Patterning the forebrain: FoxA4a/Pintallavis and Xvent2 determine the posterior limit of *Xanf1* expression in the neural plate

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Summary

During early development of the nervous system in vertebrates, expression of the homeobox gene *Anf/Hesx1/Rpx* is restricted to the anterior neural plate subdomain corresponding to the presumptive forebrain. This expression is essential for normal forebrain development and ectopic expression of *Xenopus Anf*, *Xanf1* (also known as *Xanf-1*), results in severe forebrain abnormalities. By use of transgenic embryos and a novel bi-colour reporter technique, we have identified a cis-regulatory element responsible for transcriptional repression of *Xanf1* that defines its posterior expression limit within the neural plate. Using this element as the target in a yeast one-hybrid system, we identified two transcription factors, FoxA4a/Pintallavis and Xvent2 (also known as Xvent-2), which are normally expressed posterior to *Xanf1*. Overexpression of normal and dominant-negative versions of these factors, as well as inhibition of their mRNA translation by antisense morpholinos, show that they actually function as transcriptional repressors of *Xanf1* just behind its posterior expression limit. The extremely high similarity of the identified *Anf* cis-regulatory sequences in *Xenopus*, chick and human, indicates that the mechanism restricting posterior expression of *Anf* in *Xenopus* is shared among vertebrates. Our findings support Nieuwkoop's activation-transformation model for neural patterning, according to which the entire neurectoderm is initially specified towards an anterior fate, which is later suppressed posteriorly as part of the trunk formation process.

Key words: Homeobox, Fluorescent proteins, Neural plate, Regulation of spatial expression, Transgenic embryos, Anterior posterior patterning, *Xenopus*

Introduction

One of the earliest events in the process of neural plate subdivision is defining the anterior-most domain, demarcated by expression of the homeobox gene *Anf/Hesx1/Rpx* (Zaraisky et al., 1992; Zaraisky et al., 1995; Thomas et al., 1995; Hermesz et al., 1996; Kazanskaya et al., 1997). The posterior limit of *Anf* expression domain corresponds to the caudal limit of the prospective forebrain while the anterior boundary coincides with the rostral edge of the neural plate (Hermesz et al., 1996; Kazanskaya et al., 1997; Knoetgen et al., 1999; Chapman et al., 2002). As neurulation proceeds, *Anf* expression is progressively reduced and is entirely lost within neural tissue by the end of neurulation (Kazanskaya et al., 1997). A crucial role for regionalised expression of *Anf* can be demonstrated by mouse and human mutations (Dattani et al., 1998) and by ectopic expression of the *Xenopus Anf*, *Xanf1* (also known as *Xanf-1*), which elicits numerous forebrain abnormalities, including brain outgrowths and reduction of eyes (Ermakova et al., 1999).

Recently, we demonstrated by a luciferase reporter assay that deleting a 14 bp element in the *Xanf1* promoter results in increased reporter expression within the prospective hindbrain (Eroshkin et al., 2002). By use of transgenic embryos in conjunction with a novel bi-colour fluorescent reporter technique, we show now that this posterior increase in reporter expression is a result of expansion of the expression posterior to normal expression zone of *Xanf1*. Using the crucial cis-regulatory element as the target in yeast one-hybrid system, we identified two transcription factors, FoxA4a/Pintallavis (Knochel et al., 1992; Ruiz i Altaba et al., 1993; Kaestner et al., 2000) and Xvent2 (also known as Xvent-2) (Onichtchouk et al., 1997), that can bind to the element and act as *Xanf1* repressors posterior to its natural expression limit.

Materials and methods

Bi-colour reporter vector and transgenic embryos

To prepare the bicolour reporter vector, the RFP cassette, including multiple cloning site, RFP cDNA and SV-40 PolyA sequence, was excised by cutting with *Bgl*II and *Afl*II (blunted) from the pDsRed1-1 vector (Clontech) and subcloned into the pEGFP-1 vector cut by *Bgl*II and *Sal*I (blunted). Restriction sites between *Sal*I and *Bam*HI in the pDsRed1-1 vector were deleted by first cutting at *Sal*I and *Bam*HI sites, blunt ending and self-ligating the resulting fragment. A fragment of the *Xanf1* gene from –2200 to +25, containing a part of the 5′-flanking region and the first 25 nucleotides of the non-transcribed 5′-part of the first exon, was obtained by PCR with the following pair of

primers: primer at the 5′-end of the fragment (here and below sequence matches with that of the *Xanf1* promoter are in capital letters) was 5′-gctaaggtaccTAGCTGACTAGTCAGAGA; and the reverse primer from region +25 to +3 was 5′-ttaggatc-CTCAACTGAGACTTTGT. The fragment obtained was cut with *Kpn*I and *Bam*HI and cloned into *Kpn*I and *Bam*HI sites of EGFP cassette of the bicolour reporter vector. The 5′-nested deletion fragments of 5′-flanking region of *Xanf1* were obtained by PCR with the same primer for the non-transcribed 5′-part of the first exon described above and the following primers: (–510) 5′-gtgaagcttcGGGCATTTCATCAGGATTA; (–320) 5′-gtgaagctTCGG-CGGTTCTTAACTTCT; (–203) 5′-gtgaagctTGCTAATTACACA-CCAAAC; (–189) 5′-gtgaagcttCAAACAAATAAACAATTAACTC; (–167) 5′-gtgaagctTCCTTGCCTCTCCG; (–133) 5′-gtgaagctTGCT-AAAACTTTCTAATGAAAG.

The resulting PCR fragments were cut with *Hin*dIII and *Bam*HI (blunted), and cloned into *Hin*dIII and *Eco*RI (blunted) sites of the RFP cassette of the bi-colour vector. To generate the one-reporter vector, the same fragments of the *Xanf1* promoter were cloned into the pEGFP-1 promoter reporter vector (Clontech).

All the reporter vectors were linearised with *Sfi*I and purified on Qiagen columns. Transgenic embryos bearing these vectors were prepared by the nuclear transplantation technique as described (Offield et al., 2000).

Yeast one-hybrid screening

To prepare the reporter strain bearing the target sequence, the following oligomers containing this sequence, along with flanking restriction sites, were annealed and sequentially inserted three times into pHisi and pLacZi reporter vectors (Clontech Laboratories) upstream of the reporters HIS3 and *lacZ*, respectively: 5′-aattcatgtcgacTGCTAATTACACACCAAACAAATAAACAATTAAC and 5′-tcGAGTTAATTGTTTATTTGTTTGGTGTGTAATTAGCA-gtcgacatg.

The resulting pHisi and pLacZi reporter plasmids were sequentially integrated into the genome of the YM4271 yeast strain (Matchmaker one-hybrid system) at HIS3 and URA3 loci, respectively. As the resulting reporter strains in YM 4271 carry deletions of wild-type genes required for activation of the Gal1 promoter used in the gastrula cDNA expression library (LexA yeast two-hybrid system), they were mated with the Egy48 strain (Ade^- and Tyr^-) from the LexA two-hybrid system that can drive expression of the library-encoded protein from the Gal1 promoter. The resulting strain could grow on a selective medium (–Ade/–His/–Tyr/–Ura) because of leaky HIS3 expression but was completely suppressed on this medium in the presence of 80 mM 3-aminothriasol (3-AT). This strain containing the 36 bp element as the target was transformed with the *Xenopus* laevis embryo stage 12 Matchmaker LexA cDNA library in a pB42 AD cloning vector (Clontech Laboratories). Transformants were plated on selective medium (–Ade/–His/–Trp/–Tyr/–Ura) containing 80 mM 3-AT that allowed growth of only those colonies in which the HIS3 reporter gene appeared to be activated by transcription factors binding with the 36 bp target sequence. To eliminate false positives, we tested *lacZ* reporter gene expression in colonies grown on the selective medium by a β-galactosidase filter assay. Plasmids containing cDNA inserts from the *Xenopus* stage 12 library were isolated from selected yeast colonies and re-transformed into *E. coli* for sequencing.

