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eFGF, *Xcad3* and Hox genes form a molecular pathway that establishes the anteroposterior axis in *Xenopus*

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SUMMARY

Classical embryological experiments suggest that a posterior signal is required for patterning the developing anteroposterior axis. In this paper, we investigate a potential role for FGF signalling in this process. During normal development, embryonic fibroblast growth factor (eFGF) is expressed in the posterior of the *Xenopus* embryo. We have previously shown that overexpression of *eFGF* from the start of gastrulation results in a posteriorised phenotype of reduced head and enlarged proctodaeum. We have now determined the molecular basis of this phenotype and we propose a role for eFGF in normal anteroposterior patterning.

In this study, we show that the overexpression of *eFGF* causes the up-regulation of a number of posteriorly expressed genes, and prominent among these are *Xcad3*, a *caudal* homologue, and the Hox genes, in particular *HoxA7*. There is both an increase of expression within the normal domains and an extension of expression towards the anterior. Application of eFGF-loaded beads to specific regions of gastrulae reveals that anterior truncations arise from an effect on the developing dorsal axis. Similar anterior truncations are caused by the dorsal overexpression of *Xcad3* or *HoxA7*. This suggests that this aspect of the *eFGF* overexpression phenotype is caused by the ectopic activation of posterior genes in anterior regions.

Further results using the dominant negative FGF receptor show that the normal expression of posterior Hox genes is dependent on FGF signalling and that this regulation is likely mediated by the activation of *Xcad3*. The biological activity of eFGF, together with its expression in the posterior of the embryo, make it a good candidate to fulfil the role of the 'transforming' activity proposed by Nieuwkoop in his 'activation and transformation' model for neural patterning.

Key words: fibroblast growth factor, *caudal*, *cdx*, anteroposterior specification, Hox genes, *Xenopus*

INTRODUCTION

In recent years, there has been considerable interest in the role of the fibroblast growth factor (FGF) family of signalling molecules in early vertebrate development. Work in avians strongly suggests that FGF signalling is required for the initiation and subsequent outgrowth of the limb bud (Niswander et al., 1994; Cohn et al., 1995, Crossley et al., 1996). Work on *Xenopus* has indicated an important role for the FGFs in the establishment and patterning of the mesoderm. Although most attention has been devoted to the process of mesoderm induction in the blastula (Amaya et al., 1991, 1993), it has now become clear that FGFs have an additional essential role in the process of anteroposterior patterning.

We have previously shown that overexpression of *eFGF* in embryos during the gastrula stages produces a characteristic phenotype comprising a suppression of the head and an enlargement of the posterior, in particular of the proctodaeum (Isaacs et al., 1994). Other recent studies show that bFGF can induce neural tissue of a posterior character (Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995). The expression of two secreted *Xenopus* FGFs in the posterior of gastrula- and neurula-stage embryos (Isaacs et al., 1992; Tannahill et al., 1992; Isaacs et al., 1995) support the suggested role for FGF in posterior development (Ruiz i Altaba and Melton, 1989a; Isaacs et al., 1994).

In this study, we present a molecular pathway for anteroposterior patterning whereby FGF signalling regulates Hox gene expression in the posterior via the transcriptional activation of *cdx* genes. We show that *eFGF* overexpression, either from an injected plasmid or as protein applied on a bead, causes an anterior extension of the expression domain of *Xcad3* and a group of posterior Hox genes, including *HoxA7* and *HoxB9*. It has been previously demonstrated that inhibition of the FGF signal transduction pathway by overexpression of a dominant negative FGF receptor down-regulates *Xcad3* and *HoxA7* expression (Northrop and Kimelman, 1994; Isaacs et al., 1994). Here we show that injection of *Xcad3* mRNA can rescue the expression of *HoxA7* in the absence of FGF signalling. This supports the view that members of the vertebrate *caudal* gene family (cdx) lie downstream of FGF signalling

and are upstream activators of Hox gene expression (Subramanian et al., 1995). We also show that dorsal overexpression of either *Xcad3* or *HoxA7* is sufficient to suppress anterior structures, indicating that the loss of these structures in embryos overexpressing eFGF results from the anterior ectopic expression of these posterior genes.

It is now widely accepted that the Hox genes are regulators of anteroposterior specification in animal groups ranging from *Drosophila* to vertebrates (Slack et al., 1993; Holland and Garcia-Fernandez, 1996). It has previously been shown that mesoderm induction by FGF leads to the activation of a number of Hox genes (Cho et al., 1990). In this paper, we show that FGF signalling also regulates the expression of these genes during gastrula and neurula stages, after the period of mesoderm induction is finished. We demonstrate the ability of eFGF to activate ectopic Hox gene expression in the ectoderm as well as in the mesoderm of the developing dorsal axis, which, together with the posterior expression domain of *eFGF* (Isaacs et al.,1995), points to an important role for FGF signalling in the establishment of the anteroposterior axis during normal development.

MATERIALS AND METHODS

RNA and DNA injections and embryo culture

RNA and DNA injections were carried out as described in Isaacs et al. (1994). Briefly, injections were done at the 2-cell or 4-cell stage where either 10 nl or 5 nl (respectively) of nucleic acid dissolved in water was injected into each blastomere. Embryos were in NAM plus 5% Ficoll for the injections and, at stage 6, they were transfered to NAM/10 plus 5% Ficoll for the remainder of the culture period. Synthetic mRNA for the dominant negative FGF receptor (XFD) was made from the plasmid used by Amaya et al. (1991). 500 pg of capped in vitro transcribed *XFD* mRNA was injected into each blastomere of 4-cell-stage embryos, for a total of 2 ng per embryo of injected mRNA. 1 ng of capped in vitro transcribed *HoxA7* (*Xhox36*) mRNA was injected into either each of the two ventral or the two dorsal blastomeres at the 4-cell stage. In lineage labeling experiments, 1 ng of HoxA7 mRNA was co-injected with 500 pg of β-gal mRNA into either each of the two ventral or the two dorsal blastomeres at the 4-cell stage. 100 pg of Xcad3 mRNA was injected into each of the four blastomeres at the 4-cell stage, for a total of 400 pg of injected mRNA per embryo. HoxA7, Xcad3, Xbra and β-gal were subcloned into the CS2+ plasmid (Rupp et al., 1994) for generating synthetic mRNA. 5 pg of *Not*I-linearised CSKA-eFGF plasmid was injected into each blastomere of the 2-cell-stage embryo (both albino and wild type), for a total of 10 pg of injected plasmid. Embryos were cultured to the appropriate stage, and then either fixed or frozen for further analysis by in situ hybridisation or RNAase protection.

RNA extraction and RNAase protection analysis

RNA extraction and RNAase protection analysis were carried out as described by Isaacs et al. (1994). Briefly, RNA was extracted in 0.1 M NaCl, 50 mM Tris(pH 8), 5 mM EDTA and 0.5% SDS followed by phenol-chloroform extraction and ethanol precipitation. RNAase protection analyses were hybridised at 45°C overnight and digested with RNAase T1 at 37°C for 50 minutes. The ubiquitously expressed *ornithine decarboxylase* (*ODC*) mRNA was used as an internal control for all RNAase protections (Isaacs et al., 1992). Markers used were prepared as follows: **Hox *gene markers:*** *HoxB1*(Godsave et al., 1994) was linearised with *Nde*I and transcribed with SP6 polymerase. *HoxB9* (*XlHbox* 6) was detected as in Sharpe and Gurdon (1990; Sma1/T7). *HoxC6* (*XlHbox1*;Carrasco and Malacinski, 1987), was detected as in Cho et al. (1991) using pRII as a probe (Pvu2/T3). *HoxA7* (*Xhox36*; Condie and Harland, 1987) was detected using *Xhox36.4 Eco*RI-*Pst*I fragment of *Xhox 36* cDNA cloned into pGEM2 (EcoR1/T7). ***Anterior markers:*** *Goosecoid* (Cho et al., 1991) was detected as in Green et al. (1992; Xba1/T3). *Xotx2* was linearised with *Nae*I and transcribed with SP6 polymerase (Pannese et al., 1995). ***Posterior markers:*** *Xcad3-400* (Northrop and Kimelman, 1994; a 400 bp *Eco*RI-*Pvu*2 fragment subcloned into Bluescript KS+) was linearised with *Eco*RI and transcribed with T7 polymerase; *Xbra* was detected as in Smith et al. (1991; Ssp1/T7). *Xpo.3* was linearised with *Nsi*I and transcribed with T3 polymerase (Sato and Sargent, 1991). *Xhox3* (Ruiz i Altaba and Melton, 1989b) was detected as in Saha and Grainger (1992; Dde1/T7). ***Neural marker:*** *NCAM* (PN2) (Balak et al., 1987) was linearised with *Eco*RI and transcribed using SP6 polymerase.

