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Regulation of Hox gene expression and posterior development by the *Xenopus* caudal homologue Xcad3

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The *caudal* gene codes for a homeodomain transcription factor that is required for normal posterior development in *Drosophila*. In this study the biological activities of the *Xenopus* caudal (Cdx) family member *Xcad3* are examined. A series of domain-swapping experiments demonstrate that the N-terminus of Xcad3 is necessary for it to activate Hox gene expression and that this function can be replaced by the activation domain from the viral protein VP16. In addition, experiments using an Xcad3 repressor mutant (XcadEn-R), which potently blocks the activity of wild-type Xcad3, are reported. Overexpression of XcadEn-R in embryos inhibits the activation of the same subset of Hox genes that are activated by wild-type Xcad3 and leads to a dramatic disruption of posterior development. We show that *Xcad3* is an immediate early target of the FGF signalling pathway and that Xcad3 posteriorizes anterior neural tissue in a similar way to FGF. Furthermore, Xcad3 is required for the activation of Hox genes by FGFs. These data provide strong evidence that Xcad3 is required for normal posterior development and that it regulates the expression of the Hox genes downstream of FGF signalling.

Keywords: anteroposterior/caudal/Cdx/FGF/Hox/*Xenopus*

Introduction

The prototype of the Cdx family of homeodomain transcription factors is the *Drosophila* caudal protein. The initial maternal expression of *caudal* mRNA is ubiquitous and a posterior to anterior gradient of the protein develops during the syncytial blastoderm stage and persists until the onset of cellularization. Zygotic expression, which commences in the cellular blastoderm stage, is also localized to the posterior in a region which gives rise to terminal abdominal structures and the hindgut. During later embryonic development, expression of *caudal* is found in the midgut, hindgut and Malpigian tubules (MacDonald and Struhl, 1986; Mlodzik and Gehring, 1987).

Caudal homologues have been identified in a wide range of animal groups. A *caudal*-related gene with a similar posterior expression pattern has been cloned from the short or intermediate germ band insect *Bombyx mori* and homologues are present in other invertebrates, including the nematode worm *Caenorhabditis elegans* and the annelid worm *Ctenodrilus serratus* (Dick and Buss, 1994; Xu *et al.*, 1994; Hunter and Kenyon, 1996). In common with many genes which are involved in the development of *Drosophila*, *caudal* homologues have also been found in vertebrates. At present, three *caudal*-related (Cdx) genes have been identified in the genomes of *Xenopus*, chick and mouse (for discussion see Marom *et al.*, 1997). Analysis of the expression patterns of the vertebrate Cdx genes reveals striking similarities with that of *Drosophila*. For example, the initial expression of the mouse *Cdx4* gene is in a posterior to anterior gradient within the developing axis during primitive streak stages, and it later continues to be expressed in the posterior neuroectoderm, presomitic mesoderm and endoderm of the hindgut (Gamer and Wright, 1993).

Functional studies in genetically tractable organisms as diverse as *Drosophila* and mouse demonstrate an evolutionarily conserved role for *caudal* genes in the development of posterior structures. Zygotic *caudal* mutants in *Drosophila* lack anal tufts and parts of the anal pads. If the maternal component of *caudal* expression is also missing, more severe abdominal segmentation defects are apparent (MacDonald and Struhl, 1986; Kuhn *et al.*, 1995). In mice, null alleles have been generated for the *Cdx1* and *Cdx2* genes. Animals homozygous for a null allele of *Cdx1* are viable but show anterior homeotic transformation of vertebral identity (Subramanian *et al.*, 1995). Mice homozygous for a *Cdx2* null allele die in embryonic life (3.5–5.5 days post coitum) while heterozygotes survive and show anterior shifts in vertebral identity and exhibit malformations of tail development (Chawengsaksophak *et al.*, 1997). The vertebral transformations in *Cdx1* mutant mice are associated with posterior shifts in the normal anterior expression boundaries of a number of Hox genes, suggesting that the Cdx proteins are involved in the normal regulation of these genes.

In this study we have examined the function of the *Xenopus* caudal homologue Xcad3 during early development. A series of domain swapping experiments have been carried out with Xcad3 which identify the N-terminus of the protein as being crucial for its ability to activate the transcription of Hox genes and that this activating function can be replaced by an exogenous activation domain. An Xcad3 repressor mutant (XcadEn-R) was also constructed which strongly inhibits the activity of wild-type Xcad3. Overexpression of XcadEn-R blocks the normal activation of the same subset of Hox genes which are activated by wild-type Xcad3 and results in a dramatic inhibition of trunk and tail development.

The FGF family of signalling molecules have been implicated in the process of anteroposterior specification.

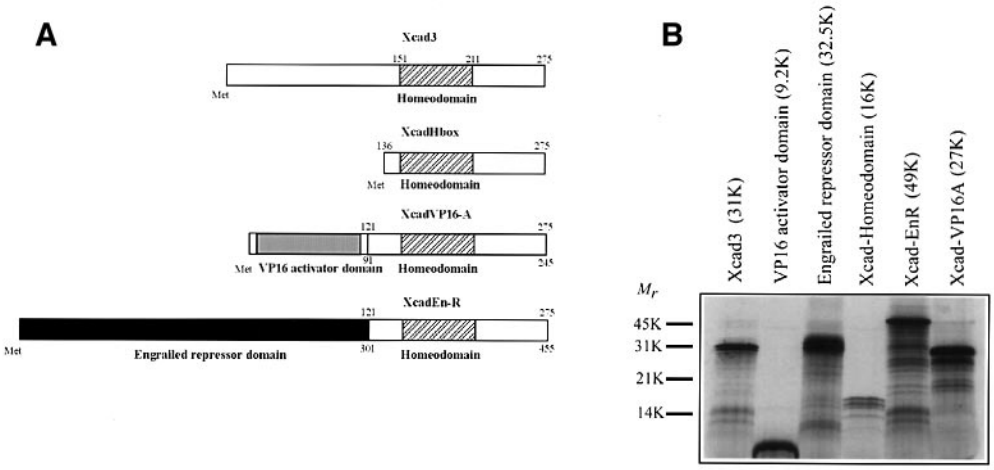

Fig. 1. *Xcad3* constructs. (**A**) A schematic diagram of the Xcad3 constructs used in this study. Numbers on top denote amino acid number of sequence derived from Xcad3. The numbers on the bottom are the position within the fusion protein. (**B**) An autoradiograph of a 15% SDS–PAGE gel showing the products of *in vitro* translation carried out on synthetic mRNA derived from the *Xcad3* constructs used in this study. The predicted molecular weight of each protein derived from conceptual translation is shown above each lane.

Inhibition of the FGF signal transduction pathway during early development leads to a dramatic disruption of posterior development (Amaya *et al.*, 1993). FGF treatment of neuralized explants activates Hox gene expression (Cox Wm and Hemmati-Brivanlou, 1995; Lamb and Hartland, 1995). Here we show that *Xcad3* is an immediate early target of the FGF signalling pathway and that *Xcad3* is able to activate Hox gene expression in neural tissue of anterior character in a similar way to that which has been shown for FGF. Furthermore, Xcad3 activity is required for the activation of Hox genes by the FGF signalling pathway. These data provide strong evidence that Cdx proteins play a crucial role in regulating the normal expression of the Hox genes and are required for posterior specification within the axis of the developing vertebrate embryo.

Results

The N-terminal region of Xcad3 is required for transcriptional activation

It has been shown previously that injection of synthetic *Xcad3* mRNA is able to activate the transcription of *HoxA7* in *Xenopus* embryos (Pownall *et al.*, 1996). Here we show that the N-terminal region of the Xcad3 protein is required for the transcriptional activation of *HoxA7 in vivo*, and that this activating function can be replaced by fusing the C-terminal regions of Xcad3, containing the homeodomain, with the activation domain from the *Herpes simplex* VP16 protein. Figure 1A shows schematic diagrams of the *Xcad3*-based constructs used in this study. The molecular weight of proteins produced from synthetic mRNAs coding for these fusions was confirmed by *in vitro* translation (Figure 1B). Figure 2 shows the results of an RNase protection analysis and demonstrates that injection of 100 pg *Xcad3* mRNA or as little as 50 pg mRNA coding for XcadVP16-A, in which the N-terminus has been replaced by the activation domain from the VP16 protein, leads to the precocious activation of *HoxA7* expression at the early gastrula stage 10.5. However, the injection of as much as 400 pg mRNA coding for an

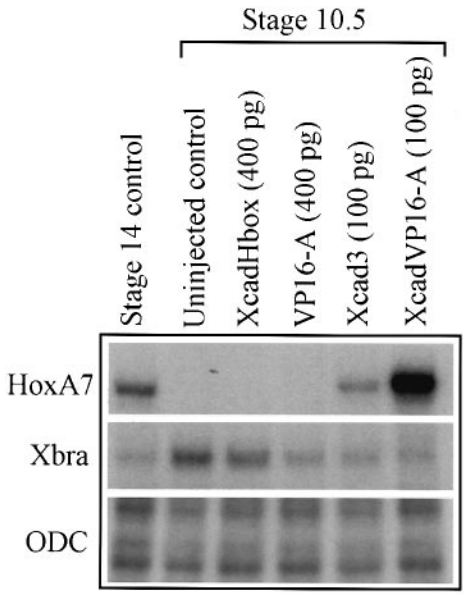

Fig. 2. XcadVP16-A potently activates Hox gene expression. An autoradiograph of an RNase protection analysis gel carried out on gastrula stage 10.5 embryo (5 μg total RNA/hybridization) following injection with synthetic mRNA coding for the Xcad3 homeodomain (XcadHbox), the VP16 *Herpes simplex* transcriptional activation domain (VP16-A), wild-type Xcad3 (Xcad3) and a fusion of VP16-A and the Xcad homeodomain (XcadVP16-A).