Gel-shift analysis

Xenopus laevis oocytes microinjected with mRNA encoding EGFP, FoxA4a/Pintallavis or Xvent2 (10 ng/oocyte) and incubated 48 hours in OR-2 solution were disrupted by pipetting in EMSA buffer (100 mM KCl, 0.25 mM EDTA, 0.2 mM EGTA, 20% glycerol, 100 mg/ml BSA, 20 mM HEPES pH 7.9 and a protease inhibitor cocktail containing chymostatin, leupeptin, pepstatin and PMSF each at final concentration of 10 μg/ml) in a volume of 10 μl/oocyte and centrifuged at 13,400 ***g*** for 5 minutes at 4°C. The oocyte extract (10 μl) was diluted with EMSA buffer (sevenfold and twofold for FoxA4a/Pintallavis and Vent2 respectively) were mixed with 2 μg of Poly(dI-dC) and incubated at 4°C for 15 minutes. The following double stranded oligomers were used for the EMSA: 5′-tctgtcccaTGCTAATTACACAC (‘14 bp element’); 5′-tctgcatgtcga-CAAACAAATAAACAATTAACTCga (‘22 bp element’). Mutated oligomers were: 5′-tctgtcccaTGCTACGTCCACAC and 5′-tctgtcccaTGCTATTTACACAC for the 14 bp element; 5′-tctgcatgtcgaCAAACAAATAAACAAGTAACTCga and 5′-tctgcatgtcgaCAAAAAAAAAACAATTAACTCga for the 22 bp element. Each oligomer (50 pmol) was ^{32}P-end-labelled with T4 polynucleotide kinase, added to oocyte extract (15,000 cpm for one reaction) and incubated at 4°C for 40 minutes. The final volume was adjusted to 15 μl with EMSA buffer. The reaction products were immediately loaded on a 6% polyacrylamide gel containing 0.5 TBE and run for 50-60 minutes at constant current (100 mA).

Preparation of constructs and mRNA for microinjections

To prepare vectors for synthesis of mRNA of FoxA4a/Pintallavis and Xvent2, their full-length coding sequences were obtained by RT-PCR with the following pairs of primers: Xvent2 forward, 5′-agaaccgctcgccaccATGACCAAAGCTTTCTCCTCAGTAG; Xvent2 reverse, 5′-ttagtcgacAGGCCAGAGACTGCCCAA; FoxA4a/Pintallavis forward, 5′-agaaccgGTGGACTCCAGAACATGCTA; FoxA4a/Pintallavis reverse, 5′-ttactcgagGGGAGCTGAGGATAGG-TCTG.

The PCR fragments were digested with *Age*I and *Xho*I and cloned into the pSP-EGFP plasmid instead of EGFP [see Ermakova et al. (Ermakova et al., 1999) for a description of pSP-EGFP].

To prepare the P40 dominant-negative mutant of Xvent2, PCR was used to obtain two overlapping parts of the Xvent2 full-length coding sequence. The following pairs of primers were used: 5′ fragment: 5′-agaaccgctcgccaccATGACCAAAGCTTTCTCCTCAGTAG and 5′-CTTCAGAAgGCTGGAGTTTGGCT; 3′ fragment, 5′-GCCAAA-CTCCAGCcTTCTGAAGTCCAGA and 5′-ccggtcgaCTTAATAGG-CCAGAGACTGCCCAAGGTGC.

The resulting PCR fragments were purified, mixed together, denatured at 96°C, annealed at 60°C and subjected to PCR with flanking primers. The resulted PCR fragment, containing a mutant codon coding for P instead of L at position 40 of the homeodomain, was digested with *Age*I and *Xho*I and cloned into pSP-EGFP (Ermakova et al., 1999). The point mutation was confirmed by sequencing.

To prepare vectors coding for fusions of FoxA4a/Pintallavis and Xvent2 with the herpes virus VP16 activation domain or repression domain from *Drosophila* engrailed, we obtained, by PCR, fragments of *FoxA4a/Pintallavis* and *Xvent2* cDNAs coding for DNA-binding regions of these proteins. The following pairs of primers were used: Xvent2 forward, 5′-agtggatCCAGCTAAAACTCCTACAACCA; Xvent2 reverse, 5′-aataagcttCTAATAGGCCAGAGACTGCCCAAG-GTGC; FoxA4a/Pintallavis forward, 5′-ttagtcgacTGCTAAATAG-AGTCAAATTGGAAA; and FoxA4a/Pintallavis reverse, 5′-cttctcgagAATGTTTAAAGGGAGCTGAGG.

In place of the Xanf1-BDGR cassette, the resulting two PCR fragments were cloned into the pSP-VP16-Xanf1-BDGR and pSP-EnR-Xanf1-BDGR plasmids [this plasmid is described elsewhere (Ermakova et al., 1999)], into *Bam*HI/*Bam*HI-*Hin*dIII/*Hin*dIII (for Xvent2) and *Bam*HI/*Bam*HI-*Xho*I/*Sal*I (for FoxA4a/Pintallavis) sites.

To prepare plasmids coding for VP16-FoxA4a/Pintallavis-BDGR and VP16-Xvent2-BDGR, PCR fragments containing DNA binding segments of FoxA4a/Pintallavis and Xvent2 were obtained with the following pairs of primers: Xvent2 forward, 5′-agtggat-CCAGCTAAAACTCCTACAACCA; Xvent2 reverse, 5′-aatctcgag-CTAATAGGCCAGAGACTGCCCAAGGTGC; FoxA4a/ Pintallavis forward, 5′-ttagtcgacTGCTAAATAGAGTCAAATTGGAAA; FoxA4a/Pintallavis reverse, 5′-ttactcgagGGGAGCTGAGGATAGGTCTG.

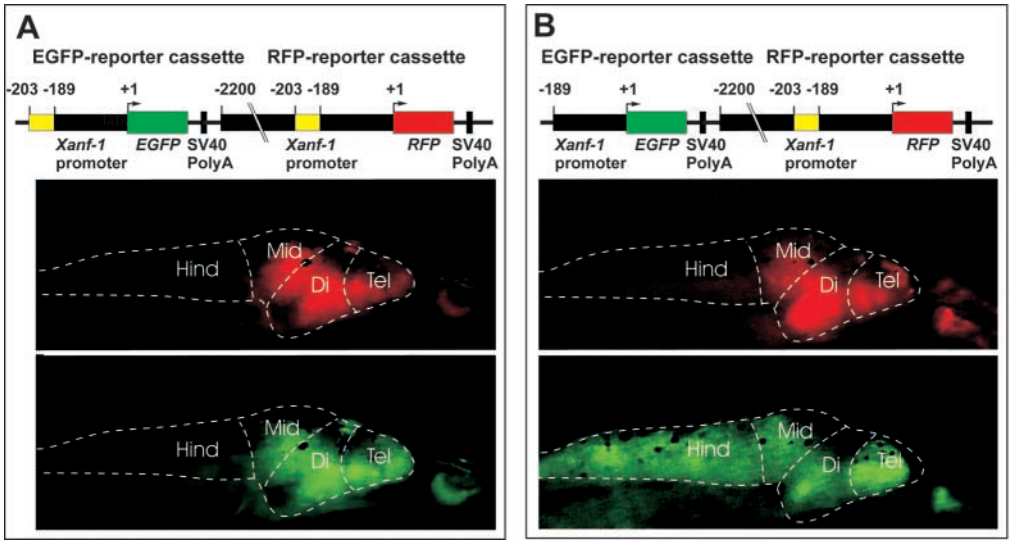

Fig. 1. A 14 bp regulatory element in the *Xanf1* promoter restricts of *Xanf1* expression to the presumptive forebrain. (A) Brain of a transgenic embryo bearing the double-reporter vector shown at the top demonstrates similar expression patterns of EGFP (green) and RFP (red) driven by promoter fragments –203 to +1 and –2200 to +1 respectively. (B) Deletion of a 14 bp promoter element (yellow), from the EGFP reporter cassette (top) results in the posterior shift in the EGFP reporter expression in the CNS of another transgenic embryo bearing this vector (bottom). RFP expression driven by the complete promoter fragment, –2200 to +1, is still localised in the forebrain (middle).

