-C* genes participate in regulating their ordered expression and function. Evidence, both in *Drosophila* and in vertebrates, indicates that 5' posterior *Hox/HOM-C* genes phenotypically dominate more 3' anterior *Hox/HOM-C* genes ('posterior prevalence') (Gonzalez-Reyes *et al.*, 1992; Duboule and Morata, 1994). There is also evidence that *Drosophila HOM-C* genes regulate their own and each other's expression. Notably, some *HOM-C* genes autoregulate their own expression (Kuziora and McGinnis, 1988; Bergson and McGinnis, 1990; Chouinard and Kaufman, 1991; Gonzalez-Reyes *et al.,* 1992; Bienz, 1994), and some repress more 3' anterior *HOM-*

Abbreviations used in this paper: RA, all trans retinoic acid; CNS, central nervous system; RT-PCR, PCR with reverse transcription.

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0214-6282/99/$15.00

Printed in Spain
www.ehu.es/ijdb

C genes (Struhl and White, 1985; Carroll *et al.*, 1986; Wirz *et al.*, 1986; Appel and Sakonju, 1993). There is still relatively little information about interactions among vertebrate *Hox* genes. It appears that autoregulatory loops which positively regulate *Drosophila Deformed (Dfd)* and *labial (lab)* (Kuziora and McGinnis, 1988; Bergson and McGinnis, 1990; Chouinard and Kaufman, 1991) are conserved among murine *Hox4* and *Hox1* paralogues respectively (Wu and Wolgemuth, 1993; Gould *et al.*, 1997; Studer *et al.*, 1998), and that these act during maintenance of *Hox* gene expression. A recent study (Studer *et al.*, 1998) also described auto-/cross-regulatory interactions among murine *Hox1* paralogues during their early expression. Other studies have revealed non-colinear positive and negative cross-regulatory interactions between murine *Hox* genes during the maintenance phase of expression. Neighbouring *Hox* genes may share enhancers (Gould *et al.*, 1997) and there may be competition for enhancers between neighbouring *Hox* gene promoters (Sharpe *et al.*, 1998). There is thus substantial evidence that interactions among *Hox/HOM-C* genes exist, but many questions remain about the nature of these interactions and their functional significance.

Here, we investigated the potential importance of *Hox-Hox* interactions for establishing the vertebrate *Hox* expression sequence by examining consequences of ectopically expressing different *Hox* genes in the very early *Xenopus* embryo (Condie and Harland, 1987; Fritz and DeRobertis, 1988; Harvey and Melton, 1988). We detected a conserved autoregulatory loop for *Hox*4 paralogues, paralleling findings in the mouse and in *Drosophila* (above).

We also detected a *Hox*7 group autoregulatory loop, which has not previously been described in vertebrates, but parallels autoregulation of *Ubx* in *Drosophila* (Christen and Bienz, 1992). More importantly, we detected a novel type of interaction: specific induction of more 5' posterior *Hox* genes by *Hoxb-4* and *HOXD4,* and by *Hoxa-7*. This phenomenon was observed clearly both *in vitro* (in neuroectoderm explants) and *in vivo* (in whole embryos and in lineage labelled clones).

Posterior induction was poorly documented till now, having been suggested in vertebrate cells (Faiella *et al.*, 1994) and shown only for induction of *Hoxb-2* by *Hoxb-1* in the mouse embryo (Maconochie *et al.*, 1997), and not being clearly established in *Drosophila*. Besides specific induction of 5' posterior *Hox* genes, we also detected specific down regulation of all anterior marker genes tested (including 3' anterior *Hox* genes), which are normally expressed anteriorly to the ectopically expressed *Hox* genes. Our results provide the first evidence that this type of repressive interaction may occur in vertebrates as well as in *Drosophila*. Additionally, our results revealed that activation of some *Hox* genes by *Hoxb-4* can be direct, whereas activation of others is indirect. Finally, we found that autoregulation and activation of 5' posterior *Hox* genes by *Hoxb-4* occurs non-cell-autonomously, as well as directly. We believe that these results are potentially important for understanding how a highly ordered *Hox* expression sequence is set up in the early vertebrate embryo.

Results

Early ectopic expression of paralogue group 4 Hox genes induces a headless phenotype

We expressed paralogue group 4 *Hox* genes ectopically by microinjecting mRNA into the zygote or a blastomere in an early *Xenopus* embryo. We also later ectopically expressed a paralogue group 7 *Hox* gene for comparison (see below). Initially, we microinjected full-length messengers of *Xenopus* and mouse *Hoxb-4*, and human *HOXD4*, as well as of a non-functional deletion construct of *Xenopus Hoxb-4* (*Xb-4BglII600*), missing the 3' part of the homeobox and 3' flanking sequences (details in legend to Fig. 1) into zygotes. Injection of functional messengers but not the mutant, had a dramatic effect on axial patterning (Fig. 2A,B). It generated anteriorly defective tadpoles, showing strong inhibition of eye and cement gland development. This phenotype superficially resembles that generated previously by ectopic expression of *Hoxa-7* (Pownall *et al.*, 1996). We show below, using an *in vitro* system, that paralogue group 4 and 7 *Hox* genes each actually generate a different specific posterior transformation. The paralogue group 4 phenotype was characterised using immunostaining with antineural (Fig. 2C,D) and antimuscle (Fig. 2E,F) antibodies. Neural staining revealed that the central nervous system (CNS) was reduced and developed abnormally at all levels anterior to the posterior hind-

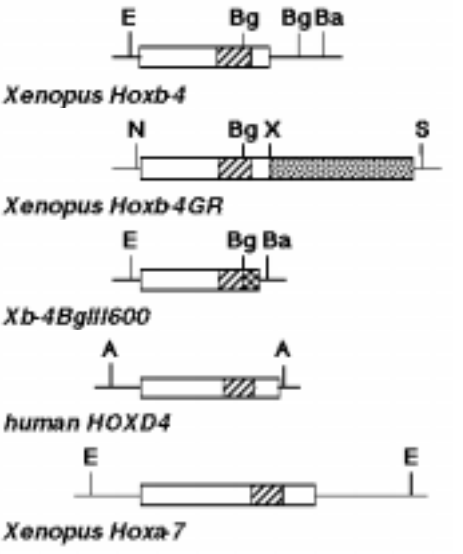

Fig. 1. Diagrammatic representation of the different plasmid constructs used. *(A=ApaI; Ba=BamHI; Bg=BglII; E=EcoRI; N=NotI; S=SpeI; X=XhoI; 5' to the left, open box is the coding region and the striped box represents the homeobox). For Xenopus Hoxb-4 overexpression, the 1 kb EcoRI/BamHI fragment of the Xhox-1A clone, 64T-c1A (Harvey et al., 1986) containing the complete coding sequence of Hoxb-4 gene, was ligated into the T7Ts vector and used as a template. The Xenopus Hoxb-4 deletion construct (Xb-4BglII600) was created by excising the 0.3 kb BglII-BglII fragment out of Xenopus Hoxb-4. Both constructs were linearised with SmaI. The Xenopus Hoxb-4 glucocorticoid receptor construct (Hoxb-4GR) was generated using a PCR amplified 0.7 kb Hoxb-4 fragment containing the complete open reading frame of Xenopus Hoxb-4. Using additionally added restriction site NotI and XhoI, this fragment was ligated in frame into a modified T7Ts vector containing the 0.8 kb hormone binding part (a.a. 512-777) of the human glucocorticoid receptor coding sequence [dotted box = 0.8 kb XhoI/SpeI fragment containing the glucocorticoid receptor coding sequence; (Gammill and Sive, 1997)]. Next, linearised with BamHI for mRNA synthesis. The human HOXD4 construct was produced by inserting the 0.9 ApaI/ApaI fragment of clone HHO.c13 (Mavilio et al., 1986) into T7Ts and subsequent linearisation by BamHI. The Xenopus Hoxa-7 construct as described previously (Pownall et al., 1996).*

brain (rostral to the anterior neural expression boundaries of paralogue group 4 *Hox* genes). The posterior CNS (posterior hindbrain and spinal cord) appeared relatively normal. Similarly, muscle staining showed strong disturbances in the head musculature and anterior somites, as was reported previously (Harvey and Melton, 1988).

