The homeobox gene, Xanf-1, can control both neural differentiation and patterning in the presumptive anterior neurectoderm of the Xenopus laevis embryo

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SUMMARY

From the onset of neurectoderm differentiation, homeobox genes of the Anf class are expressed within a region corresponding to the presumptive telencephalic and rostral diencephalic primordia. Here we investigate functions of the Xenopus member of Anf, Xanf-1, in the differentiation of the anterior neurectoderm. We demonstrate that ectopic Xanf-1 can expand the neural plate at expense of adjacent non-neural ectoderm. In tadpoles, the expanded regions of the plate developed into abnormal brain outgrowths. At the same time, Xanf-1 can inhibit terminal differentiation of primary neurones. We also show that, during gastrula/neurula stages, the exogenous Xanf-1 can downregulate four transcription regulators, XBF-1, Otx-2, Pax-6 and the endogenous Xanf-1, that are expressed in the anterior neurectoderm. However, during further development, when the exogenous Xanf-1 was presumably degraded, reactivation of XBF-1, Otx-2 and Pax-6 was observed in the abnormal outgrowths developed from blastomeres microinjected with Xanf-1 mRNA. Other effects of the ectopic Xanf-1 include cyclopic phenotype and inhibition of the cement gland, both by Otx-2-dependent and independent mechanisms. Using fusions of Xanf-1 with the repressor domain of Drosophila engrailed or activator domain of herpes virus VP16 protein, we showed that most of the observed effects of Xanf-1 were probably elicited by its functioning as a transcription repressor. Altogether, our data indicate that the repressor function of Xanf-1 may be necessary for regulation of both neural differentiation and patterning in the presumptive anterior neurectoderm.

Key words: Neurogenesis, Patterning, Forebrain, BF-1, Otx-2, Pax-6, GFP, Xanf-1

INTRODUCTION

As a result of neural induction, dorsal ectoderm of vertebrate embryos is committed to the neural fate. At the same time, it is subdivided into domains giving rise to different structures of the central neural system (CNS). Despite considerable progress achieved in understanding of genetic control of these two events, still little is known about mechanisms that co-ordinate neurogenesis and patterning.

Meanwhile, some recent data confirm tight genetic link between these two processes. Thus, a high correlation is revealed between sites of neurogenesis and expression zones of some patterning genes in the forebrain anlage of zebrafish embryo (Macdonald et al., 1994). In agreement with this finding, it is demonstrated that a homeobox gene, flh, involved in the diencephalon patterning, can regulate the expression of proneural gene, Zash-1, and generation of secondary neurones in the epiphysis primordium (Masai et al., 1997). Moreover, it is shown that a winged helix transcription factor, XBF-1, which controls formation of the telencephalic primordium, can also position neuronal differentiation (Bourguignon et al., 1998). Taken together, these data indicate that many regulatory genes thought to be involved exclusively in patterning could also participate in the control of neurogenesis.

Recently, a novel class of homeobox genes, named Anf, which are transitory expressed in the medial sector of the anterior neurectoderm, was identified in vertebrates (Zaraisky et al., 1992, 1995; Hermesz et al., 1996; Kazanskaya et al., 1997). The present work shows that the Xenopus representative of Anf, Xanf-1, is able to control not only early patterning of the forebrain primordium, but also initial steps of neural commitment of embryonic ectoderm. These data indicate that neurogenesis in vertebrates is regionally specified from the very beginning by the genetic system responsible for the neurectoderm patterning.
MATERIALS AND METHODS

Preparation of DNA vectors
For GFP-Xanf-1-BDGR cassette, coding part of Xanf-1 cDNA was obtained from the Xanf-1 DNA-containing plasmid (Zaraisky et al., 1995) by PCR with primers:

5′-ATACCCGTCGCCGCGACCTCGAGGCGTTCGAGTG-3′
5′-ATTCGCGATCCCGGACGAGCTCGAGGGCTGCTCC-3′

These primers included a PstI and a HindIII site for Xanf-1 and BDGR cDNAs that were subcloned into pGFP-C1 vector (CLONTECH).

For preparation of synthetic GFP-Xanf-1-BDGR mRNA, GFP-Xanf-1-BDGR cassette was re-cloned into pSPgsc plasmid (Niehrs et al., 1994) instead of the goosecoid cDNA.

For EnR-Xanf-1 and VP16-Xanf-1 cassettes, EnR and VP16 cDNAs and a fragment of Xanf-1 cDNA containing the homeobox were obtained by PCR with primers:

5′-TGCGATCCCGGACGAGCTCGAGGGCTGCTCC-3′
5′-CAGCGATCCCGGACGAGCTCGAGGGCTGCTCC-3′ for EnR.
5′-CAGCGATCCCGGACGAGCTCGAGGGCTGCTCC-3′ for VP16.
5′-CCGGATCCCCCGAGATGAGACGACAGC-3′
5′-ATCCGTCGACGATCCCGGACGAGCTCGAGGGCTGCTCC-3′ for Xanf-1, respectively.

The obtained cDNAs were subcloned in pSP-GFP-Xanf-1-BDGR plasmid, instead of GFP-Xanf-1.