The resulting two PCR fragments were cloned, into a modified Xanf1 plasmid, pSP-VP16-Xanf1-BDGR, at *Bam*HI/*Bam*HI –*Xho*I/*Xho*I sites. All mRNAs were synthesised from linearised plasmids using the Ambion SP6 mRNA MESSAGE MACHINE kit and purified by QIAGEN RNeasy mini-columns. To test the activity of various mRNAs in embryos 50-250 pg of mRNA was injected per blastomere.

Design and microinjections of morpholino oligonucleotides

The following morpholino oligonucleotides were designed as suggested by the manufacturer (Gene Tools, LLC) on the basis of BLAST analysis of the *Xenopus laevis* NR and EST databases: for *FoxA4a/Pintallavis* (5′-GAGGTATGGTTTCTCCAACAAGAAG) and for *Xvent2* (5′-CTTGTCTGTATTAGTCCTTGTGTTC). A water solution (2-3 nl) containing these oligonucleotides (from 1 mM to 0.01 mM in different experiments) was microinjected into animal-dorsal blastomeres of *Xenopus laevis* embryos at 8-32 blastomere stages.

Results

A small element within the *Xanf1* promoter determines restriction of its expression within the anterior neurectoderm

Previously, we used a luciferase reporter assay to identify an element from the *Xanf1* promoter, the removal of which resulted in spreading of luciferase reporter expression to the hindbrain region (Eroshkin et al., 2002). This assay has limited spatial resolution, an issue that is addressed here using transgenic embryos bearing fluorescent reporters driven by different deletion mutants of the *Xanf1* promoter, permitting one to correlate much more clearly domains of gene expression and morphology in the embryo.

First, we tested reporter expression pattern driven by the ‘full-length’ (–2200 to +1) promoter of *Xanf1* (Eroshkin et al., 2002) using transgenic embryos bearing pEGFP-1 or pDsRed reporter vectors. Ten percent of normally developing tadpoles (more than 30 embryos in a total of three experiments for each vector) demonstrated appropriate localisation of the EGFP or DsRed signals, in that part of the brain derived from the neural plate region where endogenous *Xanf1* expression domain in the forebrain and ventral part of the midbrain (not shown).

Interestingly, while *Xanf1* expression ceases in normal development by the end of neurulation, because of the high stability of EGFP and DsRed proteins in living cells (Matz et al., 1999), EGFP and DsRed reporters driven by *Xanf1* promoter, though becoming progressively weaker, could be visualised until tadpole stages, providing an integrative image of the *Xanf1* expression.

To make further promoter deletion analysis more effective, we developed a novel method using a bicolour fluorescent reporter vector (Fig. 1). These vectors containing DsRed and EGFP cDNAs under the control of ‘full-length’ (–2200 to +1) and deletion fragments (–510, –320, –203, –189, –167, –133) of the *Xanf1* promoter, respectively, were prepared and used for generation of transgenic *Xenopus tropicalis* embryos. For each construct, 7-15 embryos (obtained in two or three independent experiments) that demonstrated the expected expression patterns of DsRed driven by the ‘full-length’ promoter, were selected and analysed for spatial distribution of EGFP.

The use of this technique allowed us to confirm definitively that the removal of a short, 14 bp, element (–203 TGCTAATTACACAC –189) from the *Xanf1* promoter resulted in spreading of the EGFP reporter expression posterior to the region of the tadpole brain in which DsRed driven by the ‘full-length’ promoter is expressed (Fig. 1B). All of the promoter fragments longer than –203 bp produced spatial patterns of EGFP expression identical to that of DsRed provided by the ‘full-length’ promoter (Fig. 1A).

Identification of transcription factors binding to the *Xanf1* promoter by the yeast one-hybrid system

To identify transcription factors that could bind with the 14 bp cis-regulatory element of the *Xanf1* promoter in living embryos, we performed screening of a *Xenopus* late gastrula cDNA expression library with a yeast one-hybrid system. We used as a target the 36 bp element from the Xanf1 promoter that included, along with the 14 bp element (TGCTAATTAC-ACAC), the 22 bp element (CAAACAAATAAACAATTAA-CTC) abutting the 14 bp element from the proximal side. As it was shown previously, this 22 bp element is important for the maintenance of moderate expression levels of *Xanf1*

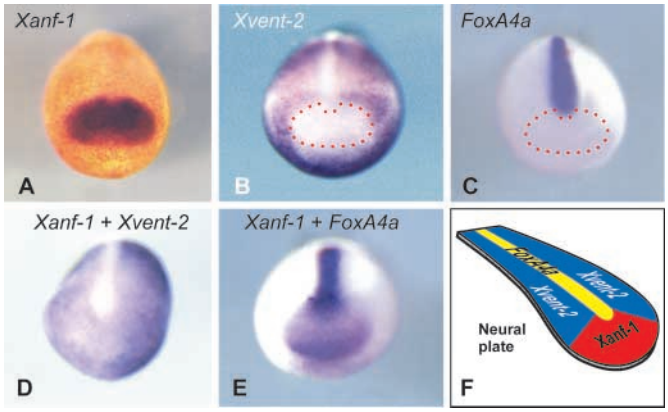

Fig. 2. Expression domains of *Xanf1*, *FoxA4a/Pintallavis* and *Xvent2* occupy complementary areas within the neural plate. Embryos at the early neurula stage are shown from the anterior, with the dorsal side upwards. (A) Expression of *Xanf1* in the anterior neuroectoderm. (B) Expression of *Xvent2* is localised within lateral parts of the neural plate, behind the expression domain of *Xanf1* (marked by the broken red line). (C) *FoxA4a/Pintallavis* is expressed along the midline of the neural plate. The anterior tip of the *FoxA4a/Pintallavis* expression domain borders on the medial part of the posterior margin of the *Xanf1* expression area (marked by the broken red line). (D,E) Double in situ hybridisation with probes to *Xanf1* and *Xvent2* (D) or *Xanf1* and *FoxA4a/Pintallavis* (E) mRNAs demonstrates lack of gaps between expression domains of these genes. (F) Diagram of complementary areas occupied by the expression domains of *Xanf1*, *FoxA4a/Pintallavis* and *Xvent2* within the neural plate.

(Eroshkin et al., 2002). Ten unique clones encoding transcription factors were identified in this screen: Dlx2, Dlx5, Hoxb9, Msx1, Nkx5.1, FoxA4a/Pintallavis, Xvent1, Xvent2, Xanf1 and Xanf2 (see Materials and methods for technical details).