Hoxb-4, Hoxa-7 and RA each induce different specific posterior transformations

We next investigated the specific effects of *Hoxb-4* overexpression on *Hox* gene expression using a well established *in vitro* system for patterning in ectoderm and neurectoderm, two tissues which show early *Hox* expression (Godsave *et al.*, 1994; Kolm and Sive, 1997) (Fig. 3). Zygotes were microinjected with mRNA for *Hoxb-4* with/without the anterior neural inducing factor noggin (Smith and Harland, 1992; Lamb *et al.*, 1993). Ectoderm or anterior neurectoderm explants were then cut from the resulting embryos at the late blastula stage (stage 9), cultured to st. 18 and analysed by RT-PCR. Our results revealed that *Xenopus Hoxb-4* strongly induced its own expression (detected using PCR primers for the endogenous 3' UTR, which is absent in the injected *Hoxb-4* mRNA). It also induced expression of each of four 5' posterior *Hoxb* genes examined (*Hoxb-5*, *Hoxb-7*, *Hoxb-8* and *Hoxb-9;* Fig. 3A). In contrast, it repressed expression of *Otx-2* as well as of *Hoxb-1*, *Hoxb-2* and *Hoxb-3* in noggin explants and in noggin/RA explants, respectively (Fig. 3B). Identical results were obtained following ectopic expression of murine *Hoxb-4*, or human *HOXD4* (not shown).

We wished to determine whether induction of 5' posterior *Hox* genes by *Hox*4 paralogues is unique to this *Hox* paralogue group or whether it is paralleled following ectopic expression of other *Hox* genes. We therefore examined *Hox* gene induction following ectopic expression of *Xenopus Hoxa-7* (Condie and Harland, 1987; Pownall *et al.*, 1996). Ectopically expressing *Hoxa-7* generates an anteriorly defective phenotype superficially resembling that generated by *Hoxb-4* (Pownall *et al.*, 1996). We discovered that, even though the morphological *Hoxa-7* phenotype is superficially similar to that induced by *Hoxb-4*, the effects of *Hoxa-7* on gene expression are, in fact, very specific. *Hoxa-7* failed to induce any of five 3' anterior *Hox* genes examined (*Hoxb-1* to *Hoxb-5*), but did activate its paralogue *Hoxb-7* as well as two 5' posterior *Hox* genes (*Hoxb-8* and *Hoxb-9)* (Fig. 4). Similarly as with *Hoxb-4*, *Hoxa-7* repressed 3' anterior *Hox* genes. In the experiment described in Figure 4, it repressed the 3' *Hox* genes, *Hoxb-4* and *Hoxb-5.* These results show that induction of 5' posterior *Hox* genes and repression of 3' anterior *Hox* genes is not unique to *Hox4* paralogues, but is paralleled by the action of another *Hox* gene, *Hoxa-7*. *Hoxb-4, HOXD4* and *Hoxa-7* each appear to activate and repress in a colinear fashion, starting with their homologous paralogue group. All-trans retinoic acid (RA) is known for its posteriorising activity in neuroectoderm (Durston *et al.*, 1989; Godsave *et al.*, 1998). RA-treatment of noggin-induced anterior neuroectoderm induced five 3' anterior *Hox* genes, *Hoxb-1, Hoxb-2, Hoxb-3, Hoxb-4,* and *Hoxb-5,* but not three 5' posterior *Hox* genes *Hoxb-7*, *Hoxb-8*, *Hoxb-9.* RA induced these genes only in neurectoderm and not in uninduced ectoderm (control) (Fig. 3B). These results show that the posterior transformations induced by *Hoxb-4* (or *HOXD4*), *Hoxa-7* and RA are each specific and different from each other.

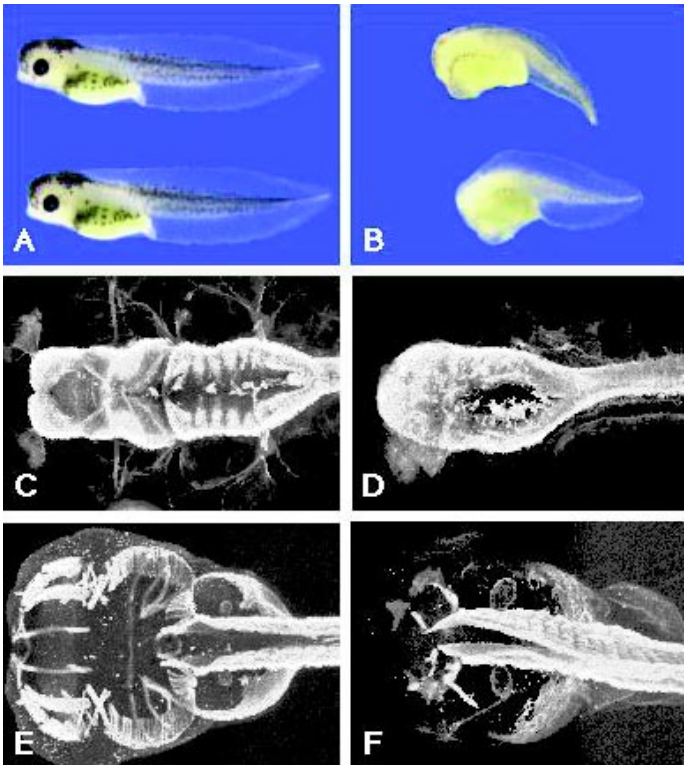

Fig. 2. Ectopic expression of *Xenopus Hoxb-4* causes severe anterior truncation in developing *Xenopus laevis* embryos. (A) *Control (Xb-4BglII600 injected; 1 ng injected at st. I) tailbud stage Xenopus embryos. Xb-4BglII600 injected embryos were identical to uninjected embryos for all aspects examined.* **(B)** *Xenopus Hoxb-4 injected embryos (1 ng, st. I). Tailbud stage embryos showing clear anterior truncation, involving inhibition of eye and cement gland development.* **(C)** *Control (Xb-4BglII600 injected; 1 ng, st. I) embryo (approx. stage 46) stained by indirect immunofluorescence with neural-specific monoclonal antibodies Xen-1 and 2G9 (Jones and Woodland, 1989; Ruiz i Altaba, 1992).* **(D)** *Xenopus Hoxb-4 injected (1 ng, st. I) embryo (approx. stage 46) stained with neural-specific monoclonal antibodies. Whole-mount immunohistochemistry and confocal analysis reveals defective development of the anterior CNS. Development of forebrain, midbrain and anterior hindbrain is severely disturbed. Development of the spinal cord appears relatively normal.* **(E)** *Control (Xb-4BglII600 injected; 1 ng, st. I) embryos (approx. stage 46). Whole-mount immunohistochemistry of muscle tissue using muscle-specific monoclonal antibody 12/101 (Kintner and Brockes, 1984).* **(F)** *Xenopus Hoxb-4 injected (1 ng, st. I) embryo (approx. stage 46). Confocal analysis reveals severe disturbance or complete lack of development of anterior muscle tissue, i.e. jaw-, eye-muscles and somites. They also show severe disorganisation of the more anterior somite muscle tissue, as observed previously (Harvey and Melton, 1988). Sections of embryos confirm these data.*

Hoxb-4 induces an identical posterior transformation in vivo and in vitro

We wished to determine whether induction of 5' posterior and repression of 3' anterior *Hox* genes by *Hoxb-4*, which was observed *in vitro*, also occurs *in vivo*. *In situ* hybridisation analysis of whole embryos developing from *Hoxb-4* injected zygotes (Fig. 5) confirmed that *Xenopus Hoxb-4* induces expression of three 5' posterior *Hox* genes examined: *Hoxb-5*, *Hoxb-7* and *Hoxb-9*. We injected different amounts of *Hoxb-4* mRNA (ranging from 10 pg

to 1000 pg) into 1 blastomere in a 16-cell stage *Xenopus* embryo, to determine whether a 5' posterior *Hox* gene can be induced locally by injecting both low and high *Hoxb-4* mRNA concentrations (Fig. 6). Assaying *Hoxb-5* expression in these embryos, we observed that ectopic expression of 10 pg of *Hoxb-4* mRNA already induces *Hoxb-5* expression in half of the injected embryos, whereas injection of 20 pg or more induces *Hoxb-5* expression in all embryos (Fig. 6). The intensity of *Hoxb-5* induction increases with increasing amount of injected *Hoxb-4* mRNA. Our results also confirmed that ectopically expressed *Hoxb-4* represses all of and only the four markers examined, including *Hoxb-1*, *Hoxb-2* and *Hoxb-3*, which are expressed anteriorly to the endogenous *Hoxb-4* expression domain (Fig. 7). Taken together with the results from explant experiments (above), these findings verify that ectopic expression of *Hoxb-4* specifically induces 5' posterior *Hox* genes and represses 3' anterior *Hoxb* genes.