For synthesis of GFP mRNA, Xanf-1-BDGR was excised from pSP-GFP-Xanf-1-BDGR. cDNAs of XBF-1, NCAM, Xtwist and Pax-6 were obtained by RT-PCR from total RNA of the late neurula stage using primers based on sequences from GenBank.

Embryos, microinjections, microsurgery and in situ hybridization
Xenopus eggs were fertilised in vitro and dejellied in 2% cysteine at pH 7.8. Microinjections of mRNA (200 pg/blastomere) were done at the 8-cell stage in 0.1x MMR, 5% Ficoll and, after 2 hours, the injected eggs were transferred to 0.1x MMR for further incubation.

Injected embryos were staged according to Nieuwkoop and Faber (1967). Embryos were operated using microknife and a fused glass capillary on plastic dishes coated with 1% agarose. Tissue explants were excised by forceps in 1 hour, then cultured in 0.5% MMR, 5% Ficoll and, after 2 hours, the tissue explant was labelled by hydroxyurea/aphidicolin solution.

If Xanf-1 was activated by DEX before the midgastrula stage (stage 11.5), the neural plate was frequently enlarged on the injected side (58%, n=120). In all cases, the enlarged side was occupied by a Pglc of the GFP labelled cells (PGLC; Fig. 1D). No abnormalities were seen without DEX treatment (100%, n=130).

The enlargement of the neural plate was confirmed by in situ hybridization with the probe to general neural marker, NCAM mRNA (n=23; Fig. 1E,F). Importantly, this effect was not a result of suppression of dorsal convergence on the microinjected side, because an excess of the NCAM-labelled tissue was also revealed on transverse sections of embryos (100%, n=5; Fig. 1G).

We showed that the neural plate enlargement resulted from conversion of the CNS fate of the prospective cells of the neural crest and epidermis. First, we observed similar enlargement when cells division was blocked by hydroxyurea/aphidicolin treatment (Harris and Hartersten, 1991; not shown). Thus, the enlargement was not attributable to extra proliferation of PGLC cells. Second, when the neural plate was expanded, the neural fold was displaced from its normal position towards more lateral area, unlabelled by GFP (Fig. 1D arrowheads). In the cranial regions, it was accompanied by severe inhibition of the cranial neural crest marker, Xtwist (100%, n=20; Fig. 1H, arrowhead). Finally, along with the neural plate expansion,
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Fig. 1. (A) At the late gastrula stage, Xanf-1 is expressed in the anterior neurectoderm. *Xenopus* embryo hybridized in whole-mount is shown from the anterior, dorsal side up. (B) Before DEX application, GFP-Xanf-1-BDGR is uniformly distributed into the ectodermal cells of the early gastrula embryo. (C) 30 minutes after DEX treatment of the embryo shown in B, GFP-Xanf-1-BDGR is accumulated into cell nuclei. (D) Expansion of the neural plate in cranial region of the midneuralura embryo microinjected with GFP-Xanf-1-BDGR mRNA. The expanded area at the right half of the neural plate (left to the viewer) is occupied by PGLC containing the hybrid protein. Note lateral displacement of the cranial neural fold (arrowheads) at the microinjected side. Lateral borders of the neural plate are marked by dotted lines. (E) Whole-mount in situ hybridization with NCAM probe. The neural plate is expanded at the right side of the midneuralura embryo microinjected with GFP-Xanf-1-BDGR mRNA. (F) The same embryo as in E, shown alive under epifluorescence. (G) Transverse section of embryo shown in E demonstrates enlargement of the right half of the neural plate. (H) Inhibition of the expression of Xtwist in the cranial neural fold (arrow) of the midneuralura embryo microinjected with GFP-Xanf-1-BDGR mRNA. (I) The dorsal limit of expression of epidermis marker Xep-1, is shifted ventrally at the right side of the embryo microinjected in the right animal blastomere with GFP-Xanf-1-BDGR mRNA and treated by DEX. The position of the midline is marked by dashed line. (J,K) Animal cap explants of embryos microinjected with GFP-Xanf-1-BDGR mRNA or noggin mRNA and hybridized with NCAM probe at the 32 stage equivalent. No signals were observed in GFP-Xanf-1-BDGR-containing explants treated with DEX (J), in comparison with those microinjected with noggin mRNA (K). (L) In situ hybridization with type II β-tubulin mRNA probe showing suppression of primary neurones (arrowhead) on the microinjected side of the late neurula embryo.

dorsal limit of expression of epidermis marker Xep-1 (Vasiliev et al., 1997) was shifted more laterally at the microinjected side (100%, n=18; Fig. 1I). In contrast, GFP-Xanf-1-BDGR was unable to neuralise more ventral areas of the epidermis or isolated animal caps. Animal caps were extirpated from the blastula stage embryos microinjected with the *GFP-Xanf-1-BDGR* mRNA or *noggin* mRNA, treated by DEX at the early gastrula stage equivalent and cultured until the hatching stage equivalent. No expression of NCAM was detected in the caps of embryos microinjected with *GFP-Xanf-1-BDGR* mRNA both treated and untreated with DEX (Fig. 1J). In contrast, the expression of NCAM was observed in animal caps of embryos microinjected with *noggin* mRNA (Fig. 1K).