Only two of these genes, *FoxA4a/Pintallavis* and *Xvent2*, have expression patterns consistent with those of hypothetical suppressors that could be responsible for posterior restriction of the *Xanf1* expression: their expression domains in the neural plate are complementary to that of *Xanf1*. In the case of the homeobox gene *Xvent2*, the expression is along all the trunk part of the neural plate excluding the notoplate, bordering the expression zone of *Xanf1* from the posterior and lateral sides (Fig. 2B,D). The fork-head gene *FoxA4a/Pintallavis* is expressed in a complementary pattern to *Xvent2*, within the notoplate, with the anterior tip of its expression domain contacting the central part of the posterior limit of the *Xanf1* expression domain (Fig. 2C,E).

FoxA4a/Pintallavis and Xvent2 can bind with the identified regulatory elements of the *Xanf1* promoter in vitro

An electrophoretic mobility shift assay (EMSA) was used to verify whether FoxA4a/Pintallavis and Xvent2 are able to bind with the 14 bp and 22 bp elements of the *Xanf1* promoter. A crude extract of *Xenopus* oocytes, microinjected with mRNAs of these proteins, causes retardation of movement of both 14 bp and 22 bp elements in polyacrylamide gels. The specificity of binding was confirmed by reduction of the EMS signal when oocyte extracts microinjected with *FoxA4a/Pintallavis* or *Xvent2* mRNA were diluted 10 times with the extract of control oocytes, and the entire lack of a gel shift in the case of extract solely from control oocytes (Fig. 3).

Interestingly, although two sequences, CTAATTA and CAATTAA (opposite strand: TTAATTG), which precisely match the consensus binding motifs of Xvent2 (Trindade et al., 1999), were found in the 14 bp and 22 bp elements, respectively, a site that matches the consensus motif for FoxA4a/Pintallavis well, (G/A)TAAA(T/C)A (Kaufmann et al., 1995), was revealed only in the 22 bp element: ATAAACA (Fig. 3D). However, the complementary strand of the 14 bp element contains the sequence GTAATTA (Fig. 1D), which has only one mismatch with the consensus core site (G/A)TAAA(T/C)A. This might explain the greatly reduced binding (at least 10 times) of FoxA4a/Pintallavis with the 14 bp element in comparison with the 22 bp element (compare lanes 2 and 3 in Fig. 3A).

To further confirm specificity of binding of FoxA4a/Pintallavis with the 14 bp element, we inserted several point mutations that, according to the literature (Kaufmann et al., 1995), should totally disrupt specific binding of this factor: CTACGTC instead of CTAATTA. As a result, we observed dramatic reduction of the EMS (Fig. 3C, lane 2). Reversion of GTAATTA to the consensus FoxA4a/Pintallavis site, GTATTTA, increased the shift signal in comparison with the intact 14 bp element (Fig. 3C, lanes 3 and 4).

FoxA4a/Pintallavis and Xvent2 act as transcriptional repressors of *Xanf1*

To test if ectopic FoxA4a/Pintallavis and Xvent2 can downregulate *Xanf1* in vivo, we microinjected synthetic mRNA encoding these factors mixed with the cell tracer fluorescein-lysine-dextran (FLD) into one or two of the animal-most dorsal blastomeres of 32 cell embryos, the progeny of which give rise predominantly to the neural plate and very rarely to the underlying head mesoderm and endoderm (Bauer et al., 1994). These localised injections helped us to exclude effects of a possible indirect influence of the microinjected FoxA4a/Pintallavis and Xvent2 on the neurectoderm via effects on the inductive properties of the underlying mesoderm.

At the early neurula stage, the embryos with an FLD signal within the anterior neural plate were selected and processed for whole-mount in situ hybridisation with the probe to *Xanf1* mRNA. In order to visualise possible changes in the expression pattern of *Xanf1* more precisely, we mixed *Xanf1* probe with a probe encoding the midbrain/hindbrain boundary marker, engrailed 2.

In all of the embryos selected (37 embryos for *FoxA4a/Pintallavis* and 39 for *Xvent2* in 4 independent experiments), we observed inhibition of *Xanf1* expression in the neural plate cells containing ectopic *FoxA4a/Pintallavis* or *Xvent2* mRNAs (Fig. 4A,A′,B,B′). To assess the tissue distribution of the microinjected cells, we prepared histological sections of four embryos injected with each mRNA. In good agreement with the expected blastomere fate map, FLD-labelled cells were seen essentially exclusively within the ectoderm that always included regions where inhibition of the *Xanf1* expression was seen (Fig. 4B1,B1′). Rarely, single labelled cells were seen in the underlying anterior endomesoderm. By contrast, in the same embryos, cells expressing *Xanf1* in the normal pattern did not contain FLD and thus the microinjected

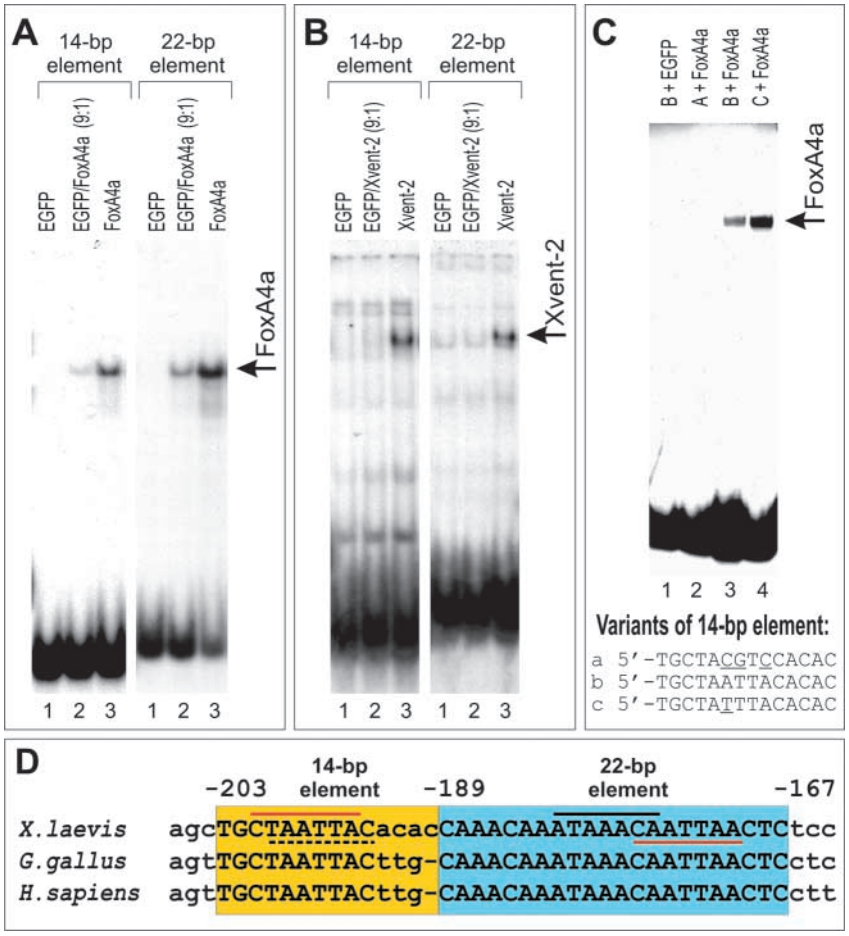

Fig. 3. Electrophoretic mobility shift assays demonstrating binding of FoxA4a/Pintallavis and Xvent2 with 14 and 22 bp elements from the *Xanf1* promoter. (A,B) The reaction mixtures are as follows: lane 1, extract from oocytes microinjected with EGFP mRNA (control); lane 2, extract from oocytes microinjected with mRNA of FoxA4a/Pintallavis (A) or Xvent2 (B) diluted ten times with extract from oocytes microinjected with EGFP mRNA; lane 3, undiluted extract from oocytes microinjected with *FoxA4a/Pintallavis* (A) or *Xvent2* (B) mRNA. (C) Lane 1, extract from oocytes microinjected with EGFP mRNA (control); lanes 2-4, extracts from oocytes microinjected with FoxA4a/Pintallavis mixed with normal (b) or two mutant (a,c) variants of the 14 bp element. Sequences of these variants are shown at the bottom of C. Sites of point mutations are underlined. (D) Core binding sites are indicated by the black and red lines for FoxA4a/Pintallavis and Xvent2, respectively; positions of binding sites located on the opposite DNA strand are underlined; possible binding site for FoxA4a/Pintallavis, which has one mismatch with the canonical binding site, is indicated by a broken line.