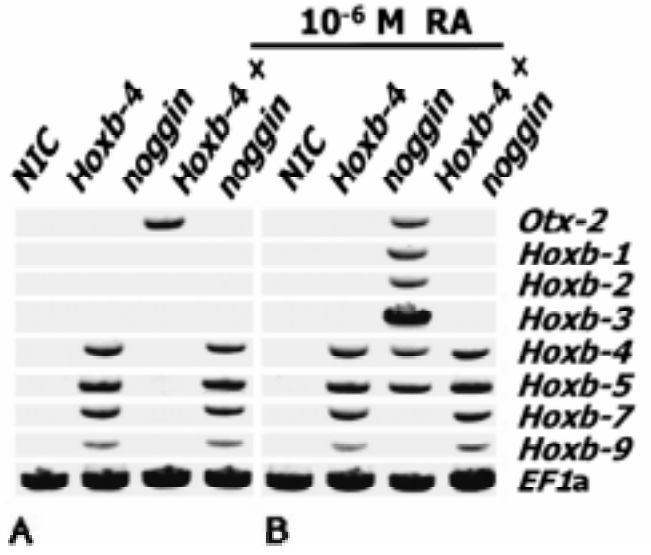

Fig. 3. RT-PCR analysis reveals strong regulatory interactions within the *Hoxb* cluster and between *Hox* clusters. *Embryos were injected with Hoxb-4 mRNA and/or noggin mRNA or not injected (controls) at stage 1. Animal caps were cut at stage 9. Total RNA was extracted from 10 animal caps at stage 18 of development (Nieuwkoop and Faber, 1967). RT-PCR was performed for Xenopus Otx-2, 7 Hoxb genes and EF1α (internal standard).* **(A)** *RT-PCR experiment of control embryos (NIC), Xenopus Hoxb-4 (1 ng, st. I; Hoxb-4), noggin (150 pg, st. I; noggin) or Hoxb-4 and noggin injected embryos (Hoxb-4+noggin). Hoxb-4 autoregulates its own expression (Hoxb-4/Hoxb-4+noggin); detected using PCR primer for the endogenous 3' UTR, which is absent in the injected Hoxb-4 mRNA), is able to induce expression of more 5' posterior Hoxb genes (Hoxb-4/Hoxb-4+noggin) and represses the expression of Otx-2 (Hoxb-4+noggin).* **(B)** *RT-PCR analysis of control embryos (NIC) or embryos injected with Xenopus Hoxb-4 (1 ng, st. I; Hoxb-4), with noggin (150 pg, st. I; noggin) or with Hoxb-4 and noggin (Hoxb-4+noggin) treated overnight with 10^{-6} M all-trans retinoic acid (RA). Hoxb-4 abolishes the RA-induced expression of 3' anterior Hoxb genes [compare (noggin) with (Hoxb-4+noggin)] and induces expression of 5' Hoxb genes (Hoxb-4/Hoxb-4+noggin). No or very low levels of expression of Hox genes were observed in the control explants: uninduced ectoderm and noggin induced neurectoderm.*

Direct Hox-Hox interactions

Another important question is whether the observed *Hox-Hox* interactions are direct or indirect. To address this, we generated an expression construct for a dexamethasone-inducible chimaera between *Hoxb-4* and the ligand binding domain of the glucocorticoid receptor (*Hoxb-4GR*) (Kolm and Sive, 1995; Gammill and Sive, 1997), and injected synthetic mRNA from this construct into the *Xenopus* zygote (Fig. 8). Dexamethasone (DEX) (Gammill and Sive, 1997) induction of *Hoxb-4* activity in ectodermal explants from the injected embryos led, after 2 h, to strongly activated expression of *Hoxb-4* and of two 5' posterior *Hox* genes, *Hoxb-5* and *Hoxb-7*. Induction of *Hoxb-4* and *Hoxb-5* proved insensitive to pretreatment with the protein synthesis inhibitor cycloheximide (CHX) (Grainger and Gurdon, 1989; Gammill and Sive, 1997), whereas induction of *Hoxb-7* was blocked totally by CHX. Notably, *Hoxb-4GR* failed to induce expression of the general neural marker *N-CAM*, making it very unlikely that the activation of 5' posterior *Hox* genes by *Hoxb-4* results from induction of neural tissue either directly on indirectly, via induction of dorsal mesoderm. Next, we examined the time course of transcriptional activation of *Hoxb-5* in CHX-treated/*Hoxb-4GR* injected embryos using Northern Blot Analysis (Fig. 9A). Stage 10.5 embryos were CHX pretreated and then DEX treated before being harvested for analysis of *Hoxb-3* and *Hoxb-5* expression at 5, 10, 30, 60, 90 and 220 min after DEX addition. Control embryos (non-injected) showed no *Hoxb-3* or *Hoxb-5* expression, whereas the *XHoxb-4GR* injected embryos already displayed *Hoxb-5* expression 5 min after addition of DEX, and this expression increased strongly during prolonged DEX treatment (Fig. 9A). We also injected *Hoxb-4GR* mRNA locally into one blastomere in a 4-cell stage embryos and assayed for *Hoxb-5* expression using *in situ* hybridisation (Fig. 9B). In this case, we detected strongly localised, time-dependent CHX insensitive *Hoxb-5* expression, starting within 15 min of DEX addition. *Hoxb-5* induction is thus very rapid and localised to the site of *Hoxb-4* injection, as would be expected if it were direct. *Hoxb-3* (also assayed in these experiments) showed no detectable expression. Based on the CHX insensitivity of DEX induction of *Hoxb-4* and *Hoxb-5,* and the rapid and localised nature of CHX insensitive *Hoxb-5* induction, following DEX treatment, we conclude that *Hoxb-4* and *Hoxb-5* are direct *Hoxb-4* targets, activated independently of protein synthesis, while *Hoxb-7* activation by *Hoxb-4* is indirect, requiring protein synthesis.

Non-cell-autonomous Hox-Hox interactions

An important question which arises is whether the *Hox-Hox* interactions reported above are cell-autonomous, so that spatiotemporal patterns of *Hox* gene expression are determined solely by external signals, or whether changes in a cell's *Hox* code itself can also induce intercellular signalling and thus be communicated from cell to cell. To test these possibilities, *Xenopus Hoxb-4* mRNA was targeted, together with a lineage label, to the anterior part of the central nervous system (CNS). *Hoxb-4* mRNA was coinjected with *LacZ* mRNA into one of the b-ring blastomeres in each of a number of 32 cell stage albino embryos (Dale and Slack, 1987) and the embryos were then analysed for *LacZ* expression at stage 15. Embryos which showed CNS localised *LacZ* expression in the anterior neural plate, as expected from lineage labelling of the progeny of a b-1 blastomere, were selected for analysis of *Hox* gene expression (*Hoxb-4* and *Hoxb-5*).

In situ hybridisation revealed expression of *Xenopus Hoxb-4* (Fig. 10A) and *Hoxb-5* (Fig. 10B). Strikingly, these genes were often expressed outside the lineage labelled zone, involving expression in lateral non-neural tissue, as well as in the CNS. In contrast to the localised *LacZ* expression, the *Hoxb* expression domains, particularly those of *Hoxb-4*, could be extensive, covering a large part (up to one quarter) of the total surface of the embryo, exceeding by far the predictable lineage domain from any one 32-cell stage b-ring blastomere (Dale and Slack, 1987). As a control, we injected mRNA from a non-functional deletion mutant of *Hoxb-4 (Xb-4BglII600)* together with *LacZ* mRNA. In this case, the localised extent of *LacZ* staining and *Hoxb-4 in situ* staining matched each other perfectly, and remained localised in the *LacZ* lineage-labelled domain, the extent of which was predictably as expected for progeny of a b1 blastomere (Fig. 10C). These results emphasise firstly that (local) ectopic expression of *Hoxb-4* can induce expression of 5' posterior *Hox* genes and autoregulation of *Hoxb-4.* Secondly, considering the localised fate of the *LacZ* lineage labelled cells, and the far more extensive expression of the *Hoxb* genes (*Hoxb-4* and *Hoxb-5*), which could be well outside the lineage restricted domains, they suggest strongly that there is non-cell-autonomous induction of *Hoxb-4* and *Hoxb-5* in neighbouring cells by *Hoxb-4* expressing cells. In a second approach, we combined two gastrula stage (stage 10) animal caps one loaded with *Hoxb-4* mRNA and one loaded with *LacZ* mRNA ('sandwich'-experiment). We then observed extensive *Hoxb-4* expression within the *LacZ* labelled animal cap, but no *LacZ* expression in the *Hoxb-4* loaded animal cap (data not shown).