As Xanf-1 could promote neural plate expansion, we tested its influence on differentiation of primary neurones. Using a neural-specific type II β-tubulin gene as a marker (Oschwald et al., 1991), we revealed suppression of neuronal differentiation in embryos with exogenous *Xanf-1* (49%, n=39; Fig. 1L).

The neural plate enlargements induced by Xanf-1 develop into abnormal outgrowths of the CNS walls
When PGLC protruded out of the presumptive neural plate in the cranial region, the expanded areas of the neural tissue differentiated as abnormal brain outgrowths (Fig. 2A-C). The frequency and severity of the abnormalities gradually decreased when GFP-Xanf-1-BDGR was activated from the early to late gastrula stages, and the abnormalities were not seen, if DEX was applied after gastrulation (Table 1).

In all cases (n=43), cells of the abnormal outgrowths contained the GFP label (Fig. 3D,E). In contrast, unlabelled regions of CNS or labelled regions in embryos untreated with DEX (Fig. 3F,G) always looked normal (n=32). This indicated that the observed abnormalities were caused by cell-autonomous activity of GFP-Xanf-1-BDGR within the ectodermal cells, but were not induced by some external influence, for example, by influences from underlying mesoderm.

To test the latter possibility more directly, we performed a series of transplantation experiments. Small pieces of the anterior area of the presumptive neurectoderm (which was not yet underlaid by dorsal mesoderm) were extirpated from the early gastrula embryos microinjected with *GFP-Xanf-1-BDGR* mRNA and homotopically transplanted to the uninjected embryos of the same stage. GFP-Xanf-1-BDGR was activated by DEX at the midgastrula stage. During neurulation, all embryos were examined for locations of
transplants, and then placed each into an individual chamber for further incubation.

As a result, we obtained embryos in which GFP-Xanf-1-BDGR was present only in cells of a restricted area of the neurectoderm, but obviously was absent in cells of the underlying mesoderm. In many cases (41%, n=68), the embryos carrying transplants demonstrated the similar CNS outgrowths as seen in the embryos microinjected with GFP-Xanf-1-BDGR mRNA (Fig. 6J-L). No abnormalities were seen in the operated embryos untreated with DEX (n=52). These data further argued that the abnormalities were elicited by cell-autonomous activity of GFP-Xanf-1-BDGR in the ectoderm.

Xanf-1 can regulate early patterning of the anterior neural plate

To analyse the role of Xanf-1 in the early patterning of anterior neurectoderm, we investigated the influence of ectopic Xanf-1 on expression of three other genes known to control the anterior patterning, namely, *BF-1, Otx-2* and *Pax-6* (Pannese et al., 1995; Papalopulu and Kintner, 1996; Hirsch and Harris, 1997).

A common feature of the *Otx-2* and *Pax-6* during gastrula/neurula transition is downregulation in the medial sector of the anterior neurectoderm, in areas overlapped with the expression zone of *Xanf-1* (Fig. 3A,D,G). As a result, by the neurula stage, *Otx-2* and *Pax-6* are expressed complimentary to the expression domain of *Xanf-1*, in a ring-shaped and arched domains, respectively (Fig. 3B,E,H).

In contrast, at the end of gastrulation, the expression of telencephalic marker *XBF-1* was localised in the same cells of deep layer of the anterior neural fold that expressed *Xanf-1* (Fig. 4E). Noteworthy, cells of the superficial layer located above the *XBF-1* expression domain did not express *Xanf-1* (Fig. 4A,E,G). Another transverse band of cells expressing *XBF-1* appeared at the beginning of neurulation in the superficial layer, adjacent to the first expression domain from the posterior (Figs 3K, 4F,G). Careful analysis showed that the anterior border of this second band corresponded to the anterior border of the *Xanf-1* expression domain in the superficial cell layer (both borders were 13-14 cell diameters apart from the

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**Table 1. The ability of the exogenous Xanf-1 to induce abnormal phenotypes gradually decreases in the course of embryogenesis**