mRNA (Fig. 4B2,B2′). All of these data confirm that the observed inhibition of *Xanf1* by exogenous FoxA4a/Pintallavis and Xvent2 was not the result of their indirect influence via underling inducing tissue but was caused by direct action of these proteins within neurectodermal cells.

To test further if FoxA4a/Pintallavis and Xvent2 can downregulate expression of *Xanf1* by acting as transcriptional repressors, we fused their DNA-binding domains with the repressor domain from the *Drosophila* engrailed gene (EnR) (Jaynes and O'Farrell, 1991). When mRNA of the repressor fusions, EnR-FoxA4a/Pintallavis or EnR-Xvent2, were microinjected into medial animal blastomeres of 32-cell embryos, inhibition of *Xanf1* expression was observed in regions populated by FLD labeled cells (34 and 37 embryos with these properties were scored, respectively, for EnR-FoxA4a/Pintallavis and EnR-Xvent2 injections in three independent experiments) (Fig. 4C,C′,D,D′). Histological sections of some of these embryos (four embryos for each type of microinjected mRNA) also confirmed that the effect was due to the activity of the fusion proteins within neuroectodermal but not mesodermal cells (not shown). Thus, the strong repressive domain EnR cannot alter the effect exerted by the wild type FoxA4a/Pintallavis and Xvent2 on expression of *Xanf1* indicating that both factors are normally acting as transcriptional repressors of the latter gene.

To further confirm that FoxA4a/Pintallavis and Xvent2 play roles as *Xanf1* transcriptional repressors, we microinjected mRNAs coding for dominant-negative versions of these genes. Two types of dominant negatives were used. First, we prepared mRNA coding for fusions of FoxA4a/Pintallavis or Xvent2 with the activation domain of the herpes virus VP16 protein. Strong positive transcriptional activation as a result of the latter domain should result in the reversion of the repressor function of FoxA4a/Pintallavis and Xvent2. Second, in the case of Xvent2, we also generated mRNA coding for the protein with the point mutation, L/P, in position 40 of the homeodomain (Xvent-P40). As shown previously, this mutant was able to neutralise activity of the normal Xvent2 by forming a non-functional dimer with it, preventing DNA binding (Onichtchouk et al., 1998; Trindade et al., 1999).

In these experiments, we observed an expansion of the *Xanf1* expression zone within the areas occupied by descendants of medial animal blastomeres of the 32-cell embryos microinjected with mRNA of *FoxA4a/Pintallavis* and *Xvent2* dominant-negative mutants (Fig. 4E,E′,F,F′,G,G′). However, cells ectopically expressing *Xanf1* occupied only a part of the area populated by the microinjected cells (as evaluated in 31, 34 and 26 embryos in three independent experiments, respectively, for the VP16-FoxA4a/Pintallavis, Vent-P40 and VP16-Xvent2 constructs). Thus, within the neural plate, ectopic expression of *Xanf1* expanded posteriorly only up to the prospective rostral part of the hindbrain, which was marked by *engrailed 2* expression [Fig. 4E-G, compare positions of the Xanf1 expression posterior limits marked by the red (area of expanded expression) and black (area of normal expression) arrows].