In situ hybridisation 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 hour 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 done for 10 minutes at room temperature with 10 μg/ml of Proteinase K. Hybridisation was carried out overnight at 60°C in 50% Formamide, 5× SSC, 1 mg/ml rRNA, 100 mg/ml heparin, 1× Denhardts, 0.1% Tween, 0.1% CHAPS, 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 hours 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 temperture in MAB by a colour reaction using BM purple precipitating alkaline phosphatase detection system (Boehringer Mannheim). Probes for in situ hybridisation were transcribed using 10× DIG RNA labelling mix (Boehringer Mannheim) from linearised plasmids: *HoxB1* (*Nde*I/SP6, 366 bases); *HoxB9* (*Eco*RI/SP6, 520 bases); *HoxA7* (*Xhox36*.1, *Bam*HI/SP6, 1.35 kbases); *Xbra* (*Cla*I/T7, 1.4 kbases); Xcad3 (Cs2-Xcad3, *Eco*RV/T7, 1.1 kbases); *Xpo* (*Bam*HI/SP6, 900 bases); and *Xotx2* (*Eco*RI/SP6, 863 bases) *eFGF* (*Eco*RI/SP6, 300 bases).

β-galactosidase detection

β-galactosidase activity was analysed in embryos injected with β-gal mRNA as a lineage tracer. For detection, embryos were fixed in MEMFA (0.1 M MOPS, 2 mM EDTA, 1 mM $MgSO_4$, 3.7% formaldehyde) for 1 hour at room temperature and then washed twice with PBSAT. The enzyme detection was assayed in staining buffer (20 mM $K_3Fe(CN)_6$, 20 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% deoxycholate, 0.02% NP-40) plus 1 mg/ml X-gal at 37°C for 15 minutes to an hour.

eFGF bead implants and explant cultures

Heparin acrylamide beads (Niswander et al., 1994) were washed repeatedly in PBS and then incubated overnight in 500 μg/ml eFGF protein (Isaacs et al., 1992). The beads were then washed in NAM before being implanted. After implantation the embryos were left in NAM for 30 minutes to allow healing and then transfered to NAM/10 for culturing. In the explant experiments, tissues combined with eFGF beads were held together under a glass bridge and allowed to heal in NAM, after which they were transferred to NAM/10 in a Terasaki dish. All experiments were also performed with control beads, which were prepared by incubation in PBS instead of eFGF.

RESULTS

Overexpression of *eFGF* up-regulates the expression of *HoxA7*, *HoxB9* and *HoxC6* during gastrula and neurula stages

The overexpression of *eFGF* results in embryos with reduced heads, often with no eyes and enlarged proctodaea (see description in Isaacs et al., 1994). In a few cases, these embryos have duplicated proctodaea or a duplicated posterior axis (see Fig. 3Y,Z). In order to gain a better understanding of the activities of eFGF during gastrula stages and to elucidate the molecular events that underlie this phenotype, we have analysed the expression of a wide range of molecular markers in CSKA-eFGF embryos. These results are summarised in Fig. 1. The Hox genes are known regulators of anteroposterior specification and, significantly, the *Xenopus* homologues of the posterior Hox genes *HoxC6* (*Xlhbox 1*), *HoxA7* (*Xhox36*), and *HoxB9* (*Xlhbox 6)* are all up-regulated in CSKA-eFGF embryos. This up-regulation of Hox genes occurs during gastrula and neurula stages, the time during development at which the anteroposterior axis is patterned (Slack and Tannahill, 1992; Saha and Grainger, 1992). However, not all Hox genes are affected this way, for example *HoxB1*, which has a very restricted anterior domain of expression (Godsave et al., 1994), is not up-regulated in CSKA-eFGF embryos.

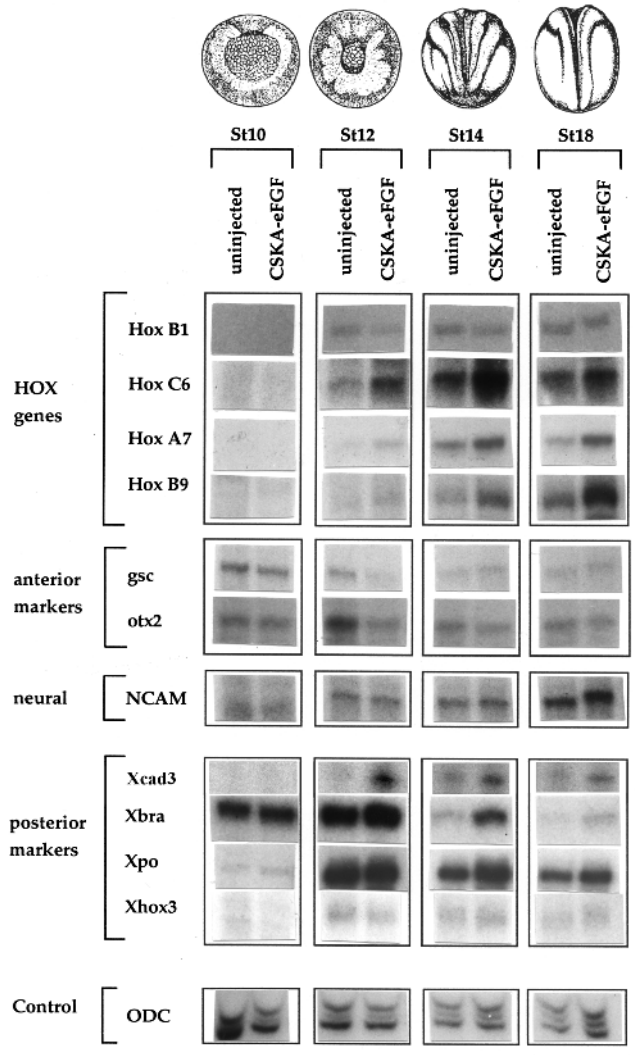

Fig. 1. Gene expression of embryos injected with CSKA-eFGF. RNAase protection analysis of embryos injected at the 2-cell stage with 10 pg of CSKA-eFGF or uninjected control embryos were cultured until early gastrula (stage 10), late gastrula (stage 12), early neurula (stage 14), or late neurula (stage18). 5 μg of total RNA was assayed by RNAase protection analysis for the expression of a panel of regional markers. All assays shown were carried out on RNA from the same experiment. The ODC loading control is a representative example.

Other posterior genes such as *Xcad3* (a *Xenopus* homologue of *Drosophila caudal*), *Xbra* (the *Xenopus* homologue of mouse *Brachyury*) and *Xpo* are also up-regulated in embryos overexpressing *eFGF*. *Xhox3* is another gene encoding a transcription factor that is expressed in the posterior; however its expression is not affected in CSKA-eFGF embryos. So although eFGF up-regulates the expression of many posterior genes, it does not up-regulate all posterior genes.

We looked at the expression of *NCAM* in order to determine whether *eFGF* could directly activate the expression of a general neural marker as opposed to up-regulating the expression of genes expressed in posterior neural regions such as *HoxB9*. *NCAM* is not detected until stage 12 and its expression does not seem to be affected by *eFGF*. We conclude that, although *eFGF* can activate genes expressed in posterior neural tissue, even genes that actively define posterior neural regions, it does not act as a direct general neural inducer in the environment of the intact embryo. Other groups who have shown direct neural induction by FGF have done so following brief disaggregation or in conditions of very low Mg^{2+} and Ca^{2+} but not, as here, in intact embryos (Kengaku and Okamoto, 1995; Lamb and Harland, 1995).

Anteriorly expressed genes respond differently to *eFGF* overexpression. The anterior markers used in this study, *goosecoid* (*gsc*) and *otx2*, encode homeobox-containing transcription factors that are expressed early in the dorsal lip of the blastopore. During subsequent development, *otx2* continues to be expressed in the anterior mesendoderm as well as anterior neurectoderm, while *gsc* expression diminishes during neurulation (Cho et al., 1991; Pannese et al., 1995). Both of these genes are down-regulated by eFGF expression during a short period between stage 12 and 14; however, during later stages, there is no apparent difference between CSKA-eFGF embryos and controls.