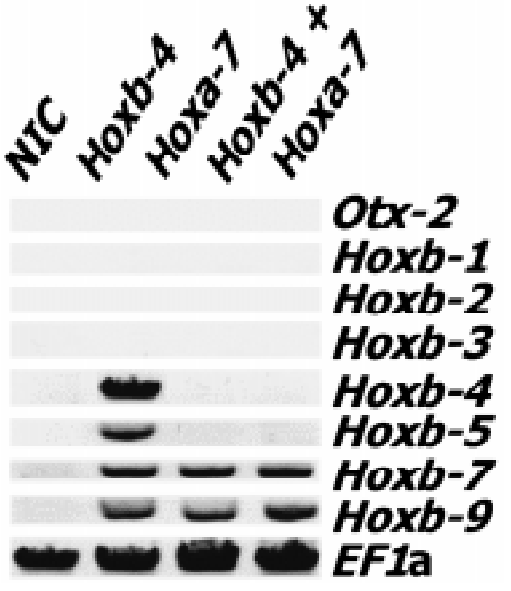

Fig. 4. RT-PCR analysis reveals that colinear induction and repression of *Hoxb* genes by *Hoxb-4* is not unique, but paralleled following ectopic expression of *Hoxa-7*. *RT-PCR analysis of control embryos (NIC) or embryos injected with Xenopus Hoxb-4 (1 ng, st. I; Hoxb-4), Hoxa-7 (1 ng, st. I; Hoxa-7) or Hoxb-4 and Hoxa-7 (Hoxb-4 + Hoxa-7). Hoxa-7 suppresses Hoxb-4 induced Hoxb-4 and Hoxb-5 expression (Hoxb-4 + Hoxa-7) and induces Hoxb-7 and Hoxb-9 expression (Hoxa-7/Hoxb-4 + Hoxa-7). No or very low levels of expression of Hox genes were observed in the control explants: uninduced ectoderm.*

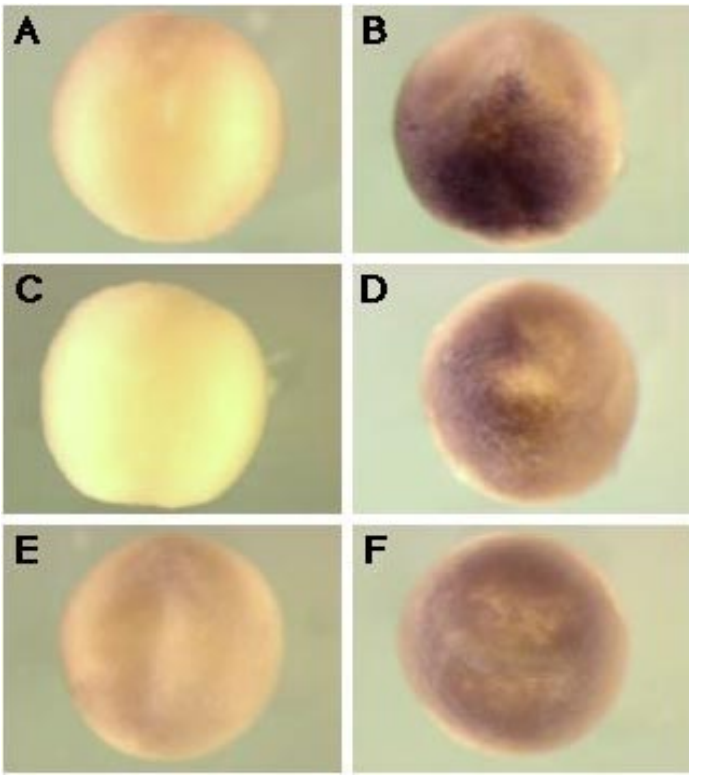

Fig. 5. *Hoxb-4* induces expression of more 5' posterior *Hoxb* genes, *in vivo,* in early *Xenopus* embryos. (A) *In situ hybridisation analysis of a stage 11 Xenopus embryos (oriented with animal side up) hybridised for Xenopus Hoxb-5.* **(B)** *Comparable stage 11 embryo ectopically expressing Xenopus Hoxb-4 (1 ng, st. I) hybridised for Xenopus Hoxb-5.* **(A)** *Hoxb-5 is normally not expressed at stage 11 of development (Godsave et al., 1994).* **(B)** *Ectopic expression of Hoxb-4 strongly induced ectopic expression of Xenopus Hoxb-5.* **(C,D,E,F)** *Hoxb-7 and Hoxb-9 in situ hybridisation analysis of control embryos and embryos overexpressing Xenopus Hoxb-4 (all embryos oriented with animal side up).* **(C,E)** *No Hoxb-7 or Hoxb-9 expression could be observed around stage 11 (Godsave et al., 1994).* **(D,F)** *Hoxb-7 and Hoxb-9 are again strongly induced in Hoxb-4 overexpressing embryos (1 ng, st. I).*

Concluding, the results of our experiments indicate that onset of *Hoxb* gene expression can be propagated from cell to cell via intercellular signalling.

Discussion

Autoregulation, posterior induction and anterior repression by Hox genes

We studied the effects on *Hox* gene expression of ectopically expressing different *Hox* genes in the very early *Xenopus* embryo. We detected three types of interactions between *Hox* genes.

First, auto- and para-regulatory interactions within two paralogue groups. *Xenopus Hoxb-4* activated expression of *Hoxb-4* itself (Figs. 3,8,10). Data obtained using the dexamethasone-inducible *Hoxb-4* glucocorticoid receptor construct (*Hoxb-4GR*) indicated that this autoregulation can be direct (cycloheximide insensitive) (Fig. 8). Direct autoactivation of group 4 *Hox* genes was previously demonstrated for *Drosophila Dfd* (Kuziora and McGinnis, 1988;

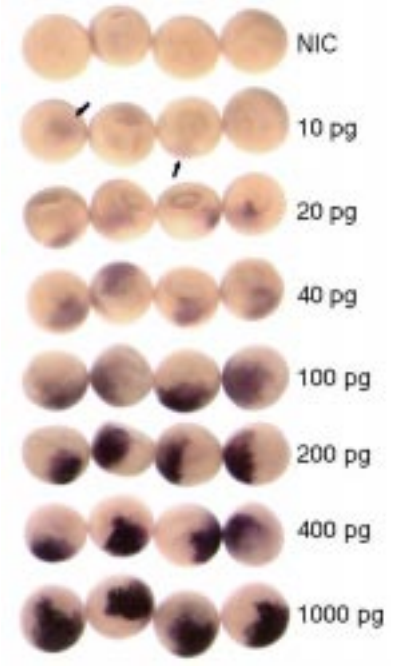

Fig. 6. Induction of *Xenopus Hoxb-5* is observed both in response to low and high *Hoxb-4* mRNA concentrations. *One blastomere of a 16-cell stage albino embryo was injected with different amounts of Hoxb-4 synthetic mRNA (ranging from 10 pg to 1000 pg). Using in situ hybridisation analysis, we observed Hoxb-5 induced expression in response to ectopic expression of Hoxb-4. 10 pg of Hoxb-4 mRNA ectopically induces Hoxb-5 in half of the embryos (arrows), whereas Hoxb-5 induction is observed in all embryos using higher Hoxb-4 mRNA concentrations (20-1000 pg). The strength of the Hoxb-5 signal increases with increasing amounts of Hoxb-4 mRNA injected. Control embryos (NIC) showed no Hoxb-5 expression.*

Bergson and McGinnis, 1990) as well as for murine *Hoxa-4*, *Hoxb-4* and *Hoxd-4* (Wu and Wolgemuth, 1993; Gould *et al.*, 1997), indicating a strongly conserved, direct autoregulatory circuit. The induction of *Xenopus Hoxb-4* by human *HOXD4* indicates that this loop is active cross cluster in *Xenopus,* as in mouse (Wu and Wolgemuth, 1993; Gould *et al.*, 1997). We also observed that *Hoxa-7* induces expression of *Hoxb-7* (Fig. 4). This is the first indication that a group 7 *Hox* autoregulatory circuit, indicated previously only by autoregulation of *Drosophila Ubx* (Christen and Bienz, 1992), is active in vertebrates.

Second, specific induction of 5' posterior *Hox* genes. Ectopic expression of *Hoxb-4* induced all more 5' *Hox* genes examined (*Hoxb-5*, *Hoxb-7*, *Hoxb-8* and *Hoxb-9*), and not more 3' *Hox* genes (*Hoxb-1, Hoxb-2, Hoxb-3*) (Figs. 3,4,5,6,8,9,10). Induction (of *Hoxb-5)* occurred following injection both of low and high *Hoxb-4* mRNA concentrations (10-1000 pg of mRNA; Fig. 6). Induction of *Hoxb-5* by *Hoxb-4* was rapid and independent of protein synthesis (CHX insensitive) (Figs. 8,9), and thus presumably direct, while induction of *Hoxb-7* by *Hoxb-4* was dependent on protein synthesis (CHX sensitive) and thus presumably indirect. Human *HOXD4* induced the same *Hoxb* genes as *Hoxb-4*, while *Hoxa-7* induced expression only of *Hoxb-7* and of *Hoxb* genes 5' posterior to it (*Hoxb-8, Hoxb-9*), not *Hox* genes 3' anterior to it (*Hoxb-1, Hoxb-2, Hoxb-3, Hoxb-4, Hoxb-5*; Fig. 4). These observations raise the possibility that a 3' to 5' activation cascade plays a role in establishing the *Hox* expression sequence. This conclusion was already suggested by a previous study on the human NT2/D1 teratocarcinoma cell line, where antisense oligonucleotides to *Hoxb-1* and *Hoxb-3* each selectively blocked expression of 5' *Hox* genes, following RA-induced, colinear induction of the *Hox* clusters (Faiella *et al.*, 1994). Activation of *Hoxb-2* by *Hoxb-1* was also observed recently following ectopic expression of *Hoxb-1* in the mouse embryo during the maintenance phase of expression (Maconochie *et al.*, 1997; Nonchev *et al.*, 1997).