N-terminal truncation of Xcad3 (XcadHbox), which still contains the whole of the homeodomain, is unable to activate this expression, indicating that the N-terminus is required for the activity of Xcad3. The effect of XcadVP16-A on *HoxA7* expression is specific and does not result from a general increase in transcription because injecting it does not lead to increased expression of the control mRNAs *Xbra* and *ornithine decarboxylase* (*ODC*).

XcadVP16-A overexpression produces anterior truncations

The phenotype that results from XcadVP16-A overexpression was examined. Figure 3A shows normal uninjected

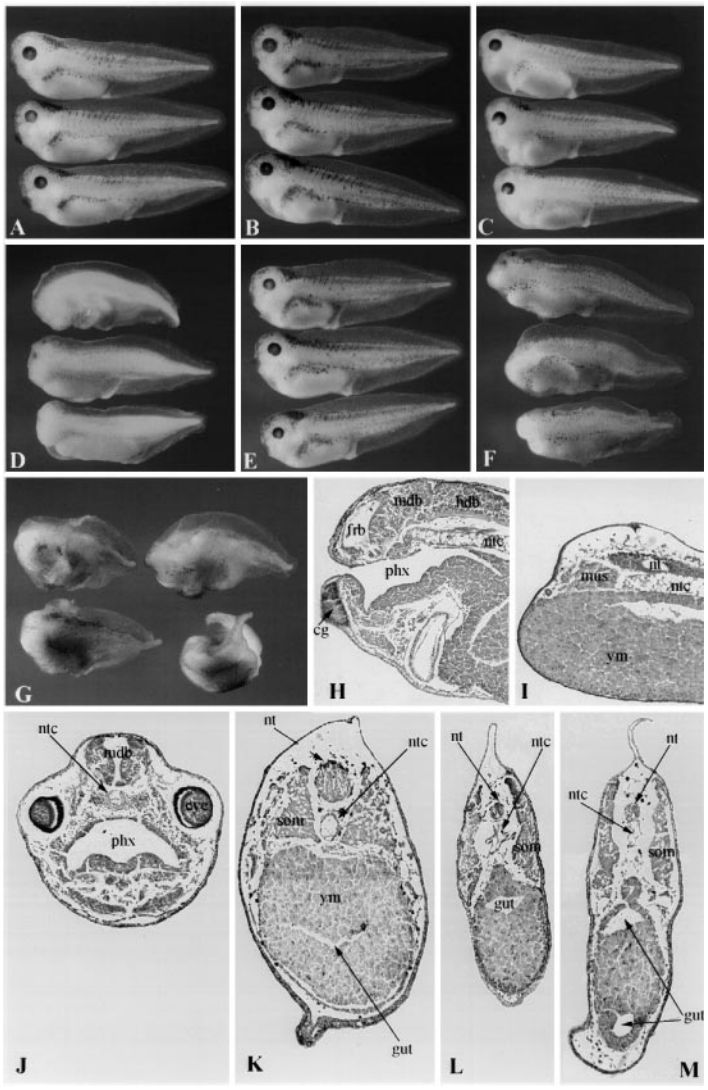

Fig. 3. Overexpression of *Xcad3* or XcadVP16-A causes anterior truncations. (A–G) Embryos at larval stage 40. (**A**, **B** and **C**) Uninjected control embryos, embryos injected with 400 pg of XcadHbox homeodomain control mRNA and embryos injected with 400 pg of VP16-A activator domain mRNA, respectively. (**D**, **E**, **F** and **G**) Embryos injected with 50 pg of XcadVP16-A, 50, 100 and 200 pg of *Xcad3* mRNA, respectively. (H–M) Histological section of stage 40 embryos. (**H**) A sagittal section through the anterior of a normal control embryo injected with 400 pg of XcadHbox. (**I**) A sagittal section through the anterior of an anteriorally truncated embryo injected with 120 pg of Xcad3. (**J**) A transverse section through the anterior of an embryo injected with 400 pg of XcadHbox. (**K**) A transverse section through the anterior of an embryo injected with 60 pg of XcadVP16-A mRNA. (**L**) A transverse section through the posterior trunk of an embryo injected with 400 pg of XcadHbox. (**M**) A transverse section through the posterior trunk of an embryo injected with 60 pg XcadVP16-A. cg, cement gland; frb, forebrain; hdb, hindbrain; mus, muscle; mdb, midbrain; nt, neural tube; ntc, notochord; phx, pharynx; som, somite; ym, yolk mass.

control embryos at larval stage 40. Most embryos injected with 400 pg XcadHbox (21 out of 29) (Figure 3B) or 400 pg VP16-A activator domain mRNA (22 out of 27) (Figure 3C) also develop normally. However, the injection of 50 pg of XcadVP16-A mRNA results in a high proportion of embryos with anterior truncations (28 out of 33) (Figure 3D), which either completely lack eyes and cement gland or have a marked reduction of these structures. This phenotype is very similar to that produced by overexpression of wild-type Xcad3.

Xcad3 overexpression reveals sharp thresholds in effects on axial development

Figure 3E, F and G shows the phenotypes of embryos resulting from the injection of *Xcad3* mRNA in the concentration range 50–200 pg. These data show that small differences in the amount of injected *Xcad3* mRNA can lead to dramatic differences in the effects on axial development. For example, 25 out of 32 embryos injected with 50 pg *Xcad3* mRNA have completely normal axial development, which is comparable with uninjected controls (21 out of 26) (Figure 3E). A 2-fold increase in the amount of injected mRNA (100 pg) results in many embryos either lacking both cement gland and eyes (11 out of 27) or having varying degrees of reduction in these structures with a concomitant tendency towards cyclopia (Figure 3F). In the majority of cases (25 out of 27) the development of trunk and tail structures is relatively normal. A further 2-fold increase of injected mRNA (200 pg) results in many embryos (18 out of 32) with extreme anterior truncations and serious effects on the posterior axis, including shortening of the embryo and disruption of normal somite patterning (Figure 3G). Eleven out of 32 embryos exhibit an almost complete absence of

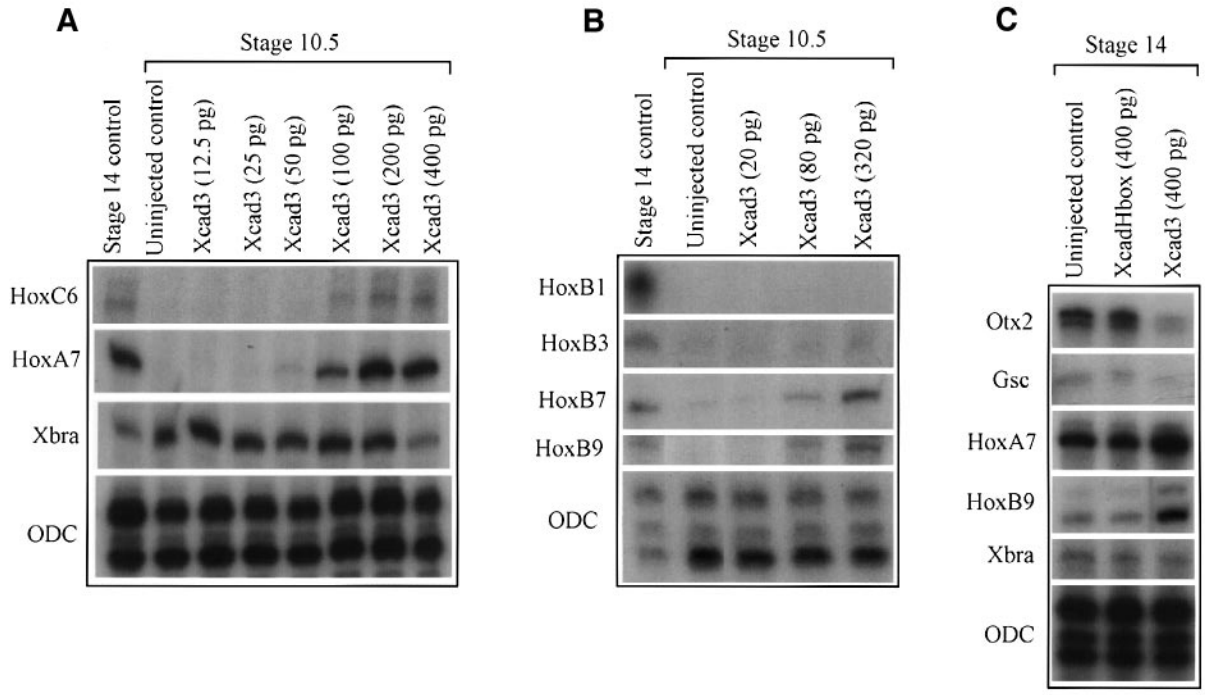

Fig. 4. Effects of *Xcad3* overexpression on anteroposterior gene expression. (**A** and **B**) RNase protection analysis for the precocious activation of a number of Hox genes carried out on early gastrula stage 10.5 embryos injected with different amounts of *Xcad3* mRNA (3 μg of total RNA/hybridization derived from embryos in the same experiment). Early neurula stage 14 embryos provide a positive control. (**C**) RNase protection analysis for the effects on the normal expression of several anteroposterior markers at early neurula stage 14 following injection with *Xcad3* mRNA (5 μg of total RNA/hybridization derived from embryos in the same experiment). The ODC loading control probe was used in each hybridization; the sets shown are representative.

axial development along with varying degrees in failure of the blastopore to close. A further 2-fold increase to 400 pg of *Xcad3* mRNA results in almost all embryos failing to gastrulate (data not shown).