In situ hybridisation shows that eFGF causes the anterior spread of Hox gene expression

CSKA-eFGF embryos were analysed by in situ hybridisation on gastrula-stage embryos in order to visualise the extent of *eFGF* expression from the injected plasmid. Fig. 2A shows the normal expression of *eFGF* in a stage11.5 embryo; there is a low level expression in the mesoderm all around the blastore and a higher level of expression in the the dorsal lip of the blastopore including the presumptive notochord. Fig. 2B,C shows *eFGF* expression in stage 11.5 embryos that have been injected at the 2-cell stage with 10 pg of CSKA-eFGF. These embryos show a massive increase of *eFGF* expression around the blastopore as well as ectopic anterior expression of *eFGF* during gastrula stages.

In order to investigate the spatial extent of the misexpression of the posterior Hox genes and other posterior

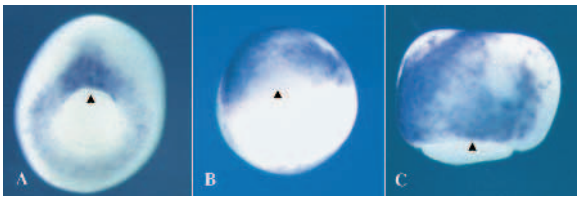

Fig. 2. Analysis of eFGF expression in embryos overexpressing eFGF from the CSKA-eFGF plasmid. (A) Control stage 11.5 embryo showing normal expression of eFGF; vegetal view. (B) CSKA-eFGF embryo at stage 11.5 showing extent of ectopic eFGF expression around the blastopore; vegetal view. (C) CSKA-eFGF embryo at stage 11.5 showing the extent of ectopic eFGF expression; dorsal view. Embryos were injected at the 2-cell stage with 10 pg of CSKA-eFGF. Arrowheads indicate the dorsal lip of the blastopore.

markers, we used whole-mount in situ hybridisation to analyse albino embryos injected with 10 pg of CSKA-eFGF as compared to uninjected control embryos. *HoxA7* is normally activated during gastrulation and expressed in a restricted region around the closing blastopore (see dorsal and side view of stage 13 embryo, Fig. 3A,B). It continues to be expressed in the posterior neural plate and mesoderm through neurula stages (Fig. 3C) and, later, in the spinal cord, pronephros and tailbud (Fig. 3D). In CSKA-eFGF embryos, *HoxA7* expression spreads massively both anteriorly and laterally in the mesoderm, neurectoderm and non-neural ectoderm during neurula stages (Fig. 3E-G). At later stages, *HoxA7* transcript levels are increased in the spinal cord and extend into the hindbrain, while expression is much higher than usual in the tailbud and pronephros (Fig. 3H).

Another posterior Hox gene, *HoxB9*, is first expressed at stage 14 in the posterior neural plate from the blastopore to about half way along the A-P axis, a position that defines the anterior limit of the future spinal cord (Godsave et al., 1994; Keller, 1976) and later continues to be expressed uniformly in the spinal cord through tailbud stages (Godsave et al., 1994) (Fig. 3I-K). In neurula and tailbud-stage embryos overexpressing *eFGF*, the expression of *HoxB9* is expanded both anteriorly along the anteroposterior axis, as well as laterally from the midline (Fig. 3L-N).

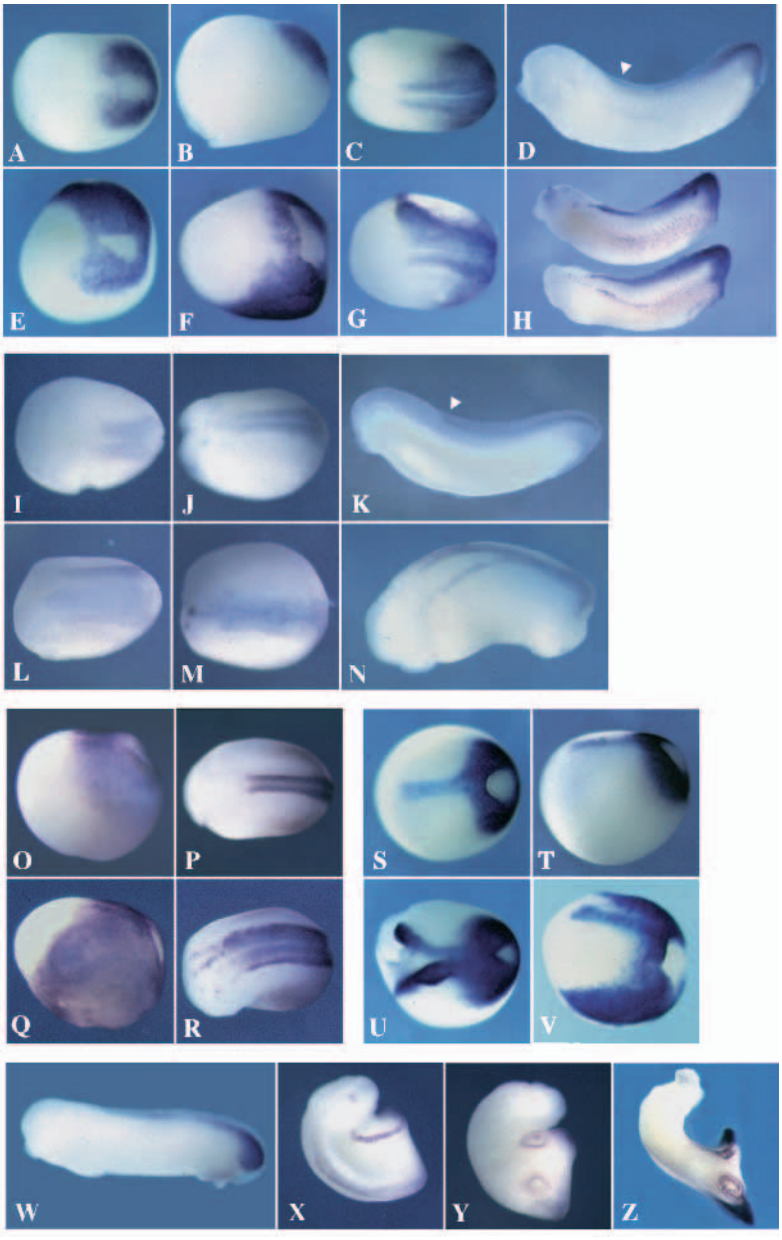

Fig. 3. In situ hybridisation analysis of albino embryos injected at the 2-cell stage with 10 pg of CSKA-eFGF or of uninjected controls. Embryos were cultured until early neurula, late neurula or tailbud stages and were then hybridised to antisense DIG labelled probes for *HoxA7* (A-H); *HoxB9* (I-N); *Xcad3* (O-P, W-Y); and *Xbra* (S-V). Controls (A-D, I-K, O-P, S-T) are shown above the corresponding CSKA-eFGF embryos (E-H, L-N, Q-R, U-V) except for the control for *Xcad3* expression in tailbud-stage embryos (W) which is shown beside the corresponding expression of *Xcad3* in CSKA-eFGF tailbud-stage embryos(X-Y). (Z) Ventral view of another CSKA-eFGF embryo with a duplicated posterior axis hybridised to *Xpo* which also marks the tailbud and proctodaea. Arrowheads indicate the anterior limit of the normal expression of *HoxA7* and *HoxB9*, respectively, at tail bud stages. Anterior is to the left is all cases.

These results show that the elevation of expression level of posterior Hox genes involves both an increase of expression in the normal domain and a spread of expression toward the anterior. Similar effects are found for *HoxB9*, whose expression is largely confined to the neurectoderm (Godsave et al., 1994), and *HoxA7*, whose expression also embraces various mesodermal tissues. This spread, during the stages at which the anteroposterior axis is being patterned, very likely underlies the characteristic posteriorised phenotype of the CSKA-eFGF embryos. This notion is supported later in this study when we show that injection of *HoxA7* mRNA results in embryos with a suppressed head.