<table>
<thead>
<tr>
<th>Stage of DEX application (activation of the exogenous Xanf-1)</th>
<th>Type of abnormality</th>
<th>8 (mid blastula)</th>
<th>10 (beginning of gastrulation)</th>
<th>11.5 (mid gastrula)</th>
<th>12.5 (the end of gastrulation)</th>
<th>13.5 (early neurula)</th>
<th>15 (mid neurula)</th>
<th>19 (late neurula)</th>
</tr>
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<tbody>
<tr>
<td>Neural tube outgrowths</td>
<td></td>
<td>35%</td>
<td>30%</td>
<td>15%</td>
<td>2%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>(n=120)</td>
<td></td>
<td>(n=133)</td>
<td>(n=110)</td>
<td>(n=115)</td>
<td>(n=120)</td>
<td>(n=100)</td>
<td>(n=75)</td>
<td></td>
</tr>
<tr>
<td>Suppression of the cement gland</td>
<td></td>
<td>55%</td>
<td>52%</td>
<td>54%</td>
<td>10%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>(n=120)</td>
<td></td>
<td>(n=133)</td>
<td>(n=110)</td>
<td>(n=115)</td>
<td>(n=120)</td>
<td>(n=100)</td>
<td>(n=75)</td>
<td></td>
</tr>
<tr>
<td>Cyclopic phenotype (reduced telencephalon, fused eyes)</td>
<td></td>
<td>21%</td>
<td>19%</td>
<td>17%</td>
<td>12%</td>
<td>5%</td>
<td>0%</td>
<td>0%</td>
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<tr>
<td>(n=120)</td>
<td></td>
<td>(n=133)</td>
<td>(n=110)</td>
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In the case of *Otx-2*, this complementation became even more evident, when a region of seeming co-expression of *Otx-2* and *Xanf-1* at the rostral margin of anterior neural fold was analysed on medial sections. From the surface view of the midneurula embryos, the expression zones of *Otx-2* and *Xanf-1* in this region looked partially superimposed (Fig. 3B,E). However, on the medial sections, these two genes were expressed in different cell layers of the neuroectoderm, namely, *Otx-2* in the superficial layer and *Xanf-1* in the deep layer (Fig. 3B,D).

![Fig. 2.](image) (A) Brain of stage-47 normal embryo. Dorsal view. Abbreviations: tel, telencephalon; di, diencephalon; mes, mesencephalon; hind, hindbrain. The telencephalic (B) and mesencephalic (C) outgrowths (arrowheads) developed in embryos microinjected with GFP-Xanf-1-BDGR mRNA and treated by DEX. (D,E) GFP label in the telencephalic outgrowth (arrowhead) of the stage-47 embryo microinjected with the mixture of GFP and GFP-Xanf-1-BDGR mRNAs and treated by DEX under white and UV light, respectively. (F,G) No brain abnormalities were observed in the embryo microinjected with the same mixture, but not treated by DEX, despite high concentration of the exogenous proteins in brain cells (arrowhead).
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Hence, the second domain of the XBF-1 expression was also located within the Xanf-1 expression zone.

These expression patterns suggest that Xanf-1 can inhibit the expression of Otx-2 and Pax-6 and activate, or at least exert no influence, on the expression of XBF-1. However, we observed downregulation of all three genes in territories occupied by PGLC that contained activated GFP-Xanf-1-BDGR (100% for preselected embryos with PGLC crossing the anterior neural plate, \( n > 20 \) for each gene). Similar suppression was also observed when PGLC were generated by transplantation of ectodermal pieces from microinjected to non-injected embryos (100%, \( n = 8, 9 \) and \( 7 \) for XBF-1, Otx-2 and Pax-6, respectively; Fig. 5).

It is known that many homeodomain proteins can regulate the transcription of their own genes. Therefore, we used the same microinjection-transplantation approaches to study the influence of GFP-Xanf-1-BDGR on the expression of endogenous Xanf-1. The obtained results indicate that Xanf-1 can inhibit its own expression (100%, \( n = 15 \) for microinjections and \( n = 7 \) for transplantations; Fig. 5G-I).

Interestingly, the expression of BF-1, Otx-2 and Pax-6, which was inhibited by exogenous Xanf-1 during gastrulation and neurulation, appeared to be re-activated at later stages. Thus, many embryos at the tailbud stage demonstrated considerable expansion of the XBF-1 expression territory (30%, \( n = 70 \); Fig. 5K,L). The same was true for Otx-2 and Pax-6, which were intensively expressed in the abnormal brain outgrowths (see Fig. 3. (A-H). Whole-mount in situ hybridization with digoxigenin-labelled probes of Xanf-1 (A-C), Otx-2 (D-F), Pax-6 (G-I) and XBF-1 (J-L) mRNAs. Embryos shown from the anterior, dorsal side up, are at the late gastrula (stage 12-12.5), midneurula (stage 14-15) and late neurula (stage 18-19), respectively. External and internal borders of the neural folds of embryos at the 15 stage are marked by dotted lines.

Fig. 4. (A,B) Expression of Xanf-1 at the late gastrula and midneurula stages, respectively, as seen on sagittal sections. Dorsal side up, anterior to the right. At both stages, the Xanf-1 expression domain in the outer layer of neuroectoderm has a very sharp boundary (arrowhead) with the Xanf-1 non-expression domain.