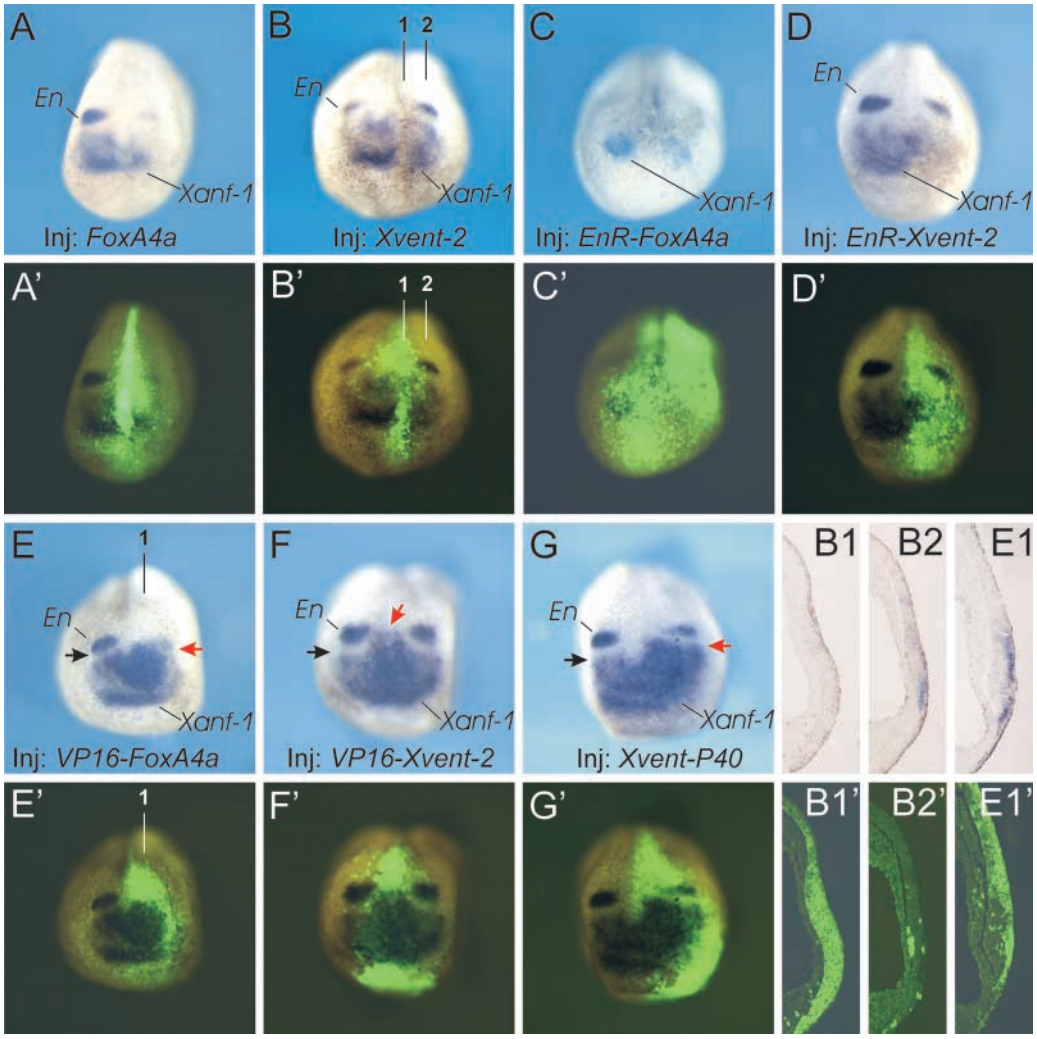

Fig. 4. Effects exerted by ectopic expression of *FoxA4a/Pintallavis* and *Xvent2*, and repression and activation constructs on the expression of endogenous *Xanf1*. All embryos were microinjected with a mixture of FLD and mRNA. (A-D) Ectopic expression of *EnR-FoxA4a/Pintallavis* and *EnR-Xvent* fusions elicit inhibition of *Xanf1* expression. Microinjection of mRNA: *FoxA4a/Pintallavis* (A,A′); *Xvent2* (B,B′); *EnR-FoxA4a/Pintallavis* (C,C′); *EnR-Xvent2* (D,D′). (B1,B1′,B2,B2′) Representative histological sections at two sagittal levels (1 and 2) of the embryo shown in B and B′ demonstrate that descendants of the microinjected blastomeres labelled with FLD are primarily localised in the neurectoderm. Only single labelled cells are found in underlying endomesoderm. (E-G) Ectopic expression of dominant-negative versions of *FoxA4a/Pintallavis* and *Xvent2* result in posterior expansion of endogenous *Xanf1* expression. Red and black arrows indicate position of the posterior border of the *Xanf1* expression domain within microinjected and non-microinjected (normal) zones, respectively. Microinjection of mRNA: *VP-16-FoxA4a/Pintallavis* (E,E′); *VP16-Xvent2* (F,F′); *Xvent2-P40* (G,G′). (E1,E1′) Representative histological section at level 1 of the embryo shown in E and E′ illustrates that microinjected blastomeres primarily populate the neurectoderm and that endogenous *Xanf1* is now expressed in these cells.

As in the cases described above, histological sections of the experimental embryos (four embryos of each type were sectioned) confirmed that the observed effects were not resulting from influence of the dominant-negative constructs via underlying mesoderm (Fig. 4E1,E1′).

To further validate the repressive role of FoxA4a/Pintallavis and Xvent2 in regulating *Xanf1*, we suppressed translation of their mRNAs by microinjections of antisense morpholino oligonucleotides. With the anti-*FoxA4a/Pintallavis* morpholino we observed posterior expansion of the *Xanf1* expression area. Notably, the expression of *Xanf1* spread toward the posterior only within the area of the prospective floor plate (arrows on Fig. 5A,A′), i.e. just in those regions where the expression of the endogenous *FoxA4a/Pintallavis* would be expected to be suppressed by the morpholino. By contrast, no expansion was seen when the morpholino-containing cells occupied the lateral parts of the neural plate, i.e. those areas in which *FoxA4a/Pintallavis* is never expressed in normal development (Fig. 5A,A′, upper row of embryos). It is noteworthy, as in the case of dominant-negative constructs, ectopic expression of *Xanf1* expanded posteriorly only up to the prospective rostral part of the hindbrain (compare with Fig. 4E-G), which might indicate the presence of some other transcription factors inhibiting expression of *Xanf1* posterior to this limit.

The effect on the *Xanf1* expression expansion was still observed when the concentration of the microinjected morpholino was decreased from 1 mM (the concentration recommended by manufacturer) to 0.3 and even 0.1 mM (respectively, 32, 24 and 12 embryos with this phenotype were scored in two independent experiments made for each of the concentrations). At the same time, no effects were seen when the commercial control morpholino (Gene Tools) was microinjected even at the concentration of 2 mM (not shown). The data taken together indicate a strong specificity for the morpholino activity.

Unfortunately, we were unable to obtain similar clear results with the anti-*Xvent2* morpholino because the microinjected embryos failed to complete gastrulation. This effect, observed with high frequency (from 80% to 95% of embryos) through a wide range of anti-*Xvent2* morpholino concentrations (from 1 to 0.1 mM), was usually accompanied by disintegration of the ectoderm into separate cells by the end of gastrulation.