Spatial extent of *Xcad3* and *Xbra* expression in embryos overexpressing eFGF

In Fig. 1, we show that overexpression of *eFGF* also up-regulates the expression of posterior markers *Xcad3* and *Xbra*. *Xcad3* is the *Xenopus* homologue of the *Drosophila caudal*, a

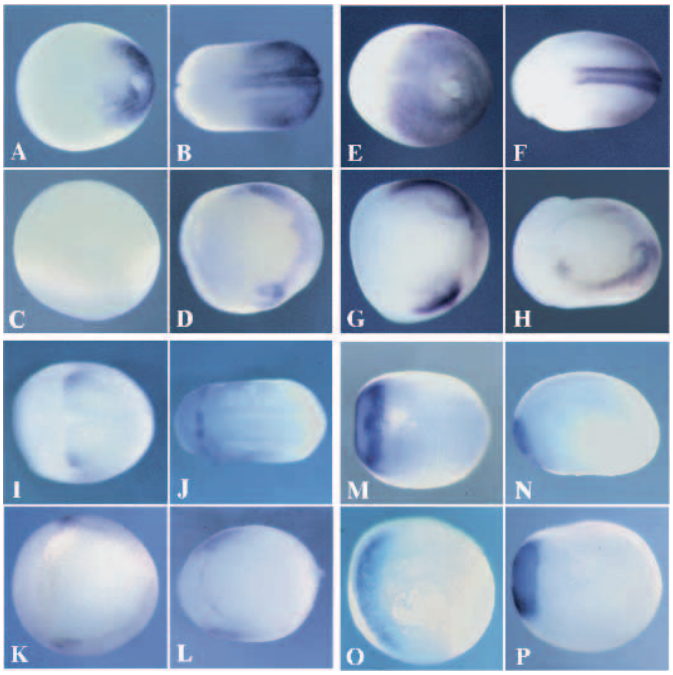

Fig. 4. In situ hybridisation analysis of regional markers in albino embryos injected with 2ng of mRNA encoding the dominant negative form of the FGF receptor. Embryos were cultured to early neurula (stage13) (A-C; E-G; I-K; M-O) and late neurula (stage 20) (B-D; F-H; J-L; N-P). Control embryos (A-B; E-F; I-J; M-N) are shown above embryos overexpressing the dominant negative form of the FGF receptor (C-D; G-H; K-L; O-P). The markers are *HoxA7* (A-D), *Xcad3* (E-H), and *HoxB1* (I-L) and *otx2* (M-P). In all cases, anterior is to the left. Embryos are viewed dorsally in all but N which is a side view, O and P which are ventral views, and E which is a dorsal-posterior view. C,D,G,H,K,L are viewed down onto the open blastopore.

homeobox gene that is expressed in the posterior of the *Drosophila* embryo (Mlodzik et al., 1985; Mlodzik and Gehring, 1987). Mutants of *caudal* lose posterior structures and *Drosophila* embryos overexpressing *caudal* show loss of head structures (McDonald and Struhl, 1986; Mlodzik et al., 1990). A mouse homologue of *caudal*, *cdx-1*, has been shown directly to activate Hox gene expression while disruption of *cdx-1* leads to the posterior shift of Hox gene expression (Subramanian et al., 1995). We present data here that supports the notion that *Xcad3* plays a similar role in regulating Hox genes in *Xenopus*, downstream of FGF signalling. *Xcad3* is normally expressed in the posterior of the embryo starting around the blastopore during early gastrula stages (Northrop and Kimelman, 1994). Fig. 3O,P shows the normal expression of *Xcad3* in neurula-stage embryos. In embryos overexpressing eFGF, the expression spreads anteriorly to encompass most of the antero-posterior axis (Fig. 3Q,R).

Brachyury is known to be essential for posterior development in mouse and zebrafish; in *T* mutants in mouse and *no tail* mutants in zebrafish, the most anterior somites form but embryos lack trunk and tail structures (Beddington et al., 1992; Schulte-Merker et al., 1994). In *Xenopus*, overexpression of a dominant interfering *Xbra* construct shows striking similarities to the genetic mutants in mouse and fish (Conlon et al., 1996). The normal expression of *Xbra* during early neurula stages is shown dorsally and from the side in Fig. 3S,T. CSKA-eFGF embryos, at this stage, often show ectopic regions of *Xbra* expression and, in many cases, the cells that are expressing *Xbra* ectopically seem to have undergone, or attempted to undertake, some convergent extension-like movements (Fig. 3U) resulting in long, ectopic, 'notochord-like' domains of *Xbra* expression. There is also a general spread of *Xbra* expression around the blastopore (Fig. 3V). We do not fully understand these streaks of ectopic *Xbra* expression, but they may represent an early stage in the formation of a duplicated posterior axis, which does not proceed to completion in these specimens. During tailbud stages, *Xcad3* is expressed in both the tailbud and the proctodaeum (Fig. 3W). Some CSKA-eFGF embryos have duplicated posterior axes and these are characterised by the expression of *Xcad3* in two tailbuds (as viewed dorsally in Fig. 3X) as well as two proctodaea (as viewed ventrally in Fig. 3Y). *Xpo* is another gene expressed in the proctodaeum and is shown here to confirm that these duplicated structures really are proctodaea (Fig. 3Z).

Expression of posterior but not anterior genes requires a functional FGF signalling pathway

Overexpression of a dominant negative form of the FGF receptor blocks FGF signalling and highlights those processes for which FGF signalling is necessary (Amaya et al., 1991, 1993; Isaacs et al., 1994). We show here that the posterior Hox genes require a functional FGF signalling pathway for their activation while certain other genes, in particular those genes involved in patterning the anterior regions, are independent of FGF signalling.

At stage 13 (Fig. 4A,C), embryos that have been injected with the dominant negative FGF receptor do not express *HoxA7* (Fig. 4C). By stage 20 (Fig. 4B,D), some expression has appeared in the posterior part of the embryo around the open blastopore (Fig. 4D), possibly due to the decay of the injected XFD mRNA and the subsequent re-establishment of an FGF signalling pathway. Alternatively, the recovery of *HoxA7* expression may be due to the activation of later regulators of *HoxA7*. The dorsal expression of *Xcad3* is also sensitive to inhibition of the FGF signalling pathway (Fig. 4E-H; Northrop and Kimelman, 1994). At both stage 13 and stage 20, embryos that have been injected with the dominant negative FGF receptor do not express *Xcad3* dorsally (Fig. 4G,H). The persistence of *Xcad3* expression in the ventral region of XFD-injected embryos has been shown to be due to the activity of additional ventral regulators, perhaps including BMP-4 (Northrop et al., 1995).

By contrast, expression of *HoxB1* and *otx2* is normal in embryos where FGF signalling is inhibited. *HoxB1* is normally expressed in an anterior stripe on both sides of the midline in the early neurula (Fig. 4I) and is refined to rhombomere 4 in the late neurula, early tail bud stage (Fig. 4J). In embryos overexpressing the dominant negative FGF receptor, although the blastopore does not close, two stripes of *HoxB1* expression can still be seen on either side of the open blastopore at both early (Fig. 4K) and late neurula stages (Fig. 4L). *otx2* is a homeobox gene that is expressed in the very anterior part of the embryo and is thought be involved in patterning the head. The normal expression of *otx2* at early neurula stage 14 is shown from a dorsal view (Fig. 4M) and a later neurula (stage 20) as viewed from the side (Fig. 4N). The embryos below (Fig. 4O,P) are

embryos of corresponding stage14 and 20 that have been injected with mRNA encoding the dominant negative FGF receptor, where the anterior domain of *otx2* expression is still apparent.

The results from these experiments are consistent with those from the overexpression experiments. Posterior genes, such as *Hox A7* and *Xcad3*, are normally dependent on FGF signalling (at least dorsally), and their expression is both elevated and spatially extended by overexpression of *eFGF*. Anterior genes (like *HoxB1* and *otx2*) behave differently in as much as they are not sensitive to inhibition of FGF signalling and are not strongly affected by overexpression of eFGF.

Anterior eFGF bead implants during early neurula stages lead to anterior ectopic expression of *HoxA7* and *Xcad3*

The application of FGF bound to a heparin acrylamide bead allows one to present ectopic eFGF in any region of the embryo at a defined time during development (Cohn et al., 1995). To look at the activity of eFGF presented later than in CSKA-eFGF-injected embryos, in which high levels of eFGF accumulate by stage 10 (Isaacs et al., 1994), we have performed a number of bead implantations into the anterior neural plate of intact late gastrula embryos. In Fig. 5A, the top embryo shows the normal expression of *HoxA7*, while the bottom embryo has had an eFGF bead implanted into the anterior neural plate at stage 12.5 and shows anterior ectopic expression of *HoxA7* associated with the eFGF bead. Embryos implanted with control PBS soaked beads show no alteration in gene expression (data not shown). *Xcad3* responds to eFGF bead implantation in much the same way as *HoxA7*. In Fig. 5B, the top control embryo shows the normal expression of *Xcad3*, while the bottom embryo shows the anterior extension of *Xcad3* expression in embryos where an eFGF bead has been implanted into the anterior neural plate.

eFGF beads activate Hox gene expression in gastrula- and neurula-stage explants

All of the experiments described so far were conducted on intact embryos. In order to reduce the complexity of the responding system, we have also studied the effects of eFGF on gastrula- and neurula-stage explants. Pieces of marginal zone were dissected out at early gastrula stages and pieces of neural plate from early neurula stages, and the expression of anterior, posterior and neural markers were analysed in response to eFGF (Fig. 5C,D). The marginal zone (stage 10) and neural plate (stage 13) explants were combined with eFGF beads, or control PBS beads, as sandwiches, in order to allow better and prolonged access of the protein to the tissue (Fig. 5C,D).