(C,D) Expression of Otx-2 at the late gastrula and midneurula stages, respectively. Whereas at the end of gastrulation Otx-2 is expressed throughout the anterior neuroectoderm (C), by the midneurula stage its expression is ceased in the region superimposed with the Xanf-1 expression domain. (E) At the end of gastrulation, XBF-1 is expressed in a single band of cells, just beneath the superficial layer of the anterior neuroectoderm. (F) During early neurula stage, the expression of XBF-1 is extended posteriorly in the superficial cell layer, where the second expression band is formed. (G) Schematic diagram of the Xanf-1, Otx-2 and XBF-1 expression patterns on sagittal sections of the midneurula.
Possibly, these genes are reactivated after the degradation of exogenous Xanf-1 at the late stages (see above for the discussion of GFP-Xanf-1-BDGR stability). Exogenous Xanf-1 can elicit a posterior shift of the expression zones of telencephalic markers As abnormal CNS outgrowths were composed of progenies of cells containing the microinjected Xanf-1, investigation of cell identity in these outgrowths could be useful for understanding the role of Xanf-1 in cell differentiation. Assuming Xanf-1 is normally expressed rostrally, it would be logical to suppose that its ectopic expression may result in an anteriorisation of cell identity. Indeed, we observed ectopic expression of the telencephalic markers, Emx-1 and XBF-1, in dorsal regions of the outgrowths developed at the diencephalic level (80%, n=17 and 75%, n=20, respectively; Fig. 6B,E). However, no expression of the more anterior marker was observed in the mesencephalic outgrowths. Thus, cells of the mesencephalic outgrowths never expressed Emx-1 and XBF-1 or Pax-6, the gene whose expression is characteristic to both di- and telencephalic cells (n=13, 12 and 12, respectively; Fig. 6C,F,H). At the same time, cells of the mesencephalic outgrowths expressed Otx-2, intensively as is normal in this region (100%, n=5 for transplants and n=11 for routine microinjections; Fig. 6L). Similarly, cells of the diencephalic outgrowth expressed Pax-6 (not shown); Pax-6 is normally expressed in the dorsal diencephalon (100%, n=15; Fig. 6G). These data indicate that the observed posterior shift of the telencephalic markers expression could occur only within the dorsal part of forebrain. However, further experiments are necessary to distinguish if this shift resulted from the true di- to telencephalic transformation or was elicited by a morphogenetic displacement of some cells predetermined to the telencephalic fate. Spatial restriction of the Xanf-1 expression is essential for the normal forebrain development In addition to CNS outgrowths, we distinguished another brain abnormality observed whenever PGLC expanded beyond the anterior margin of neural plate, namely the forebrain, associated with medial fusion of eyes (Fig. 7A-C). This cyclopic abnormality was detected only if GFP-Xanf-1-BDGR was activated by DEX before the midneurula stage (Table 1). This phenotype was never observed if PGLC did not cross the anterior neural fold or were located entirely out of the CNS primordium, at some distance from its anterior margin (not shown). Therefore, it could be associated with the restricted zone located somewhere within the anterior neural fold. To map this hypothetical zone more precisely, we made a series of transplantations of small ectodermal pieces from injected to non-injected embryos, thus, generating compact PGLC. Embryos with different positions of transplants in the
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Xanf-1 can suppress development of the cement gland

When PGLC occupied the area of presumptive cement gland and DEX was applied before the end of gastrulation, we observed suppression of the cement gland differentiation in cells containing GFP-Xanf-1-BDGR (Table 1). Depending on distribution of PGLC within the presumptive cement gland area, the effects varied from entire lack of the cement gland (Fig. 8A), to a half-sized one, or double (Fig. 8F), or several small glands (Fig. 8E). As was shown, at the tailbud stage, cells of the presumptive cement gland that contained GFP-Xanf-1-BDGR did not express the cement gland marker, XAG (Fig. 8E), producing instead the specific epidermal marker, Xep-1 (Fig. 8H). Therefore, these cells should have adopted an epidermal fate. Ability of GFP-Xanf-1-BDGR to autonomously suppress the cement gland differentiation in the ectoderm was confirmed by transplantation experiments (100%, n=10; Fig. 8F,H).

Differentiation of the cement gland is known to be controlled by Otx-2, which can induce it even in the ventral ectoderm (Gammill and Sive, 1997). As Xanf-1 could downregulate Otx-2, the revealed suppression of the gland could be a result of inhibitory influence of the microinjected Xanf-1 on the Otx-2 expression. However, it was still possible that Xanf-1 independently downregulated the cement gland differentiation.