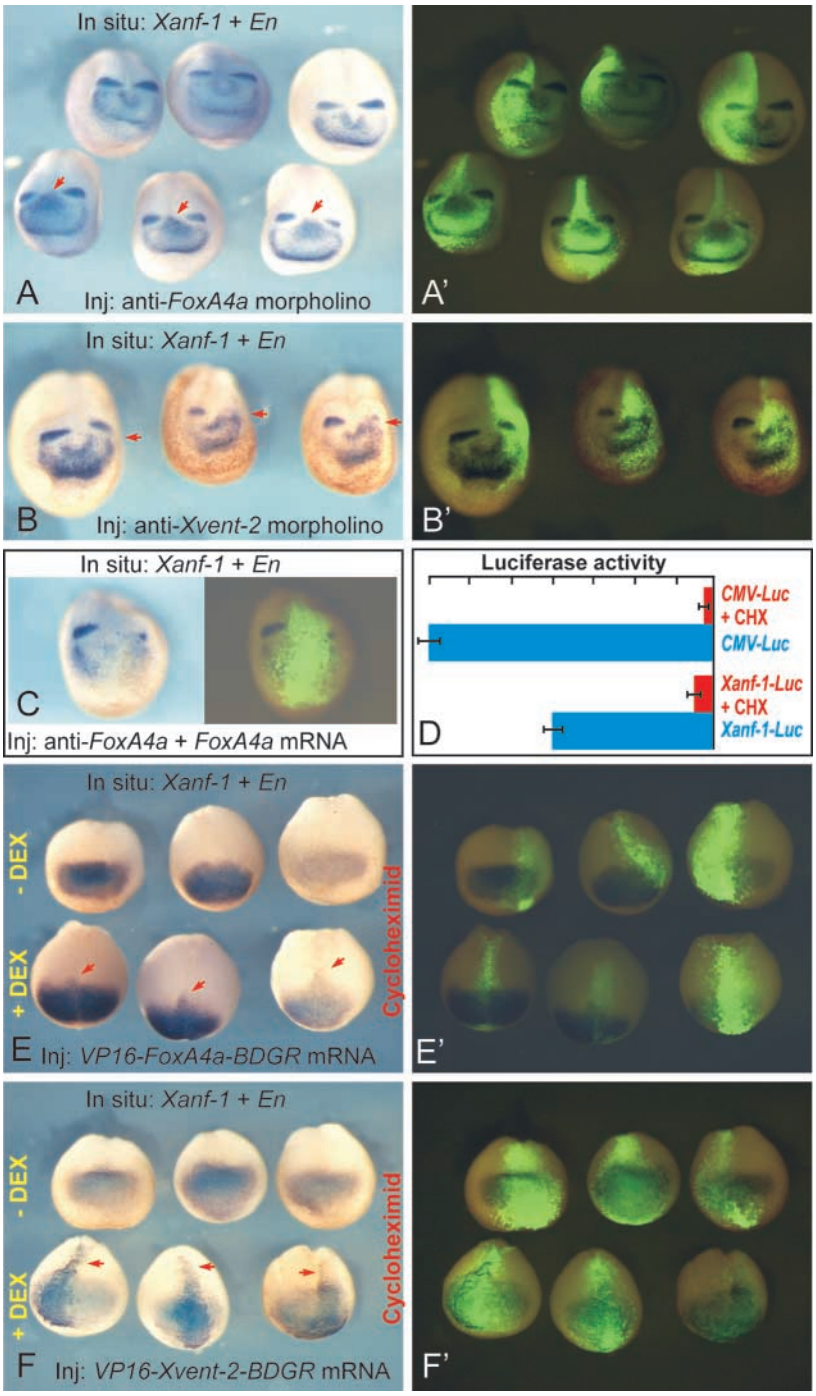

Fig. 5. (A,B) Microinjections of anti-*FoxA4a/Pintallavis* (A,A′) and anti-*Xvent2* (B,B′) morpholinos elicit short-range posterior expansion of the *Xanf1* expression. In the case of the anti-sense to *FoxA4a/Pintallavis*, the expression of *Xanf1* is expanded toward the posterior only along the midline of the neural plate, i.e. just in the region where the endogenous *FoxA4a/Pintallavis* is expressed (A, red arrows). No expansion of the *Xanf1* expression was seen in the morpholino-containing cells occupying lateral parts of the neural plate (the upper rows of embryos in A and A′). By contrast, only lateral expansion of the *Xanf1* expression was observed in embryos microinjected with the anti Xvent2 (B,B′) morpholino and these embryos demonstrated normal expression of *Xanf1* in the midline of the neural plate. (C) The mixture of anti-*FoxA4a/Pintallavis* morpholino and a synthetic *FoxA4a/Pintallavis* mRNA can interfere with the posterior expansion of endogenous *Xanf1* expression elicited by the morpholino alone. (D) To monitor the effectiveness of CHX on suppression of protein synthesis, we microinjected *Xenopus* embryos at the two blasomere stage with plasmids expressing luciferase under the control of the CMV (commercial pGL3 vector, Promega) or the 'full-length' *Xanf1* promoter (Eroshkin et al., 2002). CHX was added to one half of the microinjected embryos at the midblastula transition (*CMV-Luc*) or at the midgastrula stage (*Xanf1-Luc*). After 2 hours of incubation, luciferase activity was measured in extracts from three independently collected groups of embryos (10 embryos in each group) treated and not treated with CHX. (E,F) Experiments with the hormone-inducible versions of *FoxA4a/Pintallavis* and *Xvent2* under conditions of inhibition of protein synthesis by cycloheximide demonstrate that *Xanf1* is the direct target of these transcription factors. The dexamethasone (DEX) treatment of embryos subsequently elicits activation of *VP16-FoxA4a/Pintallavis-BDGR* (E,E′) or *VP16-Xvent2-BDGR* (F,F′), which under those conditions can activate transcription of only their own direct genetic targets, in particular, the transcription of *Xanf1*. Although only short-distance posterior expansion of the *Xanf1* expression was observed in the case of *VP16-FoxA4a/Pintallavis-BDGR* (E, red arrows), much broader spreading of the expression was seen in embryos microinjected with *VP16-Xvent2-BDGR* mRNA (F, red arrows).

Nevertheless, in a low number of cases (five embryos out of 72 surviving embryos in two independent experiments), we still observed slight posterior expansion of the *Xanf1* expression in embryos microinjected with 0.03 mM anti-*Xvent2* morpholino (Fig. 5B,B′). Interestingly, in contrast to anti-FoxA4a/Pintallavis morpholino, the expansion of the *Xanf1* expression was observed only in the lateral part of the neural plate, i.e. just in the region where endogenous *Xvent2* was expressed. No expansion was seen in the midline, even if the morpholino-containing cells occupied this region of the embryo (see central and right embryos on Fig. 5B,B′).

To assess the specificity of the morpholino effects, we tested the ability of the *FoxA4a/Pintallavis* and *Xvent2* mRNAs from which sequences complementary to their respective morpholinos had been deleted to interfere with the effect of the posterior expansion of the endogenous *Xanf1* expression elicited by the morpholinos. Because the morpholino oligonucleotides were complementary to the 5′-non-translated part of the endogenous mRNAs of *FoxA4a/Pintallavis* and *Xvent2*, we mixed them with synthetic mRNAs which contained only the reading frames of *FoxA4a/Pintallavis* and *Xvent2* (final concentrations: 1 mM morpholino + 20 ng/ml of

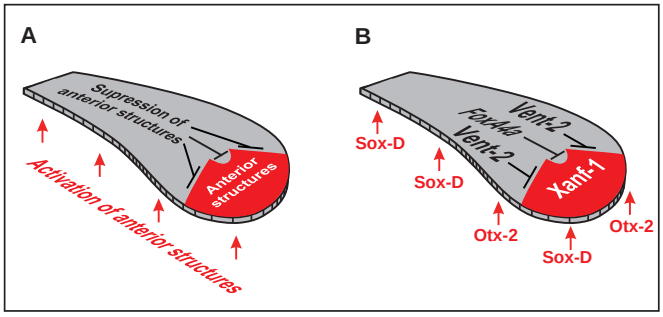

Fig. 6. *Xanf1* regulation by*FoxA4a/Pintallavis* and *Xvent2* is consistent with Nieuwkoop's two-signal model of neural induction. (A) According to the Nieuwkoop model, anterior potentials induced during the first step of the neural induction throughout the neurectoderm are realised only in the anterior part of the neural plate (red) because of suppression by a transforming signal in the posterior part of the plate (grey). (B) Expression of *Xanf1* is activated by Otx2, SoxD and possibly other transcription factors throughout the neural plate. However, during normal development the expression of *Xanf1* is inhibited in the posterior part of the neural plate because of the inhibitory influence of FoxA4a/Pintallavis, Xvent2 and some as yet unidentified factors.

RNA for *FoxA4a/Pintallavis* and 0.03 mM of morpholino + 50 ng/ml of mRNA for *Xvent2*). Microinjections of these mixtures into embryos, resulted in, instead of expansion, inhibition of *Xanf1* expression, which looked very similar to the inhibition elicited by injection of *FoxA4a/Pintallavis* and *Xvent2* mRNAs alone (Fig. 5C for *FoxA4a/Pintallavis* and not shown for *Xvent2*). Importantly, in contrast to experiments with pure anti-*Xvent2* morpholino, 50% of the microinjected embryos retained ectodermal integrity when this morpholino was microinjected, even at a concentration of 1 mM, in the mixture with the *Xvent2* mRNA (50 ng/ml). All these results taken together confirm the specificity of the morpholino effects.

FoxA4a/Pintallavis and Xvent2 act directly on *Xanf1* within embryonic cells

To verify if *Xanf1* is the direct target for FoxA4a/Pintallavis and Xvent2 within embryonic cells, we designed experiments with dexamethasone-inducible versions of these proteins. We microinjected embryos with mRNA coding for fusions of VP16-FoxA4a/Pintallavis or VP16-Xvent2 with the binding domain of the glucocorticoid receptor (BDGR). Owing to sequestration of BDGR by the hsp90 heat-shock protein complex, such fusion proteins appear to be inactivated within the embryonic cells (Gammill and Sive, 1997; Ermakova et al., 1999). At the end of gastrulation, the microinjected embryos were placed for 1 hour into a 10 mg/ml cycloheximide (CHX) solution. According to the literature (Gammill and Sive, 1997) and our own data (Fig. 5D) this period of time is enough to block the total protein synthesis by more than 90%. After this period, dexamethasone (DEX) was added to the same incubation solution at a 2 μM final concentration, which resulted in release of the previously accumulated VP16-FoxA4a/Pintallavis-BDGR and VP16-Xvent2-BDGR from the hsp90 complex. Under these conditions, only direct targets of FoxA4a/Pintallavis and Xvent2 should be activated because mRNA translation is blocked by CHX. After 2 hours of incubation with CHX and DEX, the embryos were processed for whole-mount in situ hybridisation. An expansion of the *Xanf1* expression area was observed in both cases (42 and 21 embryos with the expanded *Xanf1* expression were scored in two independent experiments for VP16-FoxA4a/Pintallavis-BDGR and VP16-Xvent2-BDGR mRNA, respectively), which verify a direct effect of VP16-FoxA4a/Pintallavis-BDGR and VP16-Xvent2-BDGR proteins on the *Xanf1* promoter (Fig. 5E,F).