Fig. 5C shows the effects of an eFGF bead on dorsal marginal zone explants. These explants do not include the dorsal lip of the blastopore, but do contain both presumptive mesoderm and neurectoderm. They were dissected out at stage 10, combined with eFGF or control beads as sandwiches and cultured to the appropriate neurula stages to assay effects on the expression of posterior Hox genes. When these explants are cultured alone or with beads soaked in PBS, there is low level expression of *HoxC6*, *A7* and *B9*. However, when these explants are exposed to eFGF, the expression of all of these posterior Hox genes is up-regulated, while the expression of *NCAM* is unaffected.

Xbra is also expressed in these marginal zone explants and is up-regulated by eFGF, and therefore it is not possible to distinguish in this experiment whether eFGF is having its effect through the mesoderm or is directly affecting ectoderm. However, we also show that neural explants which do not contain mesoderm can respond to eFGF by up-regulating the expression of posterior Hox genes (Fig. 5D). Explants from anterior/middle regions of neural plate, which are fated to form midbrain and some forebrain and hindbrain (Eagleson and Harris, 1990), were taken from early neurula and cultured as a sandwich around a bead. By stage 20, the expression of *HoxA7* and *HoxC6* was found to be increased in response to eFGF but not PBS beads (Fig. 5D). From the results of CSKA overexpression and bead implantation, both in whole embryos and in explants from gastrulae and neurulae, we conclude that eFGF can activate the expression of posterior Hox genes in anterior neurectoderm during the normal time that the anteroposterior axis is being patterned.

Implants of eFGF beads into gastrulae show distinct effects on dorsal and ventral development

We have also used heparin acrylamide beads soaked in eFGF protein implanted around the blastopore during gastrula stages to distinguish distinct dorsal and ventral roles of eFGF. When an eFGF bead is implanted into the dorsal lip of a stage 11.5 embryo, the result is a tailbud-stage embryo which shows the loss of eyes and forebrain and other anterior structures but with a normal proctodaeum (Fig. 6A). This demonstrates that the head suppression seen in CSKA-eFGF embryos is a dorsal effect of eFGF. However, when an eFGF bead is implanted into the ventral lip at stage 11.5, the result is a normal axis with normal head structures and a very enlarged proctodaeum (Fig. 6B). Therefore, the enlargement of the proctodaeum is a ventral effect. Control beads soaked in PBS and implanted around the blastopore had no effect and embryos developed normally (data not shown).

The effects of an eFGF bead implanted dorsally at stage 11.5 were analysed by in situ hybridisation to see if Hox gene expression is altered in the same way as in CSKA-eFGF-injected embryos. Fig. 6C shows expression of *HoxA7* in the top control neurula embryo, while the bottom embryo shows *HoxA7* expression in a neurula embryo where an eFGF bead was implanted into the dorsal lip of the blastopore at stage 11.5. Fig. 6D shows expression of *Xcad3* in the top control neurula-stage embryo, while the bottom embryo shows *Xcad3* expression in an embryo where an eFGF bead was implanted into the dorsal lip of the blastopore at stage 11.5. The extent of expression of *HoxA7* and *Xcad3* is expanded anteriorly in embryos implanted with an eFGF bead; as is the case with embryos overexpressing *eFGF* from a plasmid. Although at stage 11.5 much of the dorsal mesoderm has already involuted, it has not undergone the considerable elongation that occurs during later gastrula and neurula stages. It is therefore likely that the FGF released from the implanted bead still can influence much of the prospective dorsal axis.

These bead experiments indicate that there are distinct roles for eFGF in dorsal and ventral regions and that it is the dorsal activities of eFGF that regulate anteroposterior patterning of the embryonic axis. Such timed bead experiments clearly show that eFGF has the ability to activate the expression of posterior genes long after the period of mesoderm induction.

Dorsal overexpression of *HoxA7* or *Xcad3* results in anterior truncation

The above data are highly suggestive of a close relationship between the activity of eFGF and the regulation of Hox genes. Since anterior genes are largely unaffected but posterior genes are shifted anteriorly in their expression, it follows that some unnatural combinations of gene activity will be generated in anterior regions. It is not possible to predict the anatomical consequences of unnatural combinations and so we have carried out further experiments to examine the phenotype arising from overexpression of *HoxA7*, the Hox gene among our panel that is most sensitive to *eFGF* overexpression. These experiments were performed by using synthetic mRNA, rather than the CSKA plasmid, because the mosaic expression from the plasmid means that it is not suitable for overexpression of cell autonomous molecules such as transcription factors.

Microinjection of synthetic mRNA coding for *HoxA7* does result in a phenotype similar to the anterior phenotype of the CSKA-eFGF embryos. The embryos for the most part gastrulate normally and close their blastopores, while later during neurula and tailbud stages it is apparent that there are severe anterior truncations. We have compared the effects of dorsal and of ventral overexpression by making injections into both dorsal or both ventral blastomeres at the four cell stage. The dorsal injections cause anterior truncations while the ventral injections have little or no effect on the development of the embryos (Fig. 7A,B). To more clearly determine where *HoxA7* must be expressed in order to result in these anterior truncations, mRNA encoding *β–galactosidase* was co-injected with *HoxA7* either in the two dorsal (Fig. 7C,D) or ventral (Fig. 7E,F) blastomeres at the 4-cell stage. Fig. 7C shows that when *β-gal* and *HoxA7* are co-expressed in the dorsal midline, the neural plate shows delayed closure. At tadpole stages (Fig. 7D), it is clear that anterior structures are lost in embryos that express *HoxA7* in dorsal anterior regions. By contrast, embryos injected ventrally with the same dose of *HoxA7* and *β-gal* mRNA develop normally (Fig. 7E,F).

As discussed earlier, we predict that *Xcad3* is a downstream effector of FGF signalling involved in regulating Hox gene expression and, in support of this, we show here that microinjection of *Xcad3* mRNA, like that of *HoxA7*, results in anterior truncations. Fig. 7G show that when 100 pg of *Xcad3* mRNA is injected into each of the two dorsal blastomeres at the 4-cell stage, the resulting embryos show dramatic anterior truncations. This supports the notion that Xcad3, like eFGF and HoxA7, plays a role in patterning the anteroposterior axis. Furthermore, when the same dose is injected ventrally, the axis develops largely unaffected, however, in contrast to ventral *HoxA7* mRNA injections, the size of the proctodaeum is increased (Fig. 7H).

A molecular pathway regulating anteroposterior pattern

In order to examine how the targets of FGF signalling interact, we have undertaken a series of mRNA injection experiments followed by analysis by RNAase protection at gastrula and early neurula stages when the patterning of the anteroposterior axis occurs (Slack and Tannahill, 1992). The results of these experiments are summarised in Fig. 9 and support the following pathway: eFGF→Xcad3→HoxA7, while Xbra has been determined not to be directly involved in this regulatory pathway. As described previously, inhibition of the FGF signalling pathway by overexpression of a dominant negative FGF receptor (XFD) results in the down regulation of *Xcad3*, *HoxA7* and *Xbra* (Amaya et al., 1993; Isaacs et al., 1994; Northrop et al., 1994). We show here that injection of *Xcad3* mRNA does not up-regulate expression of *eFGF* or *Xbra*, but does result in high precocious levels of *HoxA7* expression at stage 10 (Fig. 8A). Not only can injection of *Xcad3* up-regulate expression of *HoxA7* in normal embryos, it but can also rescue expression of *HoxA7* in embryos where FGF signalling has been blocked (Fig. 8B), demonstrating that *Xcad3* regulates Hox gene expression downstream of FGF. In contrast, injection of *HoxA7* mRNA has no effect on expression of *eFGF*, *Xbra* or *Xcad3* (Fig. 8C) indicating that none of the genes analysed are targets of HoxA7.