Histological analysis of embryos injected with either *Xcad3* or XcadVP16-A mRNA reveals the complete absence of normal anterior structures. Figure 3H is a sagittal section through the head of a control embryo injected with 400 pg XcadHbox mRNA. The appearance is normal; note the presence of the cement gland, forebrain, midbrain and hindbrain, and the stomodeal opening of the pharynx. Figure 3I is a sagittal section through the anterior region of an embryo that has been injected with 120 pg of *Xcad3* mRNA. There is no development of brain ventricles and the remaining nervous system consists of a simple neural tube sitting dorsally to the notochord. The stomodeum is absent and there is no external opening of the foregut.

Figure 3J is a transverse section through the head of an XcadHbox-injected control embryo at the level of the anterior-most extent of the notochord, corresponding to the region of the midbrain–hindbrain junction. Note the presence of the well-differentiated eyes, brain ventricle overlying the notochord and large pharyngeal cavity. A transverse section at the level of the anterior-most extent of the notochord in an embryo injected with 60 pg XcadVP16-A mRNA is quite different (Figure 3K) and the appearance is more typical of that seen in trunk region of a control embryo (Figure 3L). Note the paired somites either side of the notochord, which is overlain by a simple neural tube. In contrast, a section through the posterior trunk region of an XcadVP16-A-injected embryo shows that in this region the pattern of the embryo is relatively unaffected (Figure 3M). The normal arrangement of neural tube, notochord and paired somites is shown in a section through the posterior trunk region of an XcadHbox injected control (Figure 3L).

It is significant that even in embryos showing severe anterior truncation following overexpression of *Xcad3*, the dorsoventral pattern of neural tube, notochord and somites of the trunk is normal. This indicates that these anterior truncations arise from an interference with anteroposterior patterning as opposed to the anterior truncations that result from UV light-induced perturbations during the establishment of dorsoventral polarity. A similar distinction has recently been drawn for effects on anteroposterior pattern that arise from the blastocoel injection of sulfatase (Wallingford *et al.*, 1997).

Xcad3 activates expression from a subset of Hox genes

We examined which Hox genes are activated by Xcad3 and the effects of *Xcad3* dose on Hox gene expression. There are suggestions from other organisms that the concentration of a given Cdx protein within a region of the embryo is significant. In *Drosophila*, a posterior to anterior gradient of caudal protein in blastoderm stages is important for the normal development of posterior structures (Kuhn *et al.*, 1995). A similar graded expression of both mRNA and protein along the developing anteroposterior axis has been noted for murine *Cdx4* (Gamer and Wright, 1993) and for *Xcad3* mRNA (Pownall *et al.*,

1996), although it is important to note that the gradient of *Cdx4* and *Xcad3* expression is within the context of the multicellular vertebrate embryo as opposed to the multinucleate syncytium of the early *Drosophila* embryo.

The precocious activation of Hox genes was examined in early gastrula embryos which have been injected with different concentrations of *Xcad3* mRNA. Figure 4A and B shows the results of the RNase protection analysis carried out on early gastrula stage 10.5 embryos that were injected with *Xcad3* mRNA in the concentration range 20–400 pg. Early neurula stage 14 embryos were used as controls for the normal expression of these genes. These data show that injection of 50 pg or more of *Xcad3* mRNA leads to activation of *HoxC6* and *HoxA7*, which are normally expressed in both the mesoderm and neuroectoderm, and *HoxB7* and *HoxB9*, which are expressed predominantly in the neuroectoderm. However, not all Hox genes behave in the same way in this assay system; at all concentrations tested, no upregulation of *HoxB1* and *HoxB3* was seen in response to Xcad3 (Figure 4B). Also note that Xcad3 does not upregulate the expression of the general mesodermal marker *Xbra*, indicating that it does not induce the formation of ectopic mesoderm.

There is a steep dose-response for the activation of the Xcad3 sensitive Hox genes (Figure 4A). A 2-fold increase in injected *Xcad3* mRNA (from 50 to 100 pg) leads to a marked increase in the expression of both *HoxA7* and *HoxC6*. Such a steep dose-response may in part explain the sharp threshold in phenotypic response resulting from *Xcad3* overexpression noted above. The present data, however, give no indication that different concentrations of Xcad3 are able to activate different cohorts of Hox gene expression.

Xcad3 expression overlaps with that of trunk Hox genes

The dichotomy in the response of Hox genes is in keeping with their normal expression patterns. *HoxA7*, *HoxB7* and *HoxB9* all have anterior limits of expression within the trunk and thus have considerable overlaps of expression with that of *Xcad3*. For example, Figure 5A and B shows double whole-mount *in situ* hybridizations for *Xcad3* and *HoxA7* at late gastrula stage 12.5 and early tailbud stage 21. These show that the expression domains of *Xcad3* and *HoxA7* are very similar both in the posterior region of the embryo, around the closed blastopore, and in the spinal cord. In contrast, the double *in situ* hybridizations in Figure 5C and D show that at the early tailbud stage 21 *HoxB1* and *HoxB3* are most highly expressed as stripes within the hindbrain in rhombomeres 3 and 4, respectively, and thus do not overlap with the expression of *Xcad3*. Note that in the double *in situ* hybridizations, Hox gene expression is magenta and *Xcad3* expression is light blue. Regions of overlapping expression are dark blue.

Xcad3 overexpression extends the anterior boundaries of expression from trunk Hox genes

Xcad3 overexpression leads to a dramatic upregulation of expression from a subset of Hox genes with anterior boundaries of expression within the trunk but the RNase protection data do not reveal whether this upregulation results from ectopic gene expression or an upregulation within the normal domains of expression. To investigate this we have looked at the effects on the spatial expression of *HoxA7* and *HoxB9* at early neurula stage 14 and early tailbud stage 20 which result from the injection of 150 pg of *Xcad3* mRNA. This amount of injected *Xcad3* mRNA consistently results in a high percentage of anterior truncations without adverse effects on blastopore closure. Figure 5E, F, G and H shows the expression of *HoxA7* in control and injected embryos. It is evident that the anterior and lateral extent of *HoxA7* expression is dramatically extended at stage 14 in *Xcad3* injected embryos. After the closure of the neural folds at stage 20, the anterior boundary of *HoxA7* expression within the neural tube of injected embryos remains considerably extended. Similar effects can be seen on the anterior extent of *HoxB9* expression (Figure 5I, J, K and L). It is important to note that the effects on both *HoxA7* and *HoxB9* expression represent extensions from their normal expression domains rather than a generalized upregulation of their expression in all regions of the embryo.

Overexpression of Xcad3 suppresses hindbrain Hox gene expression

Xcad3 overexpression results in the loss of all head structures up to and including the hindbrain. The hindbrain represents the most anterior region in which Hox gene expression can be found in normal development. We have used *HoxB1* (Figure 5M, N, O and P) and *HoxB3* (Figure 5Q, R, S and T) as markers of hindbrain development in embryos injected with *Xcad3* mRNA. These results again contrast the response of Hox genes from different paralogue groups to *Xcad3* overexpression. Hox genes with anterior limits of expression within the trunk, such as *HoxA7* and *HoxB9*, are upregulated, whereas hindbrain Hox genes, such as *HoxB1* and *HoxB3*, are downregulated in response to Xcad3 overexpression.

Both *HoxB1* and *HoxB3* are expressed in neural crest cells as they exit from rhombomeres 3 and 4, respectively. Interestingly, Hox gene expression in the migrating crest persists even in the absence of expression within the hindbrain region (Figure 5S and T), suggesting different mechanisms for regulating Hox gene expression within the hindbrain and migratory neural crest cells.

Xcad3 suppresses anterior gene expression

The ability of Xcad3 to upregulate ectopic expression of trunk Hox genes in anterior regions suggests one mechanism that could account for the perturbations in anterior development. However, *Xcad3* overexpression also leads to a downregulation of some gene expression within the hindbrain. We have looked to see whether Xcad3 also affects the early expression of genes directly involved in the development of structures more anterior to the hindbrain. Figure 4C is an RNase protection analysis at the early neurula stage 14 and shows that injection of 400 pg *Xcad3* mRNA results in a significant down regulation of *otx2* and *gsc* expression, both of which are believed to play crucial roles in anterior development (Blum *et al.*, 1992; Pannese *et al.*, 1995; Ang *et al.*, 1996).

Xcad3 can be converted into a transcriptional repressor (XcadEn-R)

Many transcription factors have a modular functional domain structure, with distinct regions of the protein