Finally, specific repression of 3' anterior *Hox* genes. Ectopic expression of *Hoxb-4* repressed all genes examined which are normally expressed anteriorly to it, including the 3' *Hox* genes *Hoxb-1, Hoxb-2* and *Hoxb-3.* It did not repress *Hoxb-4* itself, nor the 5' posterior *Hox* genes examined (Figs. 3,7). Parallel, specific, colinear repression of more 3' anterior *Hox* genes was also observed following ectopic expression of *Hoxa-7*, which repressed *Hoxb-4* and *Hoxb-5,* but not *Hoxb-7* or *Hoxb-9* (Fig. 4). These cases raise the possibility that at least certain vertebrate *Hox* genes can repress expression of more 3' anterior *Hox* genes colinearly. Colinear repressive interactions between *Hox* genes have rarely been observed previously in vertebrates. They have been observed among *Drosophila HOM-C* genes: most *HOM-C* genes show (colinear) repression of more 3' anterior genes (González-Reyes *et al.*, 1992; Appel and Sakonju, 1993) and there is clear evidence that *Drosophila HOM-C* genes can directly repress each other's expression. Our results provide the first evidence that this feature could be conserved in vertebrates.

The molecular mechanisms of these interactions require investigation.

Non-cell-autonomous interactions

When ectopically expressing *Xenopus Hoxb-4* together with the lineage label *LacZ*, we observed *LacZ* lineage labelled regions surrounded by cells expressing *Hoxb-4* and *Hoxb-5* (Fig. 10A,B). These genes were expressed outside the lineage labelled zone, involving expression in lateral non-neural tissue, as well as in the CNS. The *Hoxb-4* expression domains could be extensive, covering a large part of the total surface of the embryo, far exceeding the predicted cell lineage domains from injected cells (Dale and Slack, 1987). This effect was dependent on *Hoxb-4* function, since a non-functional deletion mutant of *Hoxb-4* (*Xb-4BglII600*) failed to generate *Hox* expression outside the lineage restricted domain, giving an *in situ* hybridisation pattern that closely matched the *LacZ* lineage labelled clone (Fig. 10C). Similarly, induction of *Hoxb-4* expression in non-injected cells was observed in animal cap 'sandwich' experiments (not shown). Our results point to the conclusion that *Hoxb-4* expressing cells are able to induce *Hox* expression in neighbouring cells via a non-cell-autonomous mechanism. Non-cell-autonomous *Hox-Hox* interactions have been reported previously in *Drosophila*, but were unknown in vertebrates. Non-cell-autonomous *Ubx* autoregulation in *Drosophila* visceral mesoderm involves signalling via the *wg* and *dpp* pathways (Bienz, 1994). Autoregulation of *Deformed*, the *Drosophila* homologue of *Hoxb-4*, is also dependent on *wg* function (González-Reyes *et al.*, 1992). Further studies should elucidate the significance of these findings and the underlying mechanisms.

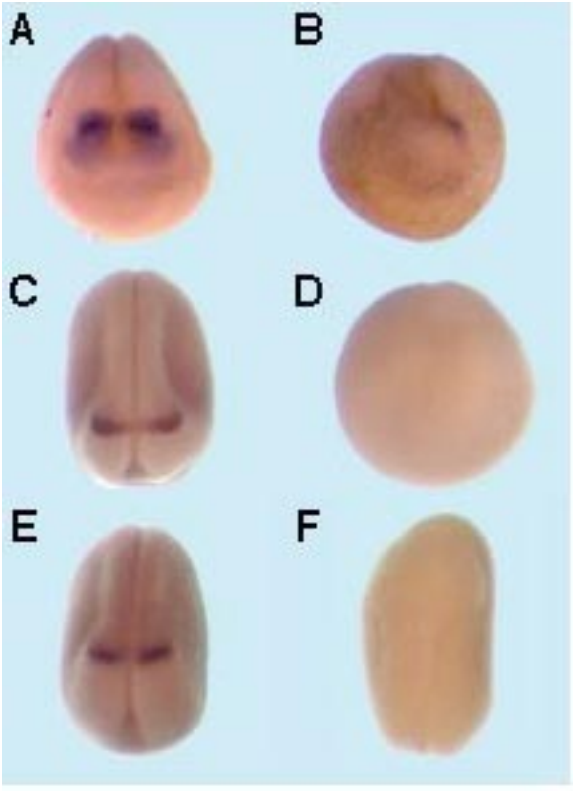

Fig. 7. Ectopic expression of *Xenopus Hoxb-4* represses expression of the more 3' anterior *Hoxb* genes, *in vivo,* in neurula stage embryos. *In situ hybridisation analysis of Xenopus embryos injected with Xenopus Hoxb-4 mRNA (1 ng, st. I) and control embryos (non-injected). Xb-4BglII600 injected (1 ng, st. I) embryos were identical to uninjected embryos. Embryos were cultured until approximately stage 18 of development (Nieuwkoop and Faber, 1967) and were then examined for expression of Xenopus Otx-2* **(A,B)**, *Hoxb-1* **(C,D)** *and Hoxb-3* **(E,F)**. **(A)** *Anterior views of a stage 18 embryo hybridised for Otx-2. Otx-2 is normally expressed in the fore- and midbrain (Pannese et al., 1995).* **(B)** *In stage 18 Hoxb-4 injected (1 ng, st. I) embryo Otx-2 expression is almost completely abolished.* **(C)** *Normal Hoxb-1 expression in a stage 18 (anterior view) embryo. Hoxb-1 is restricted in rhombomere 4 in the hindbrain at this stage (Godsave et al., 1994).* **(D)** *In embryos ectopically expressing Hoxb-4, no Hoxb-1 expression could be detected by in situ hybridisation.* **(E)** *Anterior view of a stage 18 embryo stained for Hoxb-3. Hoxb-3 is expressed most strongly immediately posterior to the otic vesicle in rhombomeres 5 and 6 at this stage (Godsave et al., 1994).* **(F)** *In Hoxb-4 injected (1 ng, st. I) embryos Hoxb-3 expression was completely lost.*

What is the significance of Hox-Hox interactions for generating the proper spatiotemporal patterns of Hox expression in the early vertebrate embryo?

We observed both direct and non-cell-autonomous induction of paralogues and 5' posterior *Hox* genes following ectopic expression of different *Hox* genes in the very early *Xenopus* embryo. Additionally, we observed repression of 3' anterior *Hox* genes following ectopic expression of *Hoxb-4* and *Hoxa-7*. At this stage of embryogenesis, coinciding with *Hox* establishment, a sequence of *Hox* expression waves follow each other in axial mesoderm and the neural plate, along the embryonic axis in the developing vertebrate embryo (Deschamps and Wijgerde, 1993; Gaunt and Strachan, 1996). It is possible that *Hox-Hox* interactions as reported here participate in establishing this colinear *Hox* expression sequence. Cell to cell relay of autoregulation of *Hox* gene expression and of induced 5' posterior *Hox* gene expression might mediate these propagating *Hox* expression waves and ensure genesis of their 3' anterior to 5' posterior nested sequence. Colinear repression of 3' anterior *Hox* genes might help mediate temporal colinearity by ensuring that the *Hox* wave sequence is unidirectional and irreversible and could limit overlap between successive *Hox* expression zones.

We suspect that a second role of non-cell-autonomous autoregulation and posterior induction is to help coordinate patterning so that the axial pattern emerges in register in different germ layers. In *Drosophila, Ubx* induced signalling does serve to coordinate axial patterning between two germ layers: endoderm and visceral mesoderm (Bienz, 1994). In vertebrates, *Hox* induced signalling could well help coordinate axial patterning between the neural plate and axial mesoderm. It may help mediate vertical signals from the axial mesoderm which imprint its axial pattern onto the developing neural plate, thus providing part of the mechanism of regional neural induction (neural transformation) and synchronising *Hox* waves in the axial mesoderm and neural plate. The idea that *Hox* patterns in axial mesoderm and the neural plate might be synchronised by non-cell-autonomous *Hox/Hox* interactions was