Xbra is a known target for FGF signalling; however, we show here that injection of *Xbra* in embryos where FGF signalling has been blocked does not rescue *HoxA7* expression (Fig. 8D), indicating that *Xbra* is not directly involved in the FGF-dependent pathway regulating Hox gene expression. The data presented in this paper has allowed us to propose a general model, discussed below, in which a common molecular pathway involving FGF signalling, *cdx* genes and Hox genes pattern the anteroposterior axis in vertebrates (Fig. 9).

DISCUSSION

Classical grafting experiments suggest that the anteroposterior axis of amphibian embryos is patterned during late gastrula and early neurula stages through inductive signals derived from the dorsal mesoderm (reviewed by Slack and Tannahill, 1992). The model of Nieuwkoop (1952) for the patterning of the neural plate has been supported by a variety of more recent experiments. It involves an initial 'activation' step in which neuroepithelium of anterior (forebrain) character is induced, followed by a 'transformation' step in which tissue at successive anteroposterior levels becomes promoted to more and more posterior character in response to signals from the posterior. The availability of molecular markers, purified growth factors and techniques such as plasmid-based overexpression, now allow us to examine the molecular nature of the signals responsible for these events.

eFGF can activate ectopic Hox gene expression during gastrula and neurula stages

The Hox genes not only provide excellent anterior and posterior markers but, moreover, are regulators of anteroposterior cell fate. Regional identity is generally thought to be conferred by the combined expression of Hox genes defining a specific anteroposterior code (see McGinnis and Krumlauf, 1992), although recent work in *Caenorhabditis elegans* has suggested other possible mechanisms for Hox gene regulation of anteroposterior pattern (Cowing and Kenyon, 1996). In any case, targeted mutagenesis of many members of the Hox gene family confirms that they have an important role in patterning the vertebrate anteroposterior axis whatever the mechanism (for review see Krumlauf, 1994).

Up regulation and ectopic expression of the posteriorly expressed Hox genes occurs both when *eFGF* is overexpressed

throughout the embryo from a plasmid, as well as in regions of embryos that have been implanted with an eFGF-loaded bead. In this study, we show eFGF can regulate the expression of Hox genes that are primarily expressed in the neurectoderm (*HoxB9*), as well as those that are expressed in both the mesoderm and neurectoderm (*HoxA7* and *HoxC6*). Furthermore, in embryos lacking a functional FGF signalling pathway, the expression of posterior Hox genes is down-regulated.

Recently the mouse homologue of *Drosophila caudal*, *cdx-1*, has been shown directly to regulate *HoxA7*, while disruption of the mouse *cdx-1* gene leads to anterior homeotic transformations of vertebrae and a posterior shift in Hox gene expression (Subramanian et al., 1995). A *Xenopus* homologue of *cdx-1*, *Xcad3*, is expressed in the posterior mesoderm and neurectoderm. We show here that injection of *Xcad3* mRNA causes up-regulation of *HoxA7* expression and results in loss of anterior structures. The response of *Xcad3* to overexpression of *eFGF* parallels that described for *HoxA7*. *Xcad3* expression is up-regulated during gastrula and neurula stages when excess eFGF is provided as a protein on a bead or overexpressed from a plasmid. Overexpression of the dominant negative FGF receptor down-regulates dorsal expression of *Xcad3* while ventral expression continues somewhat, probably due to the presence of BMP-4 signalling (Northrop et al., 1995). We also demonstrate that *Xcad3* can activate Hox gene expression and, moreover, that *Xcad3* can rescue Hox gene expression in the absence of FGF signalling.

Our studies show that the anterior neural plate, both in explant culture and within the developing embryo, can up-regulate the expression of a number of posterior genes in response to eFGF during late gastrula as well as early neurula stages. Saha and Grainger (1992) demonstrate that the A-P pattern of the developing dorsal axis is not firmly established until neurula stages. Our findings that *eFGF* can activate posterior Hox genes in anterior regions of the neurectoderm within the embryo during late gastrula and early neurula stages are in keeping with such developmental lability. The posteriorised CSKA-eFGF phenotype is very likely to arise from the increased expression of these posterior Hox genes in anterior regions of the embryo.

A regulatory pathway for vertebrate anteroposterior specification

In this paper, we demonstrate that *Xcad3* and *HoxA7* are members of a regulatory pathway downstream of FGF signalling that is involved in patterning the anteroposterior axis. Our work shows that *Xcad3* is a downstream effector of FGF signalling that regulates *HoxA7*. This is consistent with

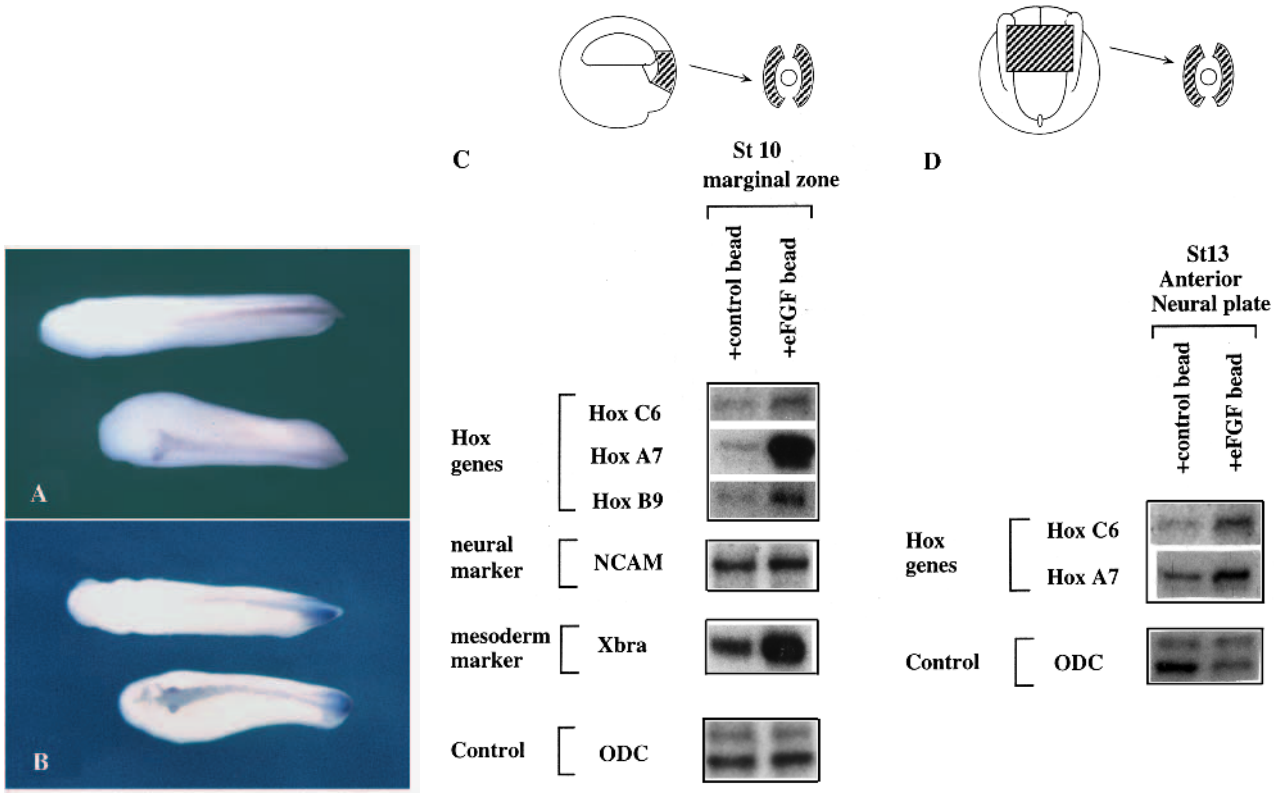

Fig. 5. eFGF beads activate anterior expression of posterior Hox genes in mesoderm and neurectoderm. (A) In situ hybridisation analysis of *HoxA7* expression in control tailbud embryo (top) or tailbud-stage embryo where an eFGF bead was implanted into the anterior neural plate at stage12.5 (bottom). Dorsal view; anterior to the left. (B) In situ hybridisation analysis of *Xcad3* expression in control tailbud embryo (top) or tailbud-stage embryo where an eFGF bead was implanted into the anterior neural plate at stage12.5 (bottom). Dorsal view; anterior to the left. (C) The dorsal marginal zone region, not including the dorsal lip, was taken from stage 10 embryos. Two explants were used to make a sandwich around an eFGF bead and were cultured until stage14 (to assay expression of *Xbra*) or stage 20 (to assay expression of *HoxC6*, *HoxA7*, *HoxB9* and *NCAM*) and processed for RNAase protection analysis. (D) The anterior and middle neural plate was taken from stage 13 embryos. Two explants were used to make a sandwich around an eFGF bead, and were cultured until stage 20 at which time they were processed for RNAase protection analysis to assay the expression of *HoxC6* and *HoxA7*.