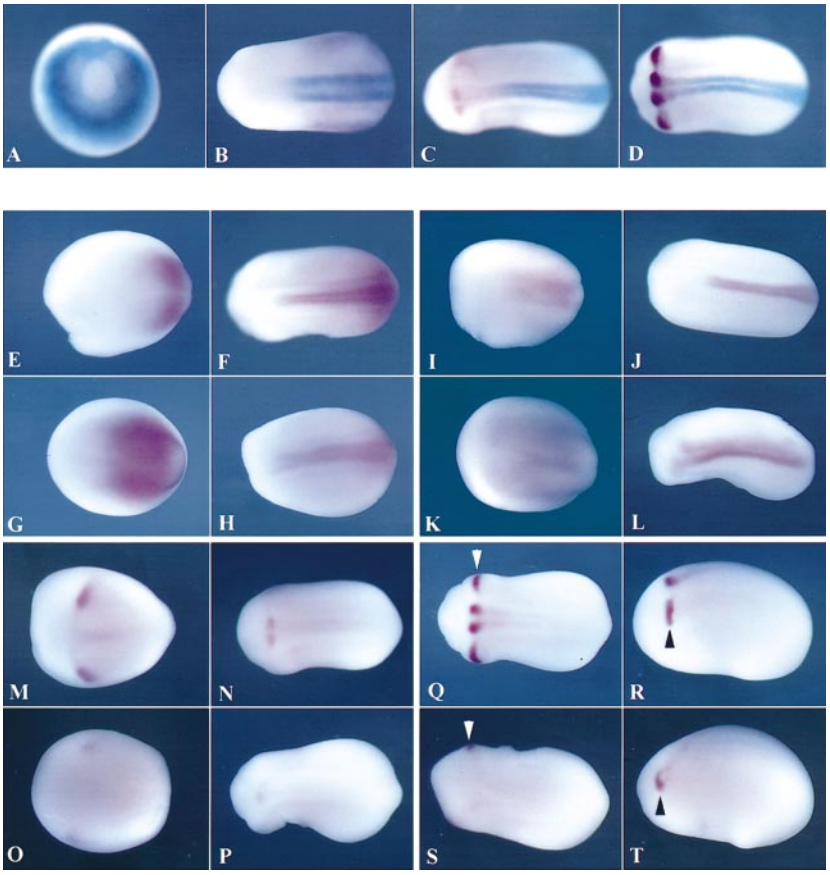

Fig. 5. Effects of *Xcad3* overexpression on the regional expression of Hox genes. (A and B) Double *in situ* hybridizations for *Xcad3* and *HoxA7*. (**A**) A view of the blastopore region of a late gastrula stage12.5 embryo (dorsal to the top). (**B**) Dorsal view of a late neurula stage 20 embryo. (**C**) Dorsal view of a stage 21 embryo hybridized with *Xcad3* and *HoxB1*. (**D**) Dorsal view of a stage 21 embryo hybridized with *Xcad3* and *HoxB3*. *Xcad3* expression is light blue, Hox gene expression is magenta and regions of overlap are dark blue. (E–H) Embryos analyzed for *HoxA7* expression. (**E** and **F**) Dorsal views of the normal expression in open neural plate stage 14 and late neurula stage 20 embryos, respectively. (**G** and **H**) Dorsal views of embryos at stage 14 and stage 20, respectively, injected with 150 pg of *Xcad3* mRNA. (I–L) Embryos analyzed for *HoxB9* expression. (**I** and **J**) Dorsal views of the normal expression in open neural plate stage 14 and late neurula stage 20 embryos, respectively. (**K** and **L**) Dorsal views of embryos at stage 14 and stage 20, respectively, injected, with 150 pg of *Xcad3* mRNA. (M–P) Embryos analyzed for *HoxB1* expression. (**M** and **N**) Dorsal views of the normal expression in open neural plate stage 14 and late neurula stage 20 embryos, respectively. (**O** and **P**) Dorsal views of embryos injected with 150 pg of *Xcad3* mRNA at stage 14 and stage 20, respectively. (Q–T) Embryos analyzed for *HoxB3* expression at stage 20. (**Q**) Dorsal and (**R**) lateral views of the normal expression. (**S**) Dorsal and (**T**) lateral views of the expression of *HoxB3* in embryos injected with 150 pg of *Xcad3* mRNA. Arrows indicate expression within the migrating neural crest.

involved in DNA binding, protein–protein interaction and activating or repressing functions. Such arrangements of independent functional units and the ability to swap these domains between proteins provides a tool for elucidating the role of these molecules in regulating gene transcription. In this study, we demonstrate the existence of an 'activator' functional unit, distinct from the DNA binding homeodomain of Xcad3. These data have allowed us to undertake further domain-swapping experiments in the analysis of Xcad3 function.

There are now several other studies reported in which putative transcriptional activators have had their activation domain removed and replaced with a strong transcriptional repressor domain. This converts a protein, which normally acts as a transcriptional activator, into a potent repressor of transcription from the protein's normal target genes. This approach has been particularly useful in *Xenopus*, where genetic ablation of gene function is not possible but mRNAs coding for domain swap mutants can easily be overexpressed and have been shown to interfere with the function of the endogenous wild-type transcription factor (Conlon *et al.*, 1996; Ryan *et al.*, 1996; Horb and Thomsen, 1997).

In this study an Xcad3 repressor mutant (XcadEn-R) was made consisting of the C-terminal region of Xcad3, which contains the homeodomain but lacks the endogenous activating function, fused to the transcriptional repressor domain of the *Drosophila* engrailed protein. Figure 1 includes a schematic diagram of XcadEn-R and shows the size of the XcadEn-R protein produced by *in vitro* translation from synthetic XcadEn-R mRNA used for injections during this study. In order to show that this construct can repress targets of Xcad3, we examined its effect on the precocious activation of *HoxA7* by Xcad3. Co-injection of as little as 25 pg of XcadEn-R mRNA dramatically reduces the precocious activation of *HoxA7* at early gastrula stage 10.5 which results from the injection of 100 pg of *Xcad3* mRNA (Figure 6A). The co-injection of 100 pg (and above) of XcadEn-R mRNA completely blocks all *HoxA7* expression. This is not just a general block on transcription, because the normal expression of *Xbra* and the loading control *ODC* are unaffected in these

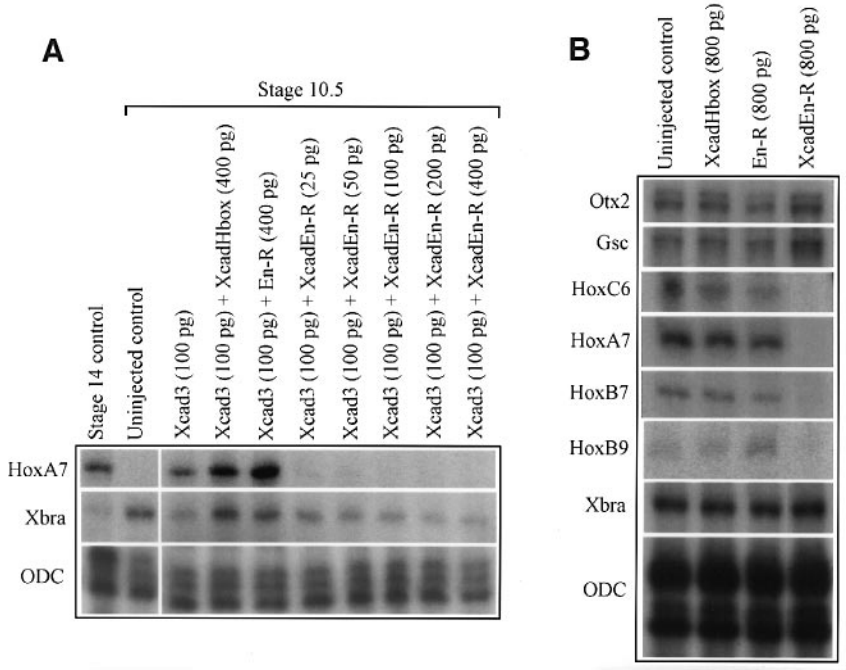

Fig. 6. The Xcad-repressor construct (XcadEn-R) blocks Hox gene activation during late gastrula stages. (**A**) RNase protection analysis carried out on early gastrula stage embryos (5 μg of total RNA/hybridization) showing that co-injection of mRNA from the Xcad3 repressor (XcadEn-R), but not the Xcad3 homeodomain (XcadHbox), is able to block the precocious activation of Hox gene that results from *Xcad3* overexpression. (**B**) RNase protection analysis for a number of Hox genes carried out on late gastrula stage 13 embryos injected with 800 pg of XcadEn-R mRNA. Five μg of total RNA derived from embryos in the same experiment were hybridized with each probe set. The ODC loading control probe was used in each hybridization; the set shown is representative.

Table I. Phenotype of embryos produced by different injected doses of XcadEn-R mRNA

	Type 0	Type I	Type II	Type III	Type IV	Other	*n*
Uninjected	43	2	0	2	0	2	49
XcadHbox							
400 pg	27	0	0	0	0	2	29
En-Rep.							
400 pg	25	0	0	2	0	2	29
XcadEn-R							
75 pg	18	5	1	8	0	4	36
175 pg	1	4	7	18	1	1	32
225 pg	0	0	0	10	9	7	26
450 pg	0	0	0	1	33	5	39

Embryos were injected with the relevant mRNA solutions into all blastomeres at the 4-cell stage and cultured to stage 41 before scoring.

embryos. The co-injection of as much as 400 pg of XcadHbox or engrailed repressor domain mRNA has no effect on the activation of *HoxA7* expression resulting from injection of *Xcad3* mRNA.

XcadEn-R overexpression disrupts the normal expression of Hox genes in the late gastrula

The demonstration that XcadEn-R is able to block activation of precocious Hox gene expression by ectopic Xcad3 suggests that XcadEn-R will also block the activity of endogenous Xcad3. In order to test this idea we examined the effect of XcadEn-R injection on the endogenous expression of those Hox genes activated by overexpression of wild-type Xcad3. Figure 6B shows RNase protections carried out on embryos that have been injected with 800 pg of XcadEn-R mRNA and shows that XcadEn-R blocks the normal activation of the trunk Hox genes *HoxC6*, *HoxA7*, *HoxB7* and *HoxB9* during late gastrula stages.

An important issue regarding the use of repressor mutants such as XcadEn-R is the binding specificity of the new molecule. It is difficult to determine whether the *in vivo* binding specificity of XcadEn-R is different to that of the wild-type source molecule (Xcad3). However, our data show that the same group of Hox genes activated by Xcad3 are also blocked by XcadEn-R. Moreover, there is good evidence that at least two of these (*HoxC6* and *HoxA7*) are legitimate direct targets of Cdx binding. Murine *HoxC6* and *HoxA7* have been shown to contain consensus binding sites for Cdx proteins and Cdx1 will transactivate expression from a *HoxA7* reporter (Subramanian *et al.*, 1995). These data suggest that XcadEn-R retains similar binding specificity to that of Xcad3. This conclusion is further supported by data in Figure 8 (see below).