To address this problem, we microinjected a mixture of the Otx-2 and GFP-Xanf-1-BDGR mRNAs into animal blastomeres. Microinjected embryos or animal caps isolated from them at the late blastula stage were treated by DEX at the midgastrula stage. As a result, a severe inhibition of cement glands was detected in embryos and explants containing the activated GFP-Xanf-1-BDGR (100%, n=21 and 35 for embryos and explants, respectively; Fig. 8J,L). Thus, in parallel to inhibition...
Fig. 8. Exogenous Xanf-1 can inhibit differentiation of the cement gland. All embryos at the tailbud stage are shown from ventral side, anterior to the top. (A-E) Inhibition of the cement gland in the embryos microinjected at the 8-cell stage with GFP-Xanf-1-BDGR mRNA in both dorsal blastomeres. (A) Almost complete suppression of the cement gland differentiation in the embryo whose head area is occupied by PGLC. (B) A very small patch of the cement gland cells is still formed just in the place where GFP labelling is absent (arrow). (C,D) In the control embryo microinjected with GFP-Xanf-1-BDGR mRNA but untreated by DEX, no signs of the cement gland inhibition are seen (C) despite wide expansion of PGLC in head area (D). (E) Hybridization with cement gland marker XAG-1 probe shows that several small patches of the cement gland cells differentiated in the embryo microinjected with GFP-Xanf-1-BDGR mRNA and treated by DEX (left), instead of a big single cement gland in the DEX-untreated embryo (right). (F,G) Transplantations of small ectodermal grafts from embryos microinjected with GFP-Xanf-1-BDGR mRNA to non-injected embryos. (F) The cement gland differentiation is inhibited in the central part of the normal cement gland area occupied by the DEX-treated graft. Two small cement glands (arrowheads) develop from those parts of the presumptive cement gland territory that are not occupied by the graft. (G) Epi-fluorescent view of the embryo shown on F reveals grafted cells. (H) Hybridization with the epidermal marker Xep-1 probe demonstrates that in the embryo shown in F and G, cells of the graft acquire epidermal fate. (I-L) Xanf-1 can suppress the cement gland differentiation induced by Otx-2. (I) The cement gland differentiation in the control embryo is revealed by XAG-1 probe. (J) Activation of GFP-Xanf-1-BDGR in embryos microinjected with the mixture of Otx-2 and GFP-Xanf-1-BDGR mRNAs results in severe reduction of the XAG-1 expression, despite the presence of Otx-2. (K,L) In animal caps isolated at the late blastula stage from embryos microinjected with the mixture of Otx-2 and GFP-Xanf-1-BDGR mRNAs, exogenous Xanf-1 demonstrates the same epistatic effect over Otx-2 as observed in the whole embryos. (K) An intense expression of XAG-1 under the influence of Otx-2 in the animal caps not treated by DEX. (L) Activation of GFP-Xanf-1-BDGR in treated caps represses the XAG-1 expression.

Fig. 9. (A-F) Microinjections of EnR-Xanf-1-BDGR mRNA (mixed with GFP mRNA) elicit the effects similar to those caused by exogenous Xanf-1. (A) Hybridization with NCAM probe demonstrates expansion of the neural plate on the microinjection side. (B) The abnormal diencephalic outgrowth in the microinjected embryo contains cells expressing the telencephalic marker, XBF-1 (arrow). (C,D) Inhibition of the Otx-2 expression (C) within the territory occupied by PGLC (D). (E,F) Inhibition of the cement gland differentiation in the medial region of cement gland territory occupied by PGLC, results in splitting into two separate cement glands. (G-L) In contrast to EnR-Xanf-1-BDGR, VP16-Xanf-1-BDGR injected into the right side of the embryo produces some effects opposite to those elicited by Xanf-1 alone: downregulation of NCAM (arrowhead G), expansion of the right Xtwist expression domain (arrowhead H), the ectopic expression of Otx-2 (arrowheads I,J), cement gland differentiation (K,L) observed in the regions occupied by cells containing the activated VP16-Xanf-1-BDGR.
of Otx-2, Xanf-1 can independently inhibit the cement gland differentiation.

The effects of Xanf-1 may result from functioning as a transcriptional repressor

The revealed inhibitory effects of Xanf-1, and also the presence of a conservative repressor domain in all known Anf proteins (Smith and Jaynes, 1996; Kazanskaya et al., 1997), suggest that Xanf-1 may be a transcription repressor. To verify this hypothesis, we fused the Xanf-1 homeodomain either with the repressor domain of Drosophila engrailed protein (EnR) or the activator domain of herpes virus VP16 protein. This approach permits one to distinguish whether a given protein operates as a transcription activator or repressor, depending on which fusion, EnR or VP16, reversed or eliminates the effects elicited by the protein (for example, see Fan and Sokol, 1997; Brewster et al., 1998).

In our case, EnR-Xanf-BDGR elicited the effects similar to those of GFP-Xanf-BDGR and with similar frequency (totally, \( n > 100 \); Fig. 9A-F). In contrast, expression of VP16-Xanf-BDGR reversed such effects typical for GFP-Xanf-1-BDGR as expansion of NCAM expression (50%, \( n = 22 \)), suppression of the Xtwist (70% of preselected embryos with PGLC crossing cranial neural folds, \( n = 17 \)), Otx-2 (100% of preselected embryos with PGLC crossing anterior neural plate, \( n = 10 \)) and the cement gland differentiation (100% of embryos with PGLC within the presumptive cement gland, \( n = 35 \); Fig. 9G-L). Moreover, in the case of VP16-Xanf-BDGR, we never observed the neural plate enlargement, suppression of primary neurogenesis, development of abnormal brain outgrowths or downregulation of Pax-6 and endogenous Xanf-1. Furthermore, in all cases (\( n = 25 \)) when PGLC containing VP16-Xanf-BDGR occupied the anterior margin of the neural plate, we observed a severe reduction of the telencephalon (data not shown). On the contrary, similarly to EnR-Xanf-GR, VP16-Xanf-BDGR elicited inhibition of XBF-1 expression during gastrulation/neurulation and cyclopic phenotypes in tadpoles. Together, these results indicate that most of the effects elicited by exogenous Xanf-1 are likely to be caused by its transcriptional repressor function.