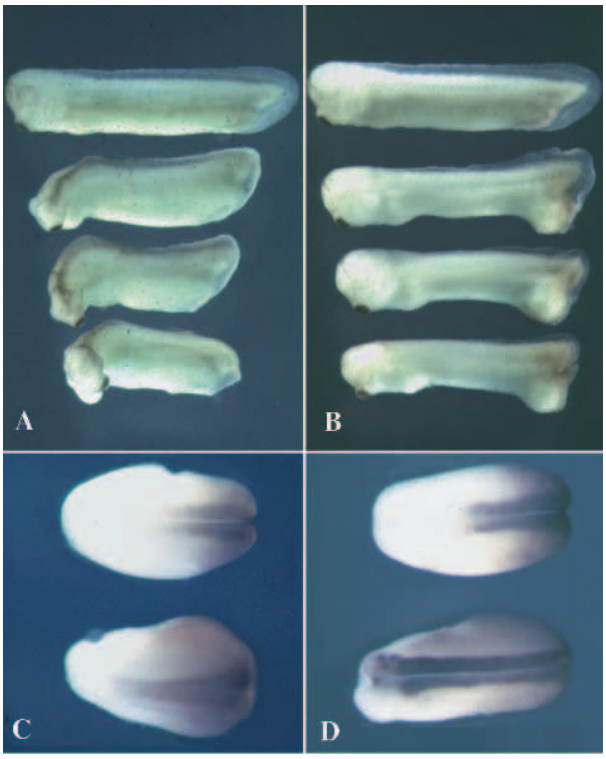

Fig. 6. Phenotypes and gene expression in embryos implanted with a heparin acrylamide bead loaded with eFGF protein. (A) Embryos were implanted with a bead into the dorsal blastopore lip at stage 11.5. The top embryo was implanted with a PBS bead, while each of the bottom three embryos were implanted with an eFGF bead and show head suppression. (B) Embryos were implanted with a bead into the ventral blastopore lip at stage 11.5. The top embryo was implanted with a PBS bead, while each of the bottom three embryos were implanted with an eFGF bead and show enlarged proctodaea. (C, D) In situ hybridisation of embryos. Both embryos were cultured to late neurula stages and hybridised to *HoxA7* probe (C) or *Xcad3* probe (D). The top embryo is a control, the bottom embryo had an eFGF bead implanted into the dorsal blastopore at stage 11.5.

findings in mouse where putative cdx-binding sites have been found in the regulatory regions of a number of Hox genes and it has been shown that cdx can transactivate a *HoxA7* reporter construct in vitro (Subramanian et al., 1995).

Our data support a model in which FGF signalling has distinct dorsal and ventral roles in regulating *cdx* and Hox genes (Fig. 9). *eFGF* is expressed in the dorsal mesoderm, specifically, in the notochord and in the posterior mesoderm around the closing blastopore (Isaacs et al., 1995). We propose that FGF signalling is required for the expression of members of the *cdx* gene family in the developing dorsal axis and that the *cdx* genes regulate expression of the posterior Hox genes during normal development.

It has been demonstrated that eFGF regulates transcription of *Xbra* during gastrula stages and that Xbra can in turn activate *eFGF* expression (Isaacs et al., 1994; Schulte-Merker and Smith, 1995). The work in this paper indicates that Xbra does not directly activate Hox gene expression. However, at the very least, Xbra clearly plays an indirect role in antero-posterior specification through its regulation of *eFGF* expression in the notochord and the posterior of the embryo.

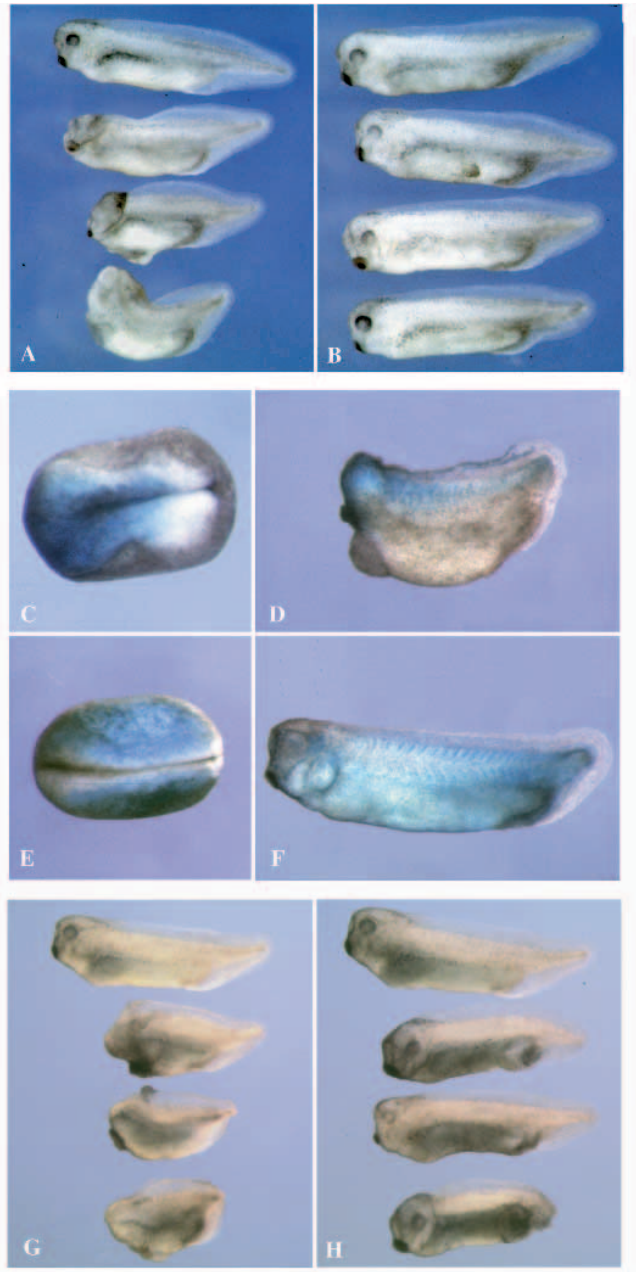

Fig. 7. Injection of *HoxA7* mRNA dorsally, but not ventrally, results in head suppression in embryos. (A) The top embryo is an uninjected control while the bottom embryos have been injected with 1ng of *HoxA7* into the dorsal two blastomeres at the four-cell stage. (B) All the embryos shown in this panel have been injected with 1 ng of *HoxA7* into the ventral two blastomeres at the four-cell stage. (C) Stage 20 embryo co-injected with 1 ng *HoxA7* and 500 pg of β-gal mRNA in to the dorsal two blastomeres at the 4-cell stage. (D) Tailbud embryo that had been co-injected with 1 ng *HoxA7* and 500 pg of β-gal mRNA in to the dorsal two blastomeres at the 4-cell stage. (E) Stage 20 embryo co-injected with 1 ng *HoxA7* and 500 pg of β-gal mRNA in to the ventral two blastomeres at the 4-cell stage. (F) Tailbud embryo that had been co-injected with 1 ng *HoxA7* and 500 pg of β-gal mRNA into the ventral two blastomeres at the 4-cell stage. (G) Top embryo is a control, while the bottom three embryos have been injected with 200 pg of *Xcad3* mRNA into the dorsal two blastomeres at the 4-cell stage. (H) Top embryo is a control, while the bottom three embryos have been injected with 200 pg of *Xcad3* mRNA into the ventral two blastomeres at the 4-cell stage.