XcadEn-R overexpression does not simply result in a general reduction in transcription. Not only are the levels of expression from anterior marker genes such as *otx2* and *gsc* unaffected in XcadEn-R injected embryos but, as was noted in Figure 6A, the levels of expression of the control mRNAs *Xbra* and *ODC* are also unaffected.

XcadEn-R overexpression disrupts posterior axial development

Injection of XcadEn-R mRNA, in contrast to the anterior truncations produced by wild-type *Xcad3* overexpression, leads to a high proportion of embryos which exhibit a reduction or complete loss of trunk and tail development. Except at the highest doses of injected mRNA, the development of anterior is quite normal.

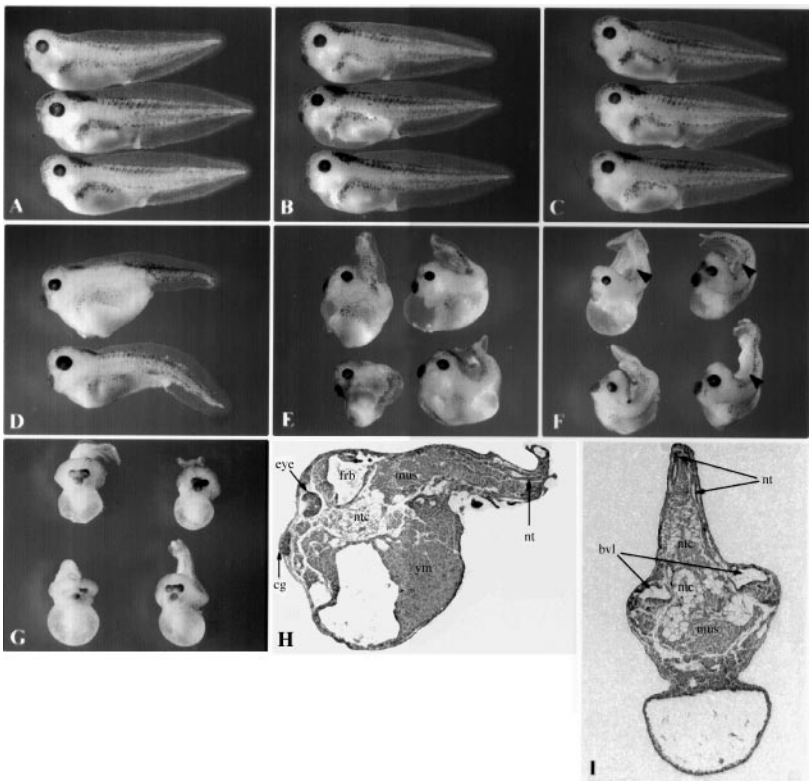

Fig. 7. Overexpression of the Xcad-repressor construct (XcadEn-R) cause posterior truncations. (A–G) Embryos at larval stage 41. (**A**, **B** and **C**) Uninjected control embryos, and embryos injected with 400 pg of homeodomain control (XcadHbox) mRNA and 400 pg of *Drosophila* engrailed repressor domain mRNA, respectively. (**D**) Type I embryos resulting from injection with 75 pg of XcadEn-R mRNA. (**E**) Type II embryos injected with 175 pg of XcadEn-R mRNA. (**F**) Type III embryos injected with 175 pg of XcadEn-R mRNA. Arrows indicate exposed yolk mass. (**G**) Type IV embryos injected with 450 pg of XcadEn-R mRNA. (**H**) Parasagittal section through a Type II embryo. (**I**) A transverse section through the posterior region of a Type III embryo. bvl, brain ventricle; cg, cement gland; frb, forebrain; mus, muscle; nt, neural tube; ntc, notochord; ym, yolk mass.

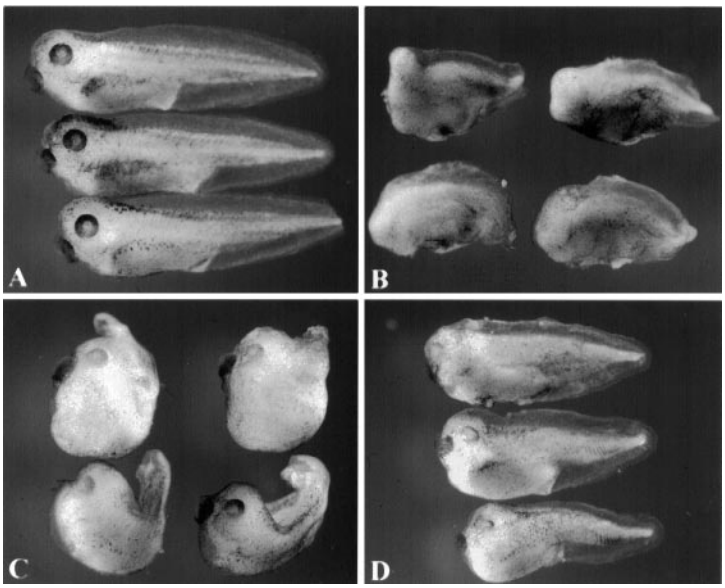

Fig. 8. XcadEn-R rescues the Xcad3 phenotype. (A–D) Larval stage 37 embryos. (**A**) Uninjected control embryos. (**B** and **C**) Embryos injected with 120 pg of Xcad3 and 120 pg of XcadEn-R mRNA, respectively. (**D**) Embryos co-injected with 120 pg of both *Xcad3* and XcadEn-R mRNA.

Table I and Figure 7A, B and C show that axial development of most embryos injected with 400 pg of either XcadHbox mRNA or engrailed repressor domain mRNA is normal. The injection of different doses of XcadEn-R mRNA produces a range of phenotypes at the larval stage 41 which have been classified according to the following criteria. Type 0 embryos show no obvious abnormalities in axial development. Type I embryos have completed blastopore closure but there is some reduction in trunk and tail development which is often associated with a somewhat 'pigeon chested' appearance (Figure 7D). Type II embryos have also closed their blastopores

but have an almost complete absence of posterior development and can have the appearance of an isolated head (Figure 7E). Type III embryos have varying degrees of open blastopore and always show a gross loss of posterior structures (Figure 7F). Type IV embryos have similar posterior defects to Type III but have additional defects including a reduction in the size of the cement gland and eyes which are often cyclopic (Figure 7G).

There is some phenotypic variability at a given concentration of injected XcadEn-R, but at higher concentrations there is clearly a higher proportion of Type III and IV phenotypes relative to Type I and II. It is somewhat difficult to determine the significance of the reduced trunk and tail structures in embryos that have failed to complete gastrulation and which retain an open blastopore. Such a disturbance of normal gastrulation is likely to affect posterior development and in severe cases might be expected to have rather non-specific effects on anterior development. However, at doses in the range 75–175 pg a significant number of embryos complete blastopore closure but still exhibit defects in posterior axial development (17 out of 68, compared with just two out of 49 for control embryos). In light of this, it seems probable that Type III and IV embryos are also subject to the same disturbance in the specification of the posterior axis as Type I and II embryos, but also suggests additional roles for Xcad3 function in controlling the cell movements of gastrulation.

Histological examination of XcadEn-R injected embryos reveals major disturbances in the normal tissue pattern, the embryos having a somewhat 'dorsalized' appearance. Importantly however, there is no tendency towards the formation of the radially symmetrical cement gland or eyes that result from the treatment of cleavage stage embryos with the lithium ion. Classically the 'lithium phenotype', which is characterized by an increase in dorsoanterior tissues, is believed to arise from effects on dorsoventral specification before the onset of gastrulation. The differences between the XcadEn-R phenotype and the lithium phenotype, and the fact that XcadEn-R overexpression does not result in the upregulation of early the dorsal marker *gsc* (Figure 6B), suggests that the effects of XcadEn-R result from an interference with anteroposterior specification rather than early effects on dorsoventral patterning.

Figure 7H shows a parasagittal section of a Type II embryo and shows that the head region contains a mass of notochord tissue underlying a large single brain vesicle. The vestigial trunk region contains a 'spike' of notochord tissue, overlain by the neural tube and surrounded by a mass of disorganized muscle running along its length. Figure 7I shows a transverse section through the extreme posterior of a Type III embryo. Again it shows a mass of disorganized notochord and muscle tissue running along the vestigial trunk region. In this case, the neural axis of the embryo has split around the open blastopore; note the brain vesicles on either side of the embryo and double neural tube in the distal regions of the trunk spike.

XcadEn-R rescues the anterior truncations caused by Xcad3 overexpression

As described above, low-dose injections of *Xcad3* mRNA result in a loss of anterior structures, including the cement gland and eyes. An important test of the specificity of XcadEn-R is that it should be able to rescue this phenotype.

Figure 8 shows the results of such a rescue experiment. The injection of 120 pg of *Xcad3* mRNA results in a large proportion of embryos lacking both eyes and cement glands (22 out of 23) (Figure 8B). The injection of 120 pg of XcadEn-R alone results in a large proportion of posterior truncations (22 out of 27) (Figure 8C). However, most of the embryos that have been co-injected with equal amounts of *Xcad3* and XcadEn-R mRNA have some degree of cement gland or eye rescue (22 out of 26) (Figure 8D).

As previously discussed, an important consideration with the construction of an interferring mutant of a transcription factor such as Xcad3 is the potential change in binding specificity that might occur. The ability of XcadEn-R to rescue the phenotypic effects of Xcad3 is perhaps the most rigorous test of the reagents specificity because it does not rely on any prior knowledge of target genes. The demonstration that XcadEnR rescues the anterior truncations produced by Xcad3 overexpression means that, at the very least, it is able to bind to the same targets of Xcad3 which are involved in the genesis of this phenotype.