DISCUSSION

Specificity of the effects of exogenous Xanf-1

Our observations of multiple inhibitory effects of the exogenous Xanf-1 raised the question whether Xanf-1 may play a role of a general inhibitor of gene expression. However, the following evidence argues for at least some specificity of Xanf-1 effects. First, we used Xanf-1 fusion to the transcription activator VP16 and observed a clear reversion of some effects elicited by Xanf-1, namely, activation of Otx-2 and Xtwist expression, and downregulation of NCAM. Second, Xanf-1 could elicit the neural plate expansion only during a certain time interval, and the percentage of affected embryos decreased abruptly if the exogenous protein was activated after the mid-gastrula stage. Importantly, other effects of Xanf-1 (inhibition of the cement gland and cyclopic phenotype) could be observed when the Xanf-1 was activated even after this stage. Finally, our unpublished data (E. M. A. and A. G. Z.) show that the ectopic expression of Xanf-1 in the neural plate did not affect the expression of at least two regulators of neurogenesis, namely, Xotch and XASH-3.

Xanf-1 may regulate early stages of neurogenesis in the anterior neurectoderm

Our results indicate that ectopic expression of Xanf-1 can expand the neural plate at the expense of adjacent tissues, namely, the presumptive neural crest and epidermis. This is confirmed by expansion of the NCAM expression territory complimentary to inhibition of the cranial neural crest marker Xtwist and epidermis marker Xep-1.

Ability of the ectopic Xanf-1 to elicit the neural plate expansion consistent with the expanded form of the anterior neural plate in which Xanf-1 is expressed in normal development. Moreover, the inhibitory influence of Xanf-1 on the neural crest differentiation corresponds to the lack of presumptive neural crest cells at the anterior margin of the neural plate, in a region where Xanf-1 expression zone reaches the neural plate border. Thus, Xanf-1 functions may be promotion of the anterior expansion of the neural plate and suppression of the neural crest differentiation.

Several factors indicate that Xanf-1 could be associated with the initial steps of neurogenesis specifically in the anterior neurectoderm. First, the Xanf-1 expression is restricted to the anterior neurectoderm as early as the midgastrula stage (Zaraisky et al., 1995). Second, the GFP-Xanf-1-BDGR can elicit the neural plate expansion only when it is activated during gastrulation, when the embryonic ectoderm is still competent to the neural induction (Servetchnick and Grainger, 1991). Third, the expression of Xanf-1 is one of the earliest responses in the ectoderm induced specifically to anterior neural development by noggin and chordin (Sasai et al., 1995; our unpublished data). Finally, it has recently been shown that disruption of the mouse ortholog of Xanf-1, Hexx1/Rpx, results in a reduction of the anterior neurectoderm derivative, the prosencephalon (Dattani et al., 1998).

Many known factors expressed in the neurectoderm can stimulate neurogenesis. Some of them, such as Neurogenin, NeuroD, ATH-3 and Zic-r1, can activate the genetic cascade leading to terminal neuronal differentiation even in the absence of additional neural inducers (Lee et al., 1995; Ma et al., 1996; Takebayashi et al., 1997; Mizuseki et al., 1998). Others, like XASH-3 and the Iroquois class homeodomain factors, Xiro, can predominantly induce early steps of neurogenesis, but inhibit terminal differentiation of neuron (Turner and Weintraub, 1994; Bellefroid et al., 1998; Gomez-Skarmeta et al., 1998).

The revealed effects of Xanf-1 resembled the latter. However, in contrast to XASH-3 and Xiro, Xanf-1 was unable to promote the expression of general neural markers in the lateral ectoderm and in the isolated animal caps. This indicates that Xanf-1 alone cannot counteract the action of the BMP neuroinhibitory cascade that is initially active in the embryonic ectoderm.

Another revealed effect of the ectopic Xanf-1 is the negative influence on differentiation of primary neurons. It is known that, in normal development, neuronal differentiation in the territory located rostrally to the prospective hindbrain-midbrain boundary is delayed in comparison with the trunk zone (Papalopulu and Kintner, 1996). Assuming anterior location of Xanf-1 expression and its ability to inhibit primary
neurogenesis, it is logical to suppose that Xanf-1 may be a factor responsible for this delay.

Although the precise role of Xanf-1 in the neurogenic cascade is still unknown, the data described above permit one to conclude that its role in neurogenesis may be specifically associated with the initial establishment of the anterior neural plate territory, but not with the promotion of neurogenesis per se.

**The repressor function of Xanf-1 may arrange the expression of other genes involved in the early patterning of the anterior ectoderm**

In our experiments, ectopic Xanf-1 was able to downregulate at least five genes, Otx-2, Pax-6, XAG-1, XBF-1 and the endogenous Xanf-1, expressed in the anterior neurectoderm. In normal development, the expression of the first three genes is also repressed to a various extent within the Xanf-1 expression zone. As shown in Xenopus and mouse, downregulation of some genes in the anterior neurectoderm, in particular Pax-6, is necessary for normal development of the ventral forebrain, and it may be based on vertical inhibitory signalling from the underlying head mesoderm (prechordal plate: Li et al., 1997; Shimamura and Rubenstein, 1997). On the contrary, the head mesoderm is able to induce the expression of Xanf-1 in the overlying neurectoderm (Zaraiisky et al., 1992). Together these data indicate that Xanf-1 may be a factor responsible for realisation of the mesodermal inhibitory signalling within the medial sector of the anterior neural plate.