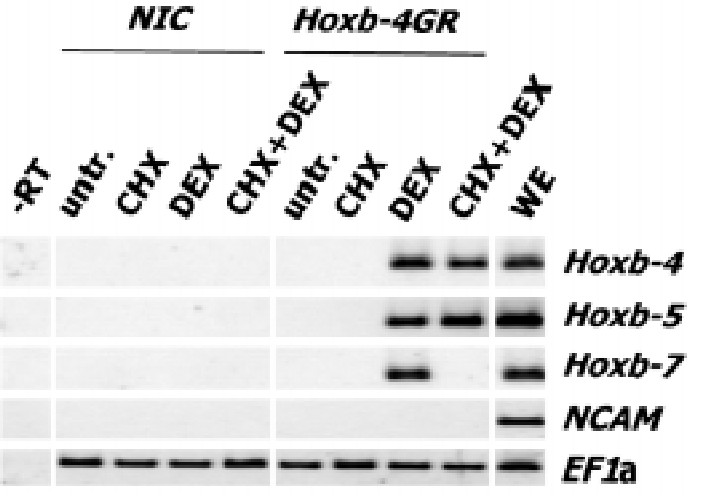

Fig. 8. Direct activation of *Hoxb-4* and the 5' posterior *Hox* gene, *Hoxb-5,* by a dexamethasone-inducible *Hoxb-4* glucocorticoid receptor (*Hoxb-4GR*) construct. *Xenopus zygotes were injected with 500 pg Hoxb-4GR mRNA and animal caps were cut at stage 9. Next, the ectodermal explants were preincubated for 30 min with or without cycloheximide (CHX), followed by 2 h treatment with or without dexamethasone (DEX). After total RNA extraction, RT-PCR was performed for 3 Xenopus 5' posterior Hox genes (Hoxb-4, Hoxb-5 and Hoxb-7), NCAM and EF1α (internal standard). -RT, control without reverse transcription. WE, RNA extracted from whole embryos. RT-PCR of explants of control embryos (NIC) showed no induction of any Hox genes or NCAM. Untreated or CHX-treated explants of Hoxb-4GR injected embryos revealed no induction of NCAM or any Hox genes. DEX- or DEX+CHX-treatment revealed that Hoxb-4 and Hoxb-5 are directly activated by Hoxb-4, whereas Hoxb-7 is an indirect Hoxb-4 target sensitive to the protein synthesis inhibitor, CHX. Hoxb-4GR is unable to either directly or indirectly (via mesoderm) induce the expression of the general neural marker, NCAM. Hoxb-9 expression was not observed (data not shown).*

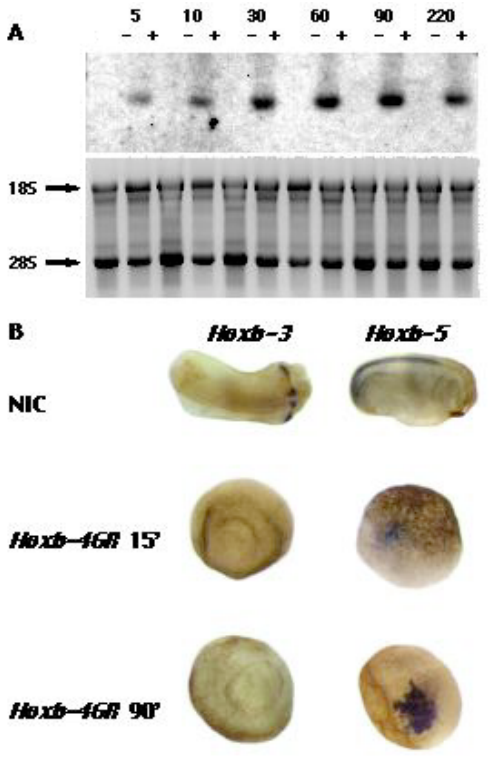

Fig. 9. Activation of *Hoxb-5* by *Hoxb-4GR* is quick and direct. (A, upper panel) *Xenopus zygotes were injected with 500 pg Hoxb-4GR mRNA (+) or not (-) and cultured up to stage 10.5. Next, the embryos were preincubated for 30 min with CHX and subsequently induced with DEX. Hoxb-3 and Hoxb-5 expression was analysed using Northern Blot Analysis 5, 10, 30, 60, 90 and 220 min after addition of DEX. Uninjected embryos (-) revealed no induction of Hoxb-3 and Hoxb-5. Hoxb-5 expression was quickly (5 min) and directly (CHX-insensitive) induced by Hoxb-4GR after DEX-treatment (+). No induction of Hoxb-3 could be detected (data not shown).* **(A, lower panel)** *Agarose gel (1xTBE, 20 mM GTC), used to run the total RNA, stained using Vistra Green (Amersham) and showing equal amounts of RNA loaded; arrows marking 18S and 28S ribosomal RNA bands.* **(B)** *One blastomere of 4-cell stage embryos was injected with 500 pg of Hoxb-4GR. Subsequently, pretreated with CHX and activated by addition of DEX. Again, Hoxb-4GR induced expression of Hoxb-5 was observed, insensitive to CHX treatment, 15 min after addition of DEX (Hoxb-4GR 15'). Activation of Hoxb-5 expression strongly increased after prolonged treatment with DEX (Hoxb-4GR 90'). No induction of Xenopus Hoxb-3 could be detected. Control embryos (NIC, Xhoxb-3 and Xhoxb-5) showed normal expression (Godsave et al., 1994).*

suggested previously by E. De Robertis and colleagues (De Robertis *et al.*, 1989). This issue is interesting, and deserves investigation.

Clearly, the overall picture is complex, but we believe that this area will richly reward further investigation.

Materials and Methods

Constructs

NogginΔ5' was transcribed from the appropriate linearised template as previously described (Smith and Harland, 1992). Details of the different *Hox* constructs used are in the legend to Figure 1. Capped mRNA was generated from the linearised templates using the appropriate T7 or Sp6 MessageMachine Kit (Ambion).

Embryos, explants and microinjection experiments

Albino and wild-type *Xenopus* embryos were obtained by *in vitro* fertilisation, dejellied and cultured as described previously (Godsave *et al.*, 1994). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Embryos were injected with 1 ng (*Xenopus Hoxb-4*, *Xb-4BglII600* or *Xenopus Hoxa-7*) or 500 pg *(Xenopus Hoxb-4GR)* synthetic *Hox* mRNA and/or 150 pg of nogginΔ5' mRNA, and/or 50 pg *LacZ* mRNA into the animal hemisphere during the first cell cycle, one blastomere at stage 3 or one of the b-ring blastomeres at stage 6 (Dale and Slack, 1987) in 4% ficoll/100% MMR (Newport and Kirschner, 1982). These messengers were injected alone or in combinations. Embryos were transferred into 1% ficoll/100% MMR for two hours and cultured in 10% MMR to appropriate stages. For animal cap experiments, stage 9 embryos were placed in 1xFlickinger medium (Flickinger, 1949) and ectodermal caps were cut. Subsequently, the caps were cultured in a 6-well plate (Costar), 15 caps per well, in 5 ml 1xFlickinger medium with or without 10^{-6} M all-trans retinoic acid (Acros). Dexamethasone (DEX) and cycloheximide (CHX) treatments were as previously described (Grainger and Gurdon, 1989; Gammill and Sive, 1997). Explants were cultured at 14º-18ºC until control embryos reached stage 18 and processed immediately for RT-PCR.

In situ hybridisation

Embryos at appropriate stages were fixed in MEMFA (0.1 M MOPS, 2 mM EDTA, 1 mM $MgSO_4$, 3.7% formaldehyde) for at least 2 h at room temperature, rocking on a nutator and were subsequently washed in 100% methanol and stored at -20ºC in fresh methanol. Digoxigenin-labelled anti-sense probes were generated by *in vitro* transcription of linearised templates, incorporating digoxigenin-11-UTP according to the manufacturers instructions (Boehringer Mannheim). Templates: *Hoxb-3* (Dekker *et al.*, 1993), *Hoxb-4* (Harvey *et al.*, 1986), *Hoxb-5* (Godsave *et al.*, 1994), *Hoxb-7* (Wright *et al.*, 1987) and *Hoxb-9* (Sharpe and Gurdon, 1990). The *in situ* hybridisation procedure was as described by Harland (Harland, 1991) with some minor modifications. Following staining and fixation in MEMFA, pigmented embryos were bleached by treatment with 0.1 M $K_2Cr_2O_7$ in 5% acetic acid for 30 min, followed by 3x10 min washes in PBSTw (PBS containing 0.1% Tween 20), and then bleaching in 4% H_2O_2 in PBSTw under a light source for 1-2 h.

Northern blot analysis

Total RNA from staged embryos was isolated using TriPure (Boehringer Mannheim). The Northern Blot Analysis was as previously described (Houtzager, 1998). Antisense DIG-labelled RNA probes for *Xenopus Hoxb-3* and *Hoxb-5* similar as for *in situ* hybridisation RNA probes. CDP-STAR chemofluorescent detection system (Promega) and X-Omat-AR (Kodak) were used for detection.

RT-PCR

Total RNA from staged embryos and animal caps was extracted using proteinase K and LiCl precipitation. Whole embryos or animal caps were homogenised in PK-buffer (50 mM NaCl, 50 mM Tris-HCl pH7.5, 5 mM EDTA, 1% SDS, 1% β-mercaptoethanol, 0.25 mg/ml proteinase K) and incubated at 50ºC for 1 h. Total RNA was extracted using phenol/chloroform and precipitation with ethanol.