XcadEn-R blocks FGF-induced Hox gene expression

In a previous study we showed that FGF overexpression during gastrula stages upregulates expression from a subset of 5′ Hox genes. Furthermore, we proposed that Xcad3 plays an important role in mediating these effects and that a regulatory pathway involving *eFGF*, *Xcad3* and Hox genes is involved in the normal development of posterior axial structures (Pownall *et al.*, 1996).

Here we show that XcadEn-R is able to block the FGF-induced activation of Hox genes, and that this is distinct from the ability of FGFs to induce the expression of other known targets such as *Xbra* (Smith *et al.*, 1991; Amaya *et al.*, 1993; Isaacs *et al.*, 1994). This lends further weight to the proposed pathway and demonstrates the existence of multiple independent downstream targets of FGF signalling.

Figure 9 shows an RNase protection analysis on animal cap explants cultured to the equivalent of mid-neurula stage 16. Explants from embryos injected with 10 pg of *eFGF* mRNA alone, as expected, express high levels of *HoxA7*, *HoxB9*, *Xcad3* and *Xbra*. The co-injection of 300 pg XcadHbox mRNA has no significant effect on the expression of any of these markers. However, the co-injection of 300 pg of XcadEn-R mRNA completely blocks the expression of *HoxA7* and *HoxB9*, while there is no reduction in the expression of the general mesodermal marker *Xbra*. Interestingly, the co-injection of XcadEn-R leads to a considerable reduction in the expression of endogenous *Xcad3*, suggesting that Xcad3 is a component of a positive feedback loop required for the normal regulation of its own expression.

Xcad3 expression is an immediate early response to FGF signalling

Further support for the FGF/Xcad3/Hox pathway is provided by the observation that the normal dorsal expression of *Xcad3* requires a functional FGF signalling pathway (Northrop and Kimelman, 1994; Pownall *et al.*, 1996).

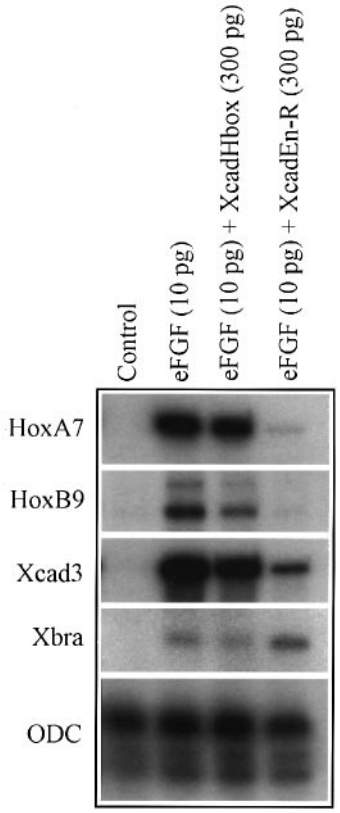

Fig. 9. XcadEn-R blocks FGF induced Hox gene expression. An RNase protection analysis carried out for a number of marker genes induced by injection of *eFGF* mRNA in animal cap explants cultured to neurula stage 16. Co-injection of XcadEn-R but not XcadHbox (homeodomain control) mRNA blocks eFGF-induced Hox gene activation. mRNA from uninjected caps provide a negative control. One μg of total RNA derived from explants in the same experiment was used in each hybridization set. The ODC loading control probe was used in each hybridization; the set shown is representative.

However, it is not known if *Xcad3* is a direct target of FGF signalling or whether the regulation of *Xcad3* by FGF requires the activation of additional intermediates.

Figure 10 shows an RNase protection analysis of animal caps at early gastrula stage 10 that have been treated with eFGF protein alone or eFGF in the presence of the protein synthesis inhibitor cycloheximide (CHX). The CHX treatment regime used in this experiment produced a 94% reduction in translation as determined by incorporation of radiolabelled methionine during the course of the experiment. *Xbra* is a known immediate early target of FGF signalling and its expression is activated by eFGF in the presence of cycloheximide (Smith *et al.*, 1991). There is a very low level of *Xcad3* expression in untreated animal caps but treatment with eFGF strongly induces *Xcad3* expression in animal caps within 2 h, even in the presence of CHX, indicating that like *Xbra*, *Xcad3* is also an immediate early target of FGF signalling. However, inhibition of translation substantially reduces the expression of both *Xcad3* and *Xbra*. These results are in keeping with a role for *Xcad3* in regulating its own expression as has been suggested for *Xbra* (Isaacs *et al.*, 1994; Schulte-Merker and Smith, 1995).

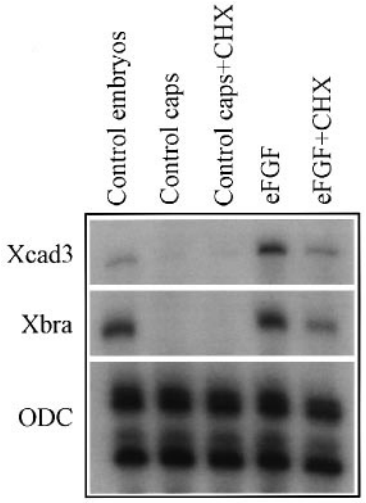

Fig. 10. *Xcad3* is an FGF immediate early response gene. RNase protection analysis of *Xcad3* and *Xbra* expression in animal cap explants cultured to early gastrula stage 10 following treatment with eFGF protein (100 ng/ml) or eFGF in the presence of the protein synthesis inhibitor CHX. Whole embryos from the same stage provide a positive control and animal caps (with and without CHX) the negative control. Total RNA (3.5 μg) derived from explants in the same experiment were used in the hybridizations.

Xcad3 activates Hox gene expression in noggin-induced anterior neural tissue

Prevailing models of nervous system patterning suggest that the initial specification of neural tissue is of a default anterior character and that the development of the full range of anteroposterior pattern requires the activity of a dominant posteriorizing signal (reviewed in Nieuwkoop *et al.*, 1985; Slack and Tannahill, 1992). There is evidence to indicate that FGF can mimic this posteriorizing signal (Cox Wm and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995) and contributes to such an activity *in vivo* (Pownall *et al.*, 1996). *Xcad3* is a downstream target of FGF signalling and, given its activities and normal expression within the developing posterior nervous system, is a good candidate to be involved in the process of neural posteriorization. Here we present data which support this hypothesis and show that Xcad3 mimics two aspects of the endogenous posteriorizing signal. Not only does Xcad3 induce posterior Hox genes in the anterior-type neural tissue that results from treatment of animal caps with the candidate neural-inducing molecule noggin, but it also suppresses the expression of anterior markers.

Figure 11 shows an RNase protection analysis for a number of anteroposterior markers carried out on animal caps at early tailbud stage 22 that have been injected with either *noggin* or *Xcad3* mRNA, or a combination of both. Consistent with previous studies, we find that injection with 100 pg of *noggin* mRNA leads to the induction of the neural marker *NCAM* and an upregulation of the anterior marker *otx2* but does not induce the expression of more posterior markers such as *HoxA7* and *HoxB9*. All (10 out of 10) of these animal caps showed the presence of cement gland tissue, which is a non-neural, ectodermal derivative that is often associated with anterior neural inductions. However, if 300 pg of *Xcad3* mRNA is co-injected with *noggin*, the expression of the spinal cord

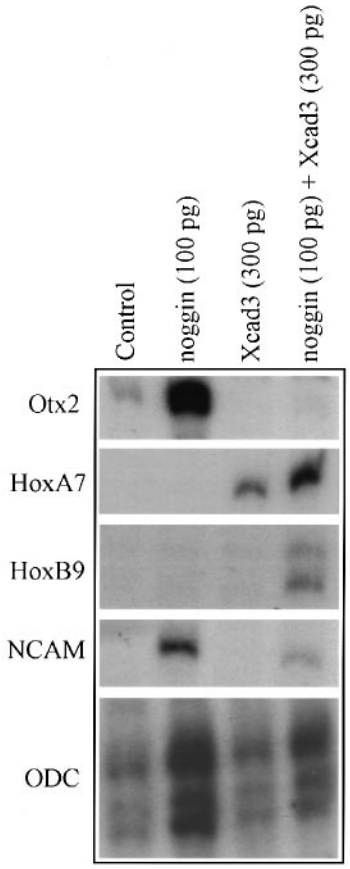

Fig. 11. Xcad3 posteriorizes anterior neural tissue. RNase protection analysis for a number of anteroposterior markers carried out on animal cap explants cultured to tailbud stage 22 from embryos injected with mRNA of the neural inducing molecule noggin or Xcad3 mRNA, or co-injected with both mRNAs. Animal caps from uninjected control embryos provide a negative control. One μg of total RNA derived from explants in the same experiment was used in each hybridization set. The ODC loading control probe was used in each hybridization; the set shown is representative.

markers *HoxA7* and *HoxB9* is activated but, as noted in the context of Xcad3 overexpression in whole embryos, the expression of the anterior marker *otx2* is suppressed. The animal caps are also different in appearance, and there is no development of cement gland (10 out of 10).

Interestingly, although the injection of *Xcad3* mRNA alone is sufficient to activate *HoxA7* expression in animal caps, it is only able to activate *HoxB9* expression in the noggin-neuralized caps. This indicates that Xcad3 requires additional tissue specific cofactors for it to regulate the expression of individual 5′ Hox genes. Further support for this idea comes from the observation that overexpression of *Xcad3* in embryos does not lead to generalized ectopic Hox gene expression but rather expands the normal expression domain (Figure 5E–L).