Interestingly, in the case of VP16-Xvent2-BDGR, the observed expansion of the *Xanf1* expression was much broader than in experiments performed without CHX treatment (compare Fig. 5F with Fig. 4F). Thus, in all embryos, in which descendants of the microinjected cells extended along the neural plate, the area of abnormal expression of *Xanf1* also spread along all the neural plate (Fig. 5F,F′). We surmise that the cycloheximide treatment resulted in rapid degradation of putative negative regulators of *Xanf1* expression, which normally operate in the trunk part of the neural plate.

Discussion

The double-reporter vector: a novel tool for the functional promoter analysis

The use of bi-colour reporter vector in combination with the technique of generation of transgenic embryos has at least three significant advantages over the techniques based on the use of only one fluorescent reporter. First, it allows one to analyse differences between expression patterns of two reporter constructs in the same embryo and even in the same cell. Second, the use of the double-reporter vector can guarantee that any pair of deletion mutants from the promoter being investigated will be inserted into the genome in an equal number of copies. Finally, if in the case of single-reporter vector it is difficult to distinguish the actual result of deletion of a cis-regulatory element from possible chromosome positional effects in transgenic animals, there is an excellent internal control in the case of the double-reporter vector: the expression pattern of DsRed under the control of the full-length promoter. Obviously, the same approach can be used in other experimental models, for example in zebrafish embryos and in cell culture.

FoxA4a/Pintallavis and Xvent2 play roles as transcriptional repressors of *Xanf1* and determine the precise position of its posterior expression limit

Previously, we presented evidence that anterior localisation of *Xanf1* expression within the neural plate might be the result of binding of transcriptional repressors, operating within more posterior areas of the neural plate, interacting with a short regulatory region of the *Xanf1* promoter (Eroshkin et al., 2002). Now we demonstrate that two transcription factors, the fork-head domain FoxA4a/Pintallavis and the homeodomain Xvent2, are sufficient and essential for precise determination of the *Xanf1* posterior expression limit. This conclusion is based on their native expression patterns, misexpression of mRNAs encoding these factors and variants containing engrailed expression domains, as well as evidence from blocking their expression both by morpholino oligonucleotides and dominant negative constructs.

Interestingly, while FoxA4a/Pintallavis and its mammalian

homologues, members of the FoxA forkhead transcription factor family, have primarily been thought to be transcriptional activators (for a review, see Kaestner, 2000), our experiments reveal that they may act as transcriptional inhibitors as well. Consistent with this proposal, it has been shown recently that mammalian homolog of FoxA4a/Pintallavis, FoxA2 can bind TLE (Wang et al., 2000) or SMAD3 (Li et al., 2002) co-repressors.

The other repressor of *Xanf1* expression identified in this study, Xvent2, was initially determined to be a transcriptional inhibitor of several dorsally expressing genes (Schuler-Metz et al., 2000). Along with its paralog, Xvent1, Xvent2 is known to be a key transcriptional regulator of the BMP4 cascade in the ventral part of embryo (Ladher et al., 1996; Schmidt et al., 1996; Onichtchouk et al., 1998). Now we show that Xvent2 is also involved in controlling the early anteroposterior patterning of the neural plate.

It is noteworthy that the nucleotide sequence of the cis-regulatory element in the *Xanf1* promoter that was studied here appears to be highly conserved in *Anf* genes among highly diverged vertebrates, including *Xenopus*, chick and human (Eroshkin et al., 2002) (Fig. 3D). This suggests that there is likely to be conservation of the mechanism of suppression of *Anf* expression by orthologues of FoxA4a/Pintallavis and Xvent2 in other vertebrates as well.

Interestingly, the closest relatives of vertebrates, hemichordates and all lower animals, seemingly have no anatomical structures homologous to the brain unit derived from the territory of the rostral neural plate in which *Anf* genes are expressed in vertebrate embryos (for a review, see Shimeld and Holland, 2000). Moreover, genomic data hint that the *Anf* type of homeobox (Kazanskaya et al., 1997) itself might also be an evolutionary invention of vertebrates as this type of homeobox is not seen among other animal groups, including the close relatives of vertebrates, the ascidians. All of these results and observations allow one to hypothesise that the appearance of *Anf* type of homeobox gene and the mechanism ensuring rostral localisation of its expression via posterior downregulation by FoxA4a/Pintallavis and Xvent2 progenitors might be crucial steps in the evolution of the forebrain in vertebrates.

Negative regulation of *Xanf1* posterior to its natural expression domain is consistent with the Nieuwkoop activation-transformation model of neural induction

In his famous model of neural induction, Nieuwkoop suggested that during the first step of the induction all the presumptive neuroectoderm is activated by the influence of the 'early' Spemann organiser in initiating neural development toward anterior fates (Nieuwkoop and Nigtevecht, 1954). At the second step, a signal emanating from the 'later', more posterior, Spemann organiser transforms the anterior potencies of the trunk part of the neural plate into posterior fates (Fig. 6A). An important finding from Nieuwkoop's experiments is that signalling responsible for the activation of the anterior potentials are still present in the posterior neural plate even at the second step of neural induction, but its influence in this region is masked by the influence of the transformation signalling.

Our results are consistent with the predictions of Nieuwkoop's activation-transformation model. Indeed, the posterior spreading of the reporter expression driven by the *Xanf1* promoter deprived of 14 bp element indicates that signalling sufficient for activation of the anterior specific marker, *Xanf1*, is indeed still present in the posterior neural plate. However, in normal development, this activation signalling is inhibited by a negative influence mediated via the cis-regulatory element of the promoter described here through the action of the two transcriptional regulators, FoxA4a/Pintallavis and Xvent2.

However, in contrast to the massive shift of the reporter expression revealed in promoter deletion experiments, all dominant-negatives of FoxA4a/Pintallavis and Xvent2, as well as their antisense morpholinos, appeared to be able to elicit the posterior shift of the *Xanf1* expression only up to the prospective midbrain-hindbrain boundary. This indicates that other repressors with much higher affinity to the same sites that bind FoxA4a/Pintallavis and Xvent2 might operate posterior to this boundary. Alternatively, these hypothetical repressors might bind to other sites within the promoter but have such a strong repressor potential that cannot be overcome by VP-16-FoxA4a/Pintallavis and VP-16-Xvent2. It is noteworthy as well that we observed a much wider posterior expansion of the *Xanf1* expression under the conditions of inhibition of the total protein synthesis by cycloheximide. These results strengthen the argument for the presence of other posteriorly operating factors that normally inhibit *Xanf1* expression in the trunk part of the neural plate.

Recently, we have shown that the *Anf* proximal promoter contains core binding sites for two potential activators of this gene, namely Otx2 and SoxD (Eroshkin et al., 2002). Consistent with this finding, Otx2 and SoxD were shown to be able to activate *Anf* in embryonic tissues of the mouse and *Xenopus* (Mizuseki et al., 1998; Rhinn et al., 1998). Normally, the expression domain of *Xanf1* overlaps with the expression areas of both these genes, which occupy broader territories in the neural plate. Therefore, without the posterior repression exerted by FoxA4a/Pintallavis, Xvent2 and possibly other factors, both Otx2 and SoxD would be able to activate the expression of *Xanf1* on a territory broader than its natural expression domain. Again, this is consistent with key features of the Nieuwkoop model, which predicts broad distribution of potential activator(s) of anterior structures throughout the neural plate (Fig. 6).

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