PCR assays with reverse transcription (RT-PCR) were carried out in the exponential phase of amplification as described (Busse and Séguin, 1993), with some minor modifications. PCR primers used were *Xenopus Otx-2* (f:GGATGGATTTGTTGCACCAGTC; r:CACTCTCCGAGCTCACTTCTC), *Hoxb-1* (f:TTCCAGAACCGGAGAATGAAGC; r:TGAAGTTCCCCTGAGAGGATGG), *Hoxb-2* (f:CTCGAACCCCGAAGATGGG; r:TAACAAGGGGCTGCTGGGG), *Hoxb-3* (f:CCCCCCTTCTGCCTA-TCCC; r:GCAGTTTGGCCATTTCCAGC), *Hoxb-4* 3' UTR (f:CTGCGGTACAAAGGCTGAACCT; r:CAGGCCCCAAACTGTGTGATC), *Hoxb-5* (f:CACCCGGTACCAGACGCTG; r:CATGGGGAGGCGACTAGAAATG), *Hoxb-7* (f:AGGGTCGGACAGGAAGAGGG; r:GCGGTTCTGGAACCAGATTTTG), *Hoxb-8* (f:GTCTGGTACAATAGCCAG;

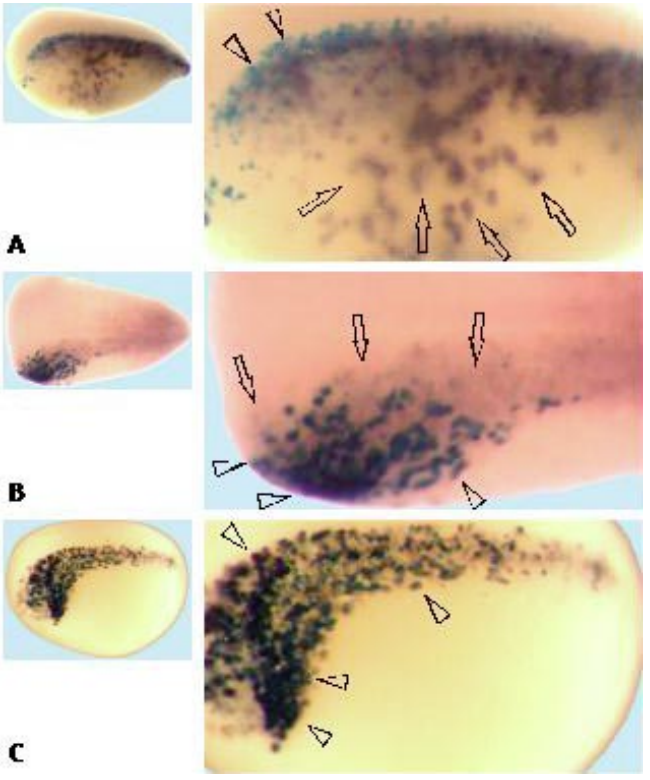

Fig. 10. Autoregulation and posterior induction by *Hoxb-4* are relayed from cell to cell. (A,B) *Stage 15 albino embryos (dorsolateral view, anterior left) previously injected with Xenopus Hoxb-4 mRNA (250 pg) and LacZ mRNA (50 pg) into one of the b-ring blastomeres at the 32-cell stage (Fig. 7A; Dale and Slack, 1987). After staining for LacZ, the embryos were probed for Xenopus Hoxb-4* **(A)** *and Hoxb-5.* **(B)** *Expression by in situ hybridisation. Ectopic Hoxb-4 expression* **(A,** *arrows***)** *could be detected far outside the LacZ staining domain (arrowheads), indicating that injected Hoxb-4 is able to induce expression of endogenous Hoxb-4 mRNA outside the lineage labelled domain containing injected mRNA. Hoxb-5* **(B)** *was also expressed ectopically (arrows) well outside the lineage labelled LacZ expressing domains (arrowheads). The entire patterns of expression were not easily photographed and weakly labelled cells were also detectable by direct observation outside the expression domains seen in the figures.* **(C)** *Stage 15 albino embryos (dorsolateral view, anterior left) previously injected with mRNA (250 pg) from the non-functional deletion construct of Xenopus Hoxb-4 (Xb-4BglII600) and LacZ mRNA (50 pg) into one of the b-ring blastomeres at the 32-cell stage (Dale and Slack, 1987). Hoxb-4 expression could only be detected in the sharply defined LacZ staining domain (arrowheads). No (induced) Hoxb-4 could be observed outside the LacZ labelled domain, in contrast with Figure 10A.*

r:GGTCA-CAGAAATCTGTCTAC) *Hoxb-9* (f:TACTTACGGGCTTGG-CTGGA; r:AGCGTGTAACCAGTTGGCTG), EF1α (f:CAGATTGGTGCT-GGATATGC; r:ACTGCCTTGATGACTCCTAG). *Otx-2*, *Hoxb-9* and EF1α (from internet at http://vize222.zo.utexas.edu).

Acknowledgments

We thank Drs. J. Deschamps, O. Destree, S. Godsave, W. Hennig, A. Otte and P. Pijnappel for critically reading the manuscript, and C. Koster, E. van den Akker, J. Narraway, T. Hoeijmaker, F. Vervoordeldonk, J. Heinen and W. Hage for excellent technical support, M. Molenaar and J. Slack for kindly providing the modified T7Ts vector and the Xenopus Hoxa-7 clone, respectively, and the E.U. Biotech and TMR programs for support.

References

APPEL, B. and SAKONJU, S. (1993). Cell-type-specific mechanisms of transcriptional repression by the homeotic gene products UBX and ABD-A in *Drosophila* embryos. *EMBO J. 12:* 1099-1109.

BERGSON, C. and McGINNIS, W. (1990). An autoregulatory enhancer element of the *Drosophila* homeotic gene *Deformed. EMBO J. 9:* 4287-4297.

BIENZ, M. (1994). Homeotic genes and positional signalling in the *Drosophila* viscera. *Trends Genet. 10:* 22-26.

BUSSE, U. and SÉGUIN, C. (1993). Molecular ananlysis of the *Wnt-1* proto-oncogene in *Ambystoma mexicanum* (axolotl) embryos. *Differentiation 53:* 7-15.

CAPECCHI, M.R. (1997). *Hox* genes and mammalian development. *Cold Spring Harbor Symp. Quant. Biol. LXII:* 273-281.

CARROLL, S.B. (1995). Homeotic genes and the evolution of arthropods and chordates. *Nature 376:* 479-485.

CARROLL, S., LAYMON, R., MCCUTCHEON, M., RILEY, P. and SCOTT, M. (1986). The localisation and regulation of *Antennapedia* gene expression in *Drosophila* embryos. *Cell 47:* 113-122.

CHOUINARD, S. and KAUFMAN, T.C. (1991). Control of expression of the *labial* (*lab*) locus of *Drosophila melanogaster*: evidence for both positive and negative autogenous regulation. *Development 113:* 1267-1280.

CHRISTEN, B. and BIENZ, M. (1992). A *cis*-element mediating *Ultrabithorax* autoregulation in the central nervous system. *Mech. Dev. 39:* 73-80.

CONDIE, B.G. and HARLAND, R. (1987). Posterior expression of a homeobox gene in the early *Xenopus* embryo. *Mech. Dev. 39:* 73-80.

DALE, L. and SLACK J.M.W. (1987). Fate map for the 32-cell stage of *Xenopus laevis. Development 99:* 527-551.

DE ROBERTIS, E.M., OLIVER, G. and WRIGHT, C.V. (1989). Determination of axial polarity in the vertebrate embryo: homeodomain proteins and homeogenetic induction. *Cell 57:* 189-191.

DEKKER, E-J., PANNESE, M., HOUTZAGER, E., BONCINELLI, E. and DURSTON, A. (1993). Colinearity in the *Xenopus laevis* Hox-2 complex. *Mech. Dev. 40:* 3-12.

DESCHAMPS, J. and WIJGERDE, M. (1993). Two phases in the establishment of *HOX* expression domains. *Dev. Biol. 156:* 473-480.

DUBOULE, D. and MORATA, G. (1994). Colinearity and functional hierarchy among genes of the homeotic complexes. *Trends. Genet 10:* 358-364.

DURSTON, A.J., TIMMERMANS, J.P.M., HAGE, W.J., HENDRIKS, H.F.J., DE VRIES, N.J., HEIDEVELD, M. and NIEUWKOOP, P.D. (1989). Retinoic acid causes an anteroposterior transformation in the developing central nervous system. *Nature 340:* 140-144.

FAIELLA, A., ZAPPAVIGNA, V., MAVILIO, F. and BONCINELLI, E. (1994). Inhibition of retinoic acid-induced activation of 3' human *HOXB* genes by antisense oligonucleotides affects sequential activation of genes located upstream in the four *HOX* clusters. *Proc. Natl. Acad. Sci. USA 91:* 5335-5339.