Discussion

A role for caudal-related proteins in the regulation of posterior Hox genes

Experiments in this study show that Xcad3 is a transcriptional activator and that inhibition of transcriptional activation by Xcad3 interferes with the normal expression of Hox genes, from at least three clusters (A, B and C), with anterior limits of expression within the trunk. These data strongly support the proposed role of caudal-related proteins in regulating Hox genes during amphibian development (Pownall *et al.*, 1996; Epstein *et al.*, 1997).

A role for *caudal*-related genes in the regulation of Hox genes is conserved in other vertebrate species. Mice homozygous for a null allele of the *Cdx1* gene exhibit posterior to anterior homeotic shifts in vertebral identity; similar shifts have been noted in mice heterozygous for a null allele of the *Cdx2* gene (Chawengsaksophak *et al.*, 1997). Interestingly, the *Cdx1* knockout mice also show posterior shifts in the boundaries of expression from a number of Hox genes, including *HoxC6* and *HoxA7* (Subramanian *et al.*, 1995). This is consistent with the anterior shifts in the expression boundaries of *HoxA7* and *HoxB9* in embryos overexpressing Xcad3 noted in the present study. It is probable that the alterations in vertebral identity, seen with both murine Cdx gene knockouts, are due to effects on Hox gene regulation.

The haplo-insufficiency phenotype that has been described for *Cdx2* is interesting because it suggests that the dose or overall levels of caudal proteins are critical during development. This notion is supported by data in this study showing sharp changes in the phenotypic response to 2-fold differences of injected *Xcad3* mRNA. These observations also highlight the significance of the posterior to anterior gradient of expression that has been noted for all vertebrate Cdx genes. One proposed explanation of these findings is that different levels of Cdx proteins along the anteroposterior axis might be involved in activating different cohorts of Hox gene expression within the trunk and tail forming region. Data in this paper for the activation of *HoxC6*, *A7*, *B7* and *B9* do not support this view. However, all of these genes have rather similar anterior boundaries of expression in *Xenopus*. A more detailed investigation of this problem will require the analysis of concentration effects on the activation of genes from a wider range of paralogue groups, in particularly groups 10–13, which have not yet been cloned in *Xenopus*.

Although in *Xenopus* it is clear that *caudal*-related genes are involved in the initial activation of Hox expression in the late gastrula, a recent study using *Xenopus* embryos carrying a XcadEn-R transgene indicates that there is a second *caudal*-independent phase of Hox gene expression during later development (Pownall *et al.*, 1998)

XcadEn-R phenotype versus the mouse Cdx gene knockout phenotype

The phenotype of posterior truncation in the XcadEn-R injected embryos is very dramatic and much more severe than the homozygous phenotype of the *Cdx1* knockout mouse or the haplo-insufficiency phenotype seen with *Cdx2*. Homozygous *Cdx2* knockout mice die at around the time of implantation. *Cdx2* is expressed within extra-embryonic tissues of the embryo and interference with *Cdx2* function within this tissue is likely to interfere with implantation. A more comprehensive analysis of the requirement for *Cdx2* in anteroposterior specification will require the generation of a conditional knockout of the locus, which allows development up to and beyond gastrula stages.

There are a number of other possible explanations for the disparity in phenotypic severity. In the mouse Cdx genes, *Cdx4* is most related by sequence identity and expression pattern to *Xcad3*, and a knockout has not yet been reported (Marom *et al.*, 1997). It is possible that the phenotype of the *Cdx4* knockout might be more similar to that of XcadEn-R-injected embryos. Another possibility is that the target specificity of the XcadEn-R repressor mutant *in vivo* is radically different from that of wild-type Xcad3 and that the severe phenotype arises from repression of transcription from non-canonic Cdx target genes. However, the fact that XcadEn-R blocks the transcription of the same genes activated by Xcad3 and that XcadEn-R can rescue the *Xcad3* overexpression phenotype suggests that this is not the case. Caudal-related proteins bind to a consenus 'TTTATG' sequence though, and it is certainly possible that XcadEn-R interferes with the function of the other *Xenopus* caudal homologues (Xcad1 and Xcad2) by binding and repressing their targets as well. In this view the XcadEn-R phenotype might be more similar to a multiple knockout of mouse Cdx genes. The possibility that XcadEn-R might interfere with the activity of Xcad1 and Xcad2 is presently under investigation.

Other functions suggested for Xcad3 during development

It is probable that the ability of Xcad3 to suppress the development of anterior structures is due in part to the expansion of posterior Hox gene expression into more anterior regions. However, in this study we show that *Xcad3* overexpression also results in a downregulation of *otx2* and *gsc* gene expression, both of which are important for normal anterior development. In addition, it has recently been reported that overexpression of *Xcad2* also leads to suppression of *otx2* expression (Epstein *et al.*, 1997). Although with regard to the posterior Hox genes Xcad3 acts as a transcriptional activator, it is possible that Xcad3 could also act as a transcriptional repressor depending on context. If this were the case, overexpression of the strong constitutive repressor XcadEn-R might also be expected to downregulate *otx2* expression. This is not the case and it is therefore probable that the repression of anterior gene expression by Xcad2 and Xcad3 is not direct but requires the activation of additional downstream target genes.

There are additional complexities to the regulation of Hox genes by caudal-related proteins. Not only does the overexpression of *Xcad3* expand the anterior expression domains of trunk Hox genes, such *HoxA7* and *HoxB9*, but it also represses expression from hindbrain Hox genes, such as *HoxB1* and *HoxB3*. These data clearly demonstrate that different mechanisms are involved in regulating the expression of genes from the 3′ region of Hox clusters such as *HoxB1* and *HoxB3*. Again, the suppression of 3′ Hox gene expression by Xcad3 may not be direct but may be an example of a more general phenomenon known as 'posterior prevalence' of Hox gene expression. In *Drosophila*, the ectopic expression of posterior Hox genes in more anterior regions can lead to the downregulation of normal anterior Hox gene expression. It is believed that this is a result of competition between Hox proteins for target sites, including the Hox genes themselves. In this way the ectopic Hox gene expression can interfere with autoregulatory loops which are important for the establishment and maintenance of Hox gene expression within anterior regions (Castelli-Gair *et al.*, 1994).

The question of to what extent the XcadEn-R phenotype can be explained solely by a disruption in Hox gene regulation remains. The phenotype is much more severe than any previously reported Hox gene knockouts, but comparison with the published data is difficult because it is probable that in XcadEn-R embryos, the expression of many Hox genes from different paralogue groups is ablated. Clearly, in this situation the phenotypic effects are likely to be more severe than can be expected from single or even double gene knockouts. However, there are indications that Xcad3 is required for more than just the regulation of Hox genes. In Type III and IV embryos overexpressing XcadEn-R, perturbations in cell movements are apparent before the normal onset of Hox gene expression at the end of gastrulation, suggesting additional roles for Xcad3 in regulating genes required for gastrulation. *Xenopus*, used in conjunction with the reagents described in this paper, provides an ideal system in which to investigate these additional Xcad targets.

A conserved role for caudal-related genes in posterior development

There is a remarkable conservation of *caudal* gene expression within the posterior of a wide range of animal groups and it is clear that there is general role for *caudal* homologues in posterior development. In *C.elegans*, the Cdx family member *pal-1* is required for posterior blastomere specification in the 4-cell embryo (Hunter and Kenyon, 1996). In *Drosophila*, caudal regulates the genes *giant*, *knirps* and *fushi tarazu* in the posterior of the embryo. Removal of both zygotic and maternal *caudal* expression results in embryos with deletions of even numbered abdominal segments (Kuhn *et al.*, 1995).

Is there any evidence that the role for *caudal*-related genes in regulating Hox genes is conserved outside the vertebrates? In *Drosophila*, homologues of the vertebrate Hox genes (Hom-C complex) are considered to be largely epistatic to caudal but there is now evidence suggesting that some aspects of expression from the HOM-C complex member *Abdominal-B* are regulated by caudal (Kuhn *et al.*, 1995). Interestingly, ectopic anterior expression of *caudal* results in a disruption of head development, part of which appears to be due to the suppression of expression from the *deformed* gene, which is also a member of the *Drosophila* Hom-C complex (Mlodzik *et al.*, 1987). Certain parallels can be seen with the posterior-promoting/anterior-suppressing activity of the *Xenopus* Xcad proteins. With regard to the role of *caudal*-related genes in other invertebrates, it has been suggested that pal-1 is involved in regulation of the *C.elegans Abd-B* homologue *mab-5* (Waring and Kenyon, 1991).

The FGF, Xcad3 and Hox gene pathways in anteroposterior specification

In a previous study we presented evidence that FGF signalling is required for anteroposterior patterning during gastrula and neurula stages (Pownall *et al.*, 1996). Data in this paper demonstrate that Xcad3 is an immediate early target of FGF signalling that is required for normal anteroposterior specification, in part through regulation of

Hox gene expression. We show that not only is Xcad3 required for FGF activation of 5′ Hox gene expression, but also Xcad3 can activate Hox gene expression in anterior neural tissue as has been previously reported for FGF (Cox Wm and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995; Kengaku and Okamoto, 1995).

This study demonstrates that the activity of a *caudal* (Cdx) homologue is required for normal development of trunk and tail regions of the amphibian embryo and underlines the importance of *caudal*-related genes in the axial development in diverse animal groups. Further studies will elucidate how the FGF/Cdx pathway interacts with other known regulators of anteroposterior specification such as retinoic acid and the wnt and otx gene families.

Materials and methods

Embryological methods and mRNA injections

Capped mRNAs were synthesized using the SP6 Megascript transcription kit (Ambion) and a modified protocol using 0.5 mM GTP and 5 mM $m^7G(5')Gppp(5')G$ cap. Following synthesis, RNAs were subject to sequential precipitation with 0.5 M ammonium acetate and 2.5 M LiCl to remove unincorporated nucleotides.