In contrast to Otx-2 and Pax-6, the revealed ability of exogenous Xanf-1 to inhibit the expression of XBF-1 seems to contradict the fact of co-expression of the endogenous Xanf-1 and XBF-1 in the same cells. This discrepancy may be explained if one supposes that ectopic Xanf-1 can inhibit some external signalling, necessary for the XBF-1 expression. We suggest that, in the anterior neural fold, the boundary between the domain of cells expressing Xanf-1 and XBF-1 and more anterior/superficial domain of cells not expressing them may be a source of such signalling. In this case, expanded expression of either Xanf-1, or VP16-Xanf-1, or EnR-Xanf-1 may destroy this boundary, stop the signalling and, thus, downregulate XBF-1. These effects were indeed observed in our experiments. However, further experiments are necessary to directly check this hypothesis.

Our data revealed a reversible character of the inhibitory influence of Xanf-1. Thus, three genes inhibited by Xanf-1 during gastrulation and neurulation, Otx-2, Pax-6 and BF-1, appeared to be re-activated in the abnormal brain outgrowths, presumably, due to degradation of the microinjected Xanf-1. Interestingly, this effect resembled the normal course of the Otx-2 and Pax-6 expression in the eye cap cells. Thus, at the end of gastrulation, cells of the prospective eye territory intensively expressed both genes (Fig. 3D,G). Then, at the beginning of neurulation, a downregulation of Otx-2 and Pax-6 was seen in the part of the prospective retina territory overlapping the Xanf-1 expression zone (Fig. 3E,H). However, downregulation of Xanf-1 by the end of neurulation is correlated with the intensive expression of Otx-2 and Pax-6 in the invaginated eye caps (Pannese et al., 1995; Hirsch and Harris, 1997).

Therefore, one of functions of Xanf-1 may be creation of a temporal pause in expression of some genes. The revealed negative feedback regulation of Xanf-1 may provide a simple way to control this function. Thus, being activated by some inducer, the expression of Xanf-1 and, hence, its repressor influence on other genes, would automatically cease when the concentration of Xanf-1 protein reaches a critical value.

**The repressor function of Xanf-1 may be necessary for the telencephalic differentiation**

The ectopic expression of Xanf-1 elicited the appearance of telencephalic cells located posteriorly to the normal telencephalon, namely, in the dorsal part of the diencephalic outgrowths. Two hypotheses can be suggested to explain this effect. First, it may result from splitting of the telencephalic field into two separate parts by abnormal morphogenetic movements. Although all brain outgrowths were entirely composed of the labelled cells derived from the Xanf-1 microinjected blastomeres, it is impossible to rule out the possibility that these cells might have initially belonged to the presumptive telencephalic field and then were moved to the diencephalic outgrowths.

The second hypothesis is that the ectopic Xanf-1 may induce a transformation of the presumptive diencephalic cells to the telencephalic identity. Previously, it has been argued that inhibition of the caudal diencephalic identity in cells of the prospective telencephalon, in particular the Otx-2 expression, might be an essential prerequisite for the normal development of the telencephalon in zebrafish embryos (Heisenberg et al., 1996; Masai et al., 1997). Assuming that Xanf-1 can downregulate the expression of Otx-2, our data may indicate that Xanf-1 is a regulator responsible for this inhibition. However, further experiments are necessary to determine if the observed ectopic telencephalic differentiation is a result of true diencephalic to telencephalic transformation.

We show that the repressor function exerted by Xanf-1 in the forebrain primordium may also be necessary to inhibit cement gland differentiation. It was demonstrated recently that, in contrast to the epidermal area, the cement gland upregulator, Otx-2, cannot induce this differentiation in the neural plate (Gammill and Sive, 1997). In the view of our data, this result may be explained by the inhibitory influence of Xanf-1 in the anterior neurectoderm.

Thus, the role of Xanf-1 may be to inhibit those types of cell differentiation that do not lead to the telencephalic development.

**Abnormalities induced by the exogenous Xanf-1 indicate that its expression should be under tight spatial control**

The results of transplantation experiments revealed a restricted zone in the anterior margin of the neural plate that appears to be critical for development of the cyclopic phenotype, when occupied by cells ectopically expressing Xanf-1. As this abnormality had non-autonomous character, we suggest that the expression of Xanf-1 in the anterior margin of the neural plate may suppress some planar morphogenetic signalling spreading into the posterior direction and patterning the rostral forebrain primordium. Possibly, suppression just of this signalling could explain inhibition of XBF-1 by the ectopic Xanf-1.

The role of anterior margin of the neural plate as a forebrain-organising centre is also supported by the results of cell-ablation experiments performed recently in mouse and zebrafish embryos. Thus, in mouse, it is shown that removal of the
Xanf-1 regulates neurogenesis and patterning