FLICKINGER, R.A. (1949). A study of the metabolism of amphibian neural crest cells during their migration in pigmentation *in vitro. J. Exp. Zool. 122:* 165-185.

FRITZ, A. and DE ROBERTIS, E.M. (1988). *Xenopus* homeobox-containing cDNAs expressed in early development. *Nucleic Acids Res. 16:* 1453-1469.

GAMMILL, L.S. and SIVE, H. (1997). Identification of *Otx2* target genes and restrictions in ectodermal competence during *Xenopus* cement gland formation. *Development 124:* 471-481.

GAUNT, S.J. and STRACHAN, L. (1996). Temporal colinearity in expression of anterior *Hox* genes in developing chick embryos. *Dev. Dyn. 207:* 270-280.

GODSAVE, S., DEKKER, E-J., HOLLING, T., PANNESE, M., BONCINELLI, E. and DURSTON, A. (1994). Expression patterns of *Hoxb* genes in the *Xenopus* embryo suggest roles in anteroposterior specification of the hindbrain and in dorsoventral patterning of the mesoderm. *Dev. Biol. 166:* 465-476.

GODSAVE, S.F., KOSTER, C.H., GETAHUN, A., MATHU, M., HOOIVELD, M., VAN DER WEES, J., HENDRIKS, J. and DURSTON, A.J. (1998). Graded retinoid responses in the developing hindbrain. *Dev. Dyn. 213:* 39-49.

GONZÁLEZ-REYES, A., MACIAS, A. and MORATA, A.M. (1992). Autocatalysis and phenotypic expression of *Drosophila* homeotic gene *Deformed*: its dependence on polarity and homeotic gene function. *Development 116:* 1059-1068.

GOULD, A., MORRISON, A., SPROAT, G., WHITE, R.A.H. and KRUMLAUF, R. (1997). Positive cross-regulation and enhancer-sharing : two mechanisms for specifying overlapping *Hox* expression patterns. *Genes Dev. 11:* 900-913.

GRAINGER, R.M. and GURDON, J.B. (1989). Loss of competence in amphibian induction can take place in single nondividing cells. *Proc. Natl. Acad. Sci. USA 86:* 1900-1904.

HARLAND, R. (1991). *In situ* hybridisation: an improved whole mount method for *Xenopus* embryos. *Methods Cell Biol. 36:* 685-695.

HARVEY, R.P. and MELTON, D.A. (1988). Microinjection of synthetic *Xhox-1A* homeobox mRNA disrupts somite formation in developing *Xenopus* embryos. *Cell 53:* 687-697.

HARVEY, R.P., TABIN, C.J. and MELTON, D.A. (1986). Embryonic expression and nuclear localization of *Xenopus* homeobox *(Xhox)* gene products. *EMBO J. 5:* 1237-1244.

HOUTZAGER, E. (1998). *The role of connexins and the function of the dorsal endoderm in embryogenesis of Xenopus laevis*. Thesis University of Utrecht.

JONES, E.A. and WOODLAND, H.R. (1989). Spatial aspects of neural induction in *Xenopus laevis*. *Development 107*: 785-791.

KINTNER, C.R. and BROCKES, J.P. (1984). Monoclonal antibodies identify blastemal cells derived from dedifferentiated muscle in newt limb regeneration. *Nature 308:* 67-69.

KOLM, P.J. and SIVE, H.L. (1995). Efficient hormone-inducible protein function in *Xenopus laevis*. *Dev. Biol. 171:* 267-272.

KOLM, P.J. and SIVE, H.L. (1997). Retinoids and posterior neural induction: a reevaluation of Nieuwkoop's two-step hypothesis. *Cold Spring Harbor Symp. Quant. Biol. 62:* 511-521.

KUZIORA, M.A. and McGINNIS, W. (1988). Autoregulation of a *Drosophila* homeotic selector gene. *Cell 55:* 477-485.

LAMB, T.M., KNECHT, A.K., SMITH, W.C., STACHEL, S.E., ECONOMIDES, A.N., STAHL, N., YANCOPOLOUS, G.D. and HARLAND R. (1993). Neural induction by the secreted polypeptide noggin. *Science 262:* 713-718.

LEWIS, E.B. (1998). The bithorax complex: the first 50 years. *Int. J. Dev. Biol. 42:* 403-415.

MACONOCHIE, M.K., NONCHEV, S., STUDER, M., CHAN, S-K., POPPERL, H., HAR SHAM, M., MANN, R.S. and KRUMLAUF, R. (1997). Cross-regulation in the mouse *hoxb* complex*:* the expression of *hoxb2* in rhombomere 4 is regulated by *hoxb1. Genes Dev. 11:* 1885-1895.

MAVILIO, F., SIMEONE, A., GIAMPAOLO, A., FAIELLA, A., ZAPPAVIGNA, V., ACAMPORA, D., POIANA, G., RUSSO, G., PESCHLE, C. and BONCINELLI, E. (1986). Differential and stage-related expression in embryonic tissues of a new human homoeobox gene. *Nature 324:* 664-668.

McGINNIS, W. (1994). A century of homeosis, a decade of homeoboxes. *Genetics 137:* 607-611.

NEWPORT, J. and KIRSCHNER, M. (1982). A major developmental transition in early *Xenopus* embryos : 1. characterization and timing of cellular changes at the midblastula stage. *Cell 30:* 675-686.

NIEUWKOOP, P.D. and FABER, J. (1967). *The normal table of Xenopus laevis (Daudin).* Second Edition. North Holland Publishing Co. Amsterdam.

NONCHEV, S., MACONOCHIE, M., GOULD, A., MORRISON, A. and KRUMLAUF, R. (1997). Cross-regulatory interactions between *Hox* genes and the control of segmental expression in the vertebrate central nervous system. *Cold Spring Harb. Symp. Quant. Biol. 62:* 313-323.

PANNESE, M., POLO, C., ANDREAZZOLI, M., VIGNALI, R., KABLAR, B., BARSACCHI, G. and BONCINELLI, E. (1995). The *Xenopus* homologue of *Otx2* is a maternal homeobox gene that demarcates and specifies anterior body regions. *Development 113:* 707-720.

POWNALL, M.E., TUCKER, A.S., SLACK, J.M.W. and ISAACS, H.V. (1996). *eFGF*, Xcad3 and *Hox* genes form a molecular pathway that establishes the anteroposterior axis in *Xenopus*. *Development 122:* 3881-3892.

RUDDLE, F.H., BARTELS, J.L., BENTLEY, K.L., KAPPEN, C., MURTHA, M.T. and PENDLETON, J.W. (1994). Evolution of *Hox* genes. *Annu. Rev. Genet. 28:* 423-442.

RUIZ I ALTABA, A. (1992). Planar and vertical signals in the induction and patterning of the *Xenopus* nervous system. *Development 115:* 67-80.

SHARKEY, M., GRABA, Y. and SCOTT, M.P. (1997). *Hox* genes in evolution: protein surfaces and paralog groups. *Trends Genet. 13:* 145-151.

SHARPE, C.R. and GURDON, J.B. (1990). The induction of anterior and posterior neural genes in *Xenopus laevis*. *Development 109:* 765-774.

SHARPE, J., NONCHEV, S., GOULD, A., WHITING, J. and KRUMLAUF, R. (1998). Selectivity, sharing and competitive interactions in the regulation of *hoxb* genes. *EMBO J. 17*: 1788-1798.

SMITH, W.C. and HARLAND, R.M. (1992). Expression cloning of noggin, a new dorsalizng factor localized to the Spemann organizer in *Xenopus* embryos. *Cell 70:* 829-840.

STRUHL, G. and WHITE, R. (1985). Regulation of the *Ultrabithorax* gene of *Drosophila* by other bithorax complex genes. *Cell 43:* 507-519.

STUDER, M., GAVALAS, A., MARSHALL, H., ARIZA-MCNAUGHTON, L., RIJLI, F.M., CHAMBON, P. and KRUMLAUF, R. (1998). Genetic interactions between *hoxa1* and *hoxb1* reveal new roles in regulation of early hindbrain patterning. *Development 125:* 1025-1036.

WIRZ, J., FESSLER, L. and GEHRING, W. (1986). Localisation of the Antennapedia protein in *Drosophila* embryos and imaginal discs. *EMBO J. 5:* 3327-3334.

WRIGHT, C.V.E., CHO, K.W.Y., FRITZ, A., BURGLIN, T.R. and DE ROBERTIS, E.M. (1987). A *Xenopus laevis* gene encodes both homeobox-containing and homeobox-less transcripts. *EMBO J. 6:* 4083-4094.

WU, K. and WOLGEMUTH, D.J. (1993). Protein product of the somatic-type transcript of the *Hoxa-4* (*Hox-1.4*) gene binds the homeobox consensus binding sites in its promoter and intron. *J. Cell. Biochem. 52:* 449-462.