Staging of embryos was according to Nieuwkoop and Faber (1967). Embryos in NAM + 5% Ficoll were injected with 5 nl of the relevant sample into each blastomere at the 4-cell stage. At the 64-cell stage, embryos for phenotypic and molecular analysis were transferred to NAM/10 + 5% Ficoll for the remainder of the culture period. Injected embryos required for animal cap explants were transferred to NAM + 5% Ficoll at the 64-cell stage. Animal cap explants were removed at late blastula stage 9 and then cultured in NAM/2 until the required stage.

The pSP64t-eFGF plasmid used was as described by Isaacs *et al.* (1992). The pSP64t–noggin construct used consists of a 740 bp PCR fragment of Xenopus *noggin* (Smith and Harland, 1992), containing the whole of the coding region, cloned into the *Bgl*II site of pSP64t (Krieg and Melton, 1984).

CS2–VP16-N consists of a 273 bp fragment, containing sequence coding for amino acids 410–490 of the *Herpes simplex* VP16 protein, cloned into the *Cla*I–*Xho*I site of the CS2+ vector (Rupp *et al.*, 1994). CS2–ENG-N consists of an 888 bp fragment coding for amino acids 1–296 of the *Drosophila* engrailed protein cloned into the *Stu*I site of CS2+. Both the CS2–VP16-N and CS2–ENG-N vectors were kind gifts of Dr Dan Kessler.

The CS2–Xcad3 plasmid used was as described by Pownall *et al.* (1996) and consists of bases 1–1070 of the published *Xcad3* cDNA sequence subcloned into the *Xho*I–*Xba*I site of CS2+ (Northrop and Kimelman, 1994). The *Xcad3* activator construct (CS2–XcadVP16-A) was made by cloning a *Pvu*II–*Nsi*I fragment from CS2–Xcad3, containing the sequence coding for amino acids 121–275 of Xcad3, into the *Xba*I–*Nsi*I site of CS2–VP16-N. The *Xho*I site was first blunted with Klenow enzyme to allow the production of an in-frame N-terminal fusion of the VP16 activator domain to the Xcad3 sequence. The Xcad3 repressor construct (CS2–XcadEn-R) was made cloning the same *Pvu*II–*Nsi*I fragment from CS2–Xcad3 into the *Xho*I–*Nsi*I site of CS2–ENG-N. The *Xho*I site was first blunted with Klenow enzyme to allow the production of an in-frame N-terminal fusion of the engrailed repressor domain to amino acids 121–275 of *Xcad3*. An Xcad3 homeobox control plasmid (CS2–XcadHbox) was constructed by digesting CS2–XcadEn-R with *Eco*RI, followed by religation. This has the effect of removing the whole of the engrailed repressor domain and allows the production of an N-terminally truncated Xcad3 protein, containing the whole of the homeodomain initiating from the methionine at position 136 of the wild-type protein, and terminating with the endogenous stop codon at position 276.

Cycloheximide and growth factor treatments

At blastula stage 8.5 animal caps were explanted (10 for each treatment) and cultured for 30 min in NAM/2 + 5 mg/ml bovine serum albumen (BSA), either with or without 10 μg/ml of cycloheximide. Animal caps were then transferred to NAM/2 + BSA, either with or without 10 μg/ml of cycloheximide and with or without recombinant 100 ng/ml of eFGF (Isaacs *et al.*, 1992), and cultured for 1.5 h. Following treatment with cycloheximide and growth factor, all animal caps were transferred to NAM/2 + BSA for a further 0.5 h. At this stage (very early gastrula equivalent) the animal caps were harvested for analysis by RNase protection. In order to test the degree of protein translation inhibition, animal caps were metabolically labelled with [^{35}S]methionine (with or without CHX) over the same culture period and TCA-precipitable counts were measured using a scintillation counter.

In vitro translation from synthetic mRNAs

The sizes of proteins produced from synthetic mRNAs used for embryo injections in this study were tested by *in vitro* translation using a rabbit reticulocyte lysate system (Promega). Synthesized proteins were labelled with [^{35}S]methionine and analyzed by SDS–PAGE and autoradiography.

RNase protection analysis

RNA from samples was purified by extracting up to six embryos in 0.4 ml of 0.1 M NaCl, 50 mM Tris (pH 8.0), 5 mM EDTA and 0.5% SDS, followed by phenol/chloroform extraction and ethanol precipitation. RNase protection analysis was carried out as described by Isaacs *et al.* (1992). [^{32}P]UTP-labelled antisense probes were synthesized and hybridized to sample RNA at 50°C overnight and digested with RNase T1 at 700 μg/ml for 1 h before running on 6% acrylamide–urea gels. Exposure time for autoradiographs using Kodak X-omat film, was from a few hours up to 10 days depending upon probe abundance. Autoradiographs were scanned using a Umax flatbed scanner and images were processed using Adobe Photoshop.

Probes used in this study were as follows. Hox gene probes: *HoxB1* was linearized with *Sty*I and transcribed with SP6 polymerase (Godsave *et al*., 1994). *HoxB3* was linearized with *Xba*I and transcribed with T3 polymerase (Papalopulu and Kintner, 1996). *HoxC6* was linearized with *Pvu*II and transcribed with T3 (Wright *et al*., 1987). *HoxB7* was linearized with *Bam*HI and transcribed with T7 polymerase (Godsave *et al*., 1994). *HoxA7* was linearized with *Eco*RI and transcribed with T7 polymerase (Condie and Harland, 1987). *HoxB9* was linearized with *Sma*I and transcribed with T7 polymerase (Sharpe and Gurdon, 1990). Anterior marker probes: *Goosecoid* was linearized with *Xba*I and transcribed with T3 polymerase (Green *et al*., 1992). *otx2* was linearized with *Nae*I and transcribed with SP6 polymerase (Pannese *et al*., 1995). The en-2 probe is derived from a 210 bp *Hin*dIII–*Sac*I subclone of the full-length *Xenopus* en-2 cDNA (Hemmati-Brivanlou *et al*., 1990), linearized with *Hin*dIII and transcribed with T7 polymerase. Posterior marker probes: *Xbra* was linearized with *Ssp*I and transcribed with T7 polymerase (Smith *et al*., 1991). *Xcad3* was linearized with *Eco*RI and transcribed with T7 polymerase (Pownall *et al*., 1996). *eFGF* was linearized with *Eco*RI and transcribed with T3 polymerase (Isaacs *et al*., 1992). Neural marker probe: *NCAM* was linearized with *Eco*RI and transcribed with SP6 polymerase (Balak *et al*., 1987). Loading control probe: in all RNase protection assays the ubiquitously expressed *ODC* gene was used as an internal loading control (Isaacs *et al*., 1992). ODC was linearized with *Bgl*II and transcribed with T7 polymerase.

In situ hybridization analysis

Albino embryos were cultured to appropriate stages and then fixed in MEMFA (0.1 M MOPS, 2 mM EDTA, 1 mM $MgSO_4$, 3.7% formaldehyde) for 1 h at room temperature and stored in 100% ethanol at –20°C until further processing. Embryos were rehydrated through a graded series of ethanols and then rinsed in PBS with 0.1% Tween. Proteinase K treatment was carried out for 10 min at room temperature with 10 μg/ml of Proteinase K. Hybridization was carried out overnight at 60°C in 50% formamide, 5× SSC, 1 mg/ml rRNA, 100 mg/ml heparin, 1× Denhardt's, 0.1% Tween, 0.1% CHAPS and 10 mM EDTA. Extensive washes in 2× SSC and 0.2× SSC at 60°C were followed by washes at room temperature with maleic acid buffer (MAB; 0.1 M maleic acid, 0.15 M NaCl, 0.1% Tween pH 7.8) and blocking in 2% Boehringer Mannheim Blocking Reagent and 20% heat-treated lamb serum for 2 h at room temperature. Embryos were then incubated with anti-DIG antibody at a dilution of 1:2000 in blocking solution at 4°C overnight. The antibody is detected after extensive washes at room temperature in MAB by a colour reaction using Magenta Phos (5-bromo-6-chloro-3-indolyl phosphate) from Molecular Probes. Probes were transcribed using 10× DIG RNA labelling mix (Boehringer Mannheim).

For double *in situ* hybridizations, the hybridization protocol remains the same, while the probes were transcribed using either 10× DIG RNA labelling mix (*HoxB1*, *HoxB3* and *HoxA7*) or 10× fluorescein RNA labelling mix (Boehringer Mannheim) (*Xcad3*). Embryos were then incubated with anti-DIG antibody at a dilution of 1:2000 in blocking solution at 4°C overnight. After extensive washes in MAB, the first antibody is detected by a colour reaction using Magenta Phos (Molecular

Probes). After heat-inactivation of the alkaline phosphatase (10 min at 65°C in MAB with 10 mM EDTA) followed by two 5 min washes in methanol, the embryos were rehydrated and blocked in 2% Boehringer Mannheim Blocking Reagent and 20% heat-treated lamb serum for 1 h at room temperature. Embryos were then incubated with anti-fluorescein antibody at a dilution of 1:1500 in blocking solution at 4°C overnight. After extensive washes in MAB, the second antibody is detected by a colour reaction using BCIP (5-bromo-4-chloro-3-indolyl phosphate). *In situ* probes: *HoxA7* was linearized with *Bam*HI and transcribed with SP6 polymerase (Condie and Harland, 1987). *HoxB1* was linearized with *Nde*I and transcribed with SP6 polymerase. *HoxB3* was linearized with *Hin*dIII and transcribed with T7 polymerase. *HoxB9* was linearized with *Eco*RI and transcribed with SP6 polymerase (Godsave *et al.*, 1994). *Xcad3* was linearized with *Eco*RV and transcribed with T3 polymerase (Northrop and Kimelman, 1994).

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