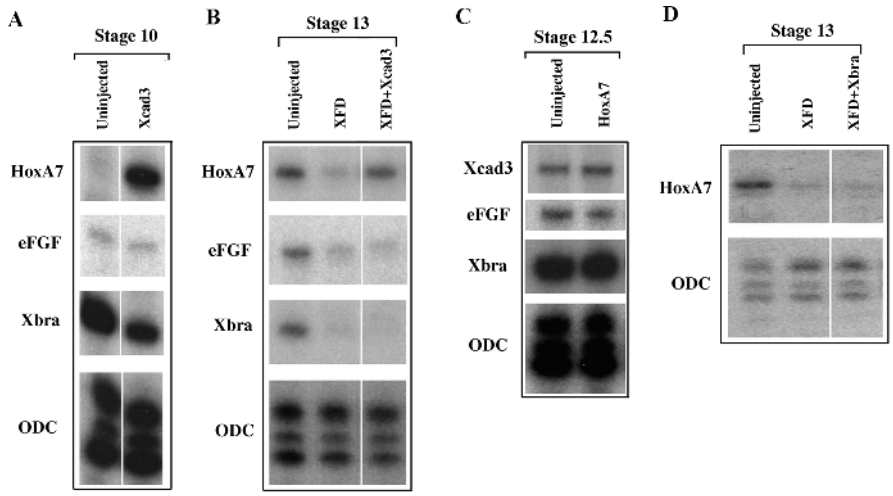

Fig. 8. *Xcad3* regulates Hox gene expression downstream of FGF signalling. (A) Injection of *Xcad3* mRNA causes the early activation of *HoxA7* expression, but does not effect the expression of *eFGF* or *Xbra*; 400pg of Xcad3 was injected at the 4-cell stage, RNA from embryos was collected for analysis at stage10. (B) Injection of Xcad3 mRNA can rescue the expression of *HoxA7* in embryos where the FGF signalling pathway has been blocked (XFD-injected). 400 pg of Xcad3 was injected at the 4-cell stage ± 2 ng of XFD mRNA. RNA from embryos was collected for analysis at stage13. (C) Injection of *HoxA7* mRNA does not affect the expression of *eFGF*, *Xbra* or *Xcad3*. 2 ng of *HoxA7* mRNA was injected at the 4-cell stage and RNA from embryos was collected for analysis at stage12.5 (D) Injection of *Xbra* mRNA does not rescue the expression of *HoxA7* in embryos where the FGF signalling pathway has been blocked (XFD-injected). 2 ng of *Xbra* mRNA was injected at the 4-cell stage ± 2 ng of XFD mRNA. RNA from embryos was collected for analysis at stage13. All hybridisations were done with 10 μg of total RNA.

Overexpression of *eFGF* leads to enlarged proctodaea when overexpressed globally by a plasmid or supplied ventrally on a bead. Ventral injection of mRNA from a member of the *cdx* gene family also results in an enlarged proctodaeum, while ventral injection of *HoxA7* mRNA does not. FGF inhibition studies show that ventral *cdx* expression requires regulators other than FGF. The work of Northrop et al. (1995) suggests that the secreted factor BMP4 is likely one of these regulators. The genes downstream of FGF and *cdx* required for proctodaea induction remain unknown, but may include *Xpo* or *HoxD13*, which are other transcription factors known to be expressed in the proctodaeum (Sato and Sargent, 1989; van der Hoeven et al., 1996; Kondo et al., 1996).

Certainly there are other members of the pathway described here, as well as other separate pathways regulating anteroposterior specification; for example, injection of *Xhox3* mRNA results in embryos with anterior truncations (Ruiz i Altaba and Melton, 1989b) similar to those seen in CSKA-eFGF. However, we know that *Xhox3* expression is not dependent on FGF signalling for its expression (Isaacs et al., 1994) and this study shows that *Xhox3* expression is not up-regulated by eFGF. This indicates that *Xhox3* is a member of a distinct pathway involved in patterning the posterior axis.

Ectopic, anterior expression of Hox genes results in suppression of the head

Head suppression is evident during later development in CSKA-eFGF embryos with the notable loss of eyes and forebrain in tailbud-stage embryos. However, the early expression of anterior markers like *otx2* and *gsc* is only slightly down-regulated during late gastrula and early neurula stages and, at later stages, their expression is largely unaffected. In addition, *otx2* expression is indifferent to ablation of FGF signalling.

One explanation for the loss of anterior stuctures in CSKA-eFGF embryos is that it is due to the ectopic anterior expression of Hox genes, such as *HoxA7*. It has been proposed that specification of the anterior regions of the head involves the expression of a combination of *otx2* and other members of this class of genes (*otx1*, *emx1* and *emx2*), which provide the region with a positional code in much the same way that the Hox genes are believed to pattern the hind brain and spinal cord (Holland et al., 1992). The anterior suppression could arise because a 'nonsense' coding is generated, consisting of expression both of the anterior genes and of the posterior Hox genes together. This is supported by Pannese et al. (1995) who have recently shown that overexpression of *otx2* results in posterior truncations and which could also be due to a nonsense coding in the posterior. In this paper, we show that overexpression of *HoxA7* in the head results in suppression of anterior structures similar to those caused by overexpression of *eFGF*.

An alternative explanation is that the repression of genes like *otx2* by FGF is responsible for the loss of anterior structures. We do see a transient reduction of *otx2* expression in CSKA-eFGF embryos during gastrulation (Fig. 1). This is in keeping

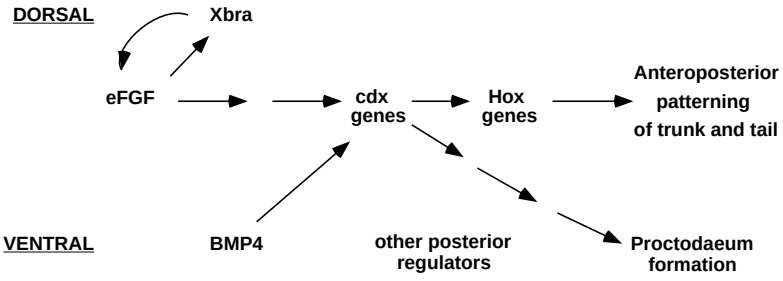

Fig. 9. A molecular pathway depicting the role of FGF signalling in patterning the anteroposterior axis during gastrula stages. FGF signalling is important in regulating the dorsal expression of members of the cdx family, which directly regulate Hox gene expression required for patterning the anterposterior axis. *Brachyury* expression is known to be dependent on FGF signalling and also feeds back to activate the expression of *FGF* during gastrula stages, however, *Brachyury* does not appear to be a direct regulator of Hox gene expression. FGF and other signals, such as BMP4, activate ventral expression of cdx and other posterior regulators which pattern ventroposterior mesoderm and induce the proctodaeum.

with a study from another group, which also shows that *otx2* expression is repressed in animal caps exposed to bFGF (Lamb and Harland, 1995).

eFGF acts as a transforming signal during anteroposterior specification

Experiments presented in this study indicate a role for eFGF in patterning the anteroposterior axis. Other recent studies have also suggested that the FGFs are involved in this process. Kengaku and Okamoto (1995) have argued that bFGF can fulfill both the role of activating signal as well as the transforming signal in Nieuwkoop's model of A-P specification. Another study (Lamb and Harland, 1995) reports that bFGF can directly induce neural tissue, and that in combination with the secreted neural inducer *noggin* (Lamb et al., 1993) can neuralise and pattern ectodermal explants. Furthermore, Cox and Hemmati-Brivanlou (1995) have shown that bFGF can mimic the in vivo transforming or posteriorising signal.

The studies showing direct neural induction by bFGF involve culturing animal cap explants in a disaggregated state or in low calcium and magnesium media, conditions which evoke the expression of *XAG-1*, a gene expressed in the cement gland, indicating that the culture conditions are not completely neutral. Consistent with this, it has been shown that complete cell disaggregation is sufficient to activate neural specification in earlier stage ectodermal cells (Grunz and Täcke, 1989; Godsave and Slack, 1991). In contrast to the above studies, we find that although eFGF has a very strong activity in up-regulating the expression of posterior genes, it does not directly activate the expression of *NCAM* in the context of the whole embryo. Our work indicates that the FGFs do not induce neural tissue directly and that the 'activating' signal of Nieuwkoop's model is probably composed of other secreted factors like noggin, follistatin or chordin (Smith and Harland, 1992; Lamb et al., 1993; Hemmati-Brivanlou et al., 1994; Holley et al., 1995).

Several members of the FGF family that have been identified in *Xenopus* are candidates to be involved in the processes described above (Kimelman et al., 1988; Isaacs et al., 1992; Tannahill et al., 1992; Song and Slack, 1996). bFGF has been used in the recent studies of a possible role for FGF in neural induction. However, the expression of *bFGF* is not spatially restricted to regions that would suggest a role in anteroposterior patterning (Song and Slack, 1994). Furthermore, bFGF does not have a recognised signal sequence and is not efficiently secreted from cells. By contrast, in this study, we use eFGF, which is secreted efficiently (Isaacs et al.,1994) and is expressed in regions, such as the posterior notochord and closing blastopore (Isaacs et al., 1995), making it a better in vivo candidate for these activities. We provide evidence here that eFGF is very likely responsible for at least part of the 'transforming' signal by regulating a molecular pathway involving the activation of expression of *cdx* and Hox genes.

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