**Xenopus Zic-related-1 and Sox-2, two factors induced by chordin, have distinct activities in the initiation of neural induction**

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**SUMMARY**

In a differential screen for downstream genes of the neural inducers, we identified two extremely early neural genes induced by Chordin and suppressed by BMP-4: Zic-related-1 (Zic-r1), a zinc finger factor related to the *Drosophila* pair-rule gene *odd-paired*, and Sox-2, a Sry-related HMG factor. Expression of the two genes is first detected widely in the prospective neuroectoderm at the beginning of gastrulation, following the onset of Chordin expression and preceding that of Neurogenin (Xngnr-1). Zic-r1 mRNA injection activates the proneural gene Xngnr-1, and initiates neural and neuronal differentiation in isolated animal caps and in vivo. In contrast, Sox-2 alone is not sufficient to cause neural differentiation, but can work synergistically with FGF signaling to initiate neural induction. Thus, Zic-r1 acts in the pathway bridging the neural inducer with the downstream proneural genes, while Sox-2 makes the ectoderm responsive to extracellular signals, demonstrating that the early phase of neural induction involves simultaneous activation of multiple functions.

Key words: Neural induction, Downstream signals, Sox, Odd-paired, Zic, Chordin, BMP-4

**INTRODUCTION**

Recently, molecular study of early vertebrate neurogenesis has made progress in two separate areas. The first concerns the mechanisms of neural induction. Neural inducers such as Noggin (Lamb et al., 1993; Zimmerman et al., 1996), Follistatin (Hemmati-Brivanlou et al., 1994; Fainsod et al., 1997) and Chordin (Chd) (Sasai et al., 1995; Piccolo et al., 1996) dorsalize the ectoderm and drive it into neural fate by binding to and inactivating the endogenous epidermalizing factor BMP-4 in the extracellular space (reviewed in Hemmati-Brivanlou and Melton, 1997; Sasai and De Robertis, 1997). BMP-4 ventralizes both ectoderm and mesoderm, while attenuation of the BMP signaling by the organizer factors results in dorsalization. In zebrafish a ventralized mutant *dino*, which has a reduced size of the CNS, is caused by mutation in the fish Chd homologue and can be rescued by *Xenopus* Chd (Schulte-Merker et al., 1997; Sasai and De Robertis, 1997). BMP-4 ventralizes both ectoderm and mesoderm, while attenuation of the BMP signaling by the organizer factors results in dorsalization. In zebrafish a ventralized mutant *dino*, which has a reduced size of the CNS, is caused by mutation in the fish Chd homologue and can be rescued by *Xenopus* Chd (Schulte-Merker et al., 1997; Sasai and De Robertis, 1997). BMP-4 ventralizes both ectoderm and mesoderm, while attenuation of the BMP signaling by the organizer factors results in dorsalization.

The second area of study concerns the regulation of neuronal differentiation involving the vertebrate homologues of *Drosophila* proneural and neurogenic genes (reviewed in Simpson, 1995). A large number of vertebrate *AS-C* and *atona* homologues have been isolated (reviewed in Lee, 1997). Together with vertebrate Delta and Notch homologues, these proneural genes are involved in temporal and spatial regulation of neuronal differentiation at multiple steps. In Xenopus neurogenin-related-1 (Xngnr-1), an atonal-class bHLH factor, is among the earliest proneural genes and is expressed in the primary neurons (Ma et al., 1996). Xngnr-1 alone is sufficient to induce neural and neuronal differentiation in the embryonic ectoderm when overexpressed, suggesting that Xngnr-1 may be a primary regulator of the primary neurons (Ma et al., 1996).

The initial specification of neuroectoderm by inhibiting BMP-class molecules and the proneural/neurogenic gene networks are well conserved during evolution. However, little is known about the pathways connecting these two steps either in fly or in vertebrates. In *Xenopus* the onsets of Chd and Xngnr-1 expression are separated by a few hours (Sasai et al., 1994; Ma et al., 1996), suggesting that one or more transcriptional steps may be involved. Here, we have carried out a systematic screen for genes that may fill in the gap between the neural inducers and the proneural/neurogenic genes.

**MATERIALS AND METHODS**

Differential screening for the Chd downstream genes

3 nl of 50 ng/µl *sog* mRNA (Holley et al., 1995) or water was injected.
into four animal blastomeres of 8-cell embryos. 1,500 animal cap explants were prepared at stage 9 and harvested for mRNA preparation at stage 11. The first strand cDNA was synthesized from mRNA of the sog-injected or control caps and each was subtracted with tenfold excess of ventral marginal zone mRNA (from 5,000 explants) in order to remove maternal and ventral mRNAs, and used as a differential probe. The subtraction and differential hybridization were performed as described previously (Sasai et al., 1994), except that we added 500 ng/ml heat-denatured pSP35-sog (from which the sog mRNA was transcribed in vitro) to the hybridization solution in order to reduce background derived from the injected mRNA. Two clones out of the 78 positives had expression in the very early neuroectoderm, Sox-2 and an odd-paired homologue. We found another frog Zic-related gene deposited in the GenBank database (U57453); this one was reportedly isolated from neurula embryos and shows higher homology to Zic2 than to Zic1, unlike our clone. Therefore, we named our clone *Xenopus* Zic-related-1 (Zic-r1). At this moment, because of high similarity between Zic-related factors in structure and distribution, it is still to be clarified whether or not Zic-r1 is the true frog orthologue of mouse Zic-1. *Xenopus* Sox-2 was also isolated independently in the screening that yielded Cerberus (Bouwmeester et al., 1996). A detailed study of late expression of this clone is available elsewhere (De Robertis et al., 1997).

**Microinjection, lineage tracing and animal cap assay**

Microinjection of mRNA was performed as described previously (Sasai et al., 1994, 1995). The coding regions of Zic-r1 and Sox-2 were amplified by PCR and subcloned into the Shi1-XhoI sites and the EcoRI-XhoI sites of pCS2 vector, respectively (pCS2-ZCR1 and pCS2-SOX2). For mRNA injection, the plasmids were linearized with KpnI (Zic-r1) or NotI (Sox-2) and the in vitro transcripts were synthesized with SP6 polymerase. Control experiments were performed with the same amount of β-gal mRNA. For lineage tracing, 25 pg of β-gal mRNA (Sasai et al., 1995) or EGFP mRNA (EGFP is an enhanced GFP from Clontech) was co-injected into one blastomere of the two-cell embryo. For GFP, neural-plate-stage embryos that had massive GFP signals on the right side but no signals on the left were selected under the fluorescence microscope and used for in situ hybridization studies. For animal cap experiments with RNA microinjection, we excised and cultured explants in 1× LCMR (66 mM NaCl, 1.33 mM KCl, 0.33 mM CaCl2, 0.17 mM MgCl2, 5 mM Hepes, pH 7.2) supplemented with 0.1% BSA and antibiotics (Lamb et al., 1993). As reported previously (Lamb and Harland, 1995), we did not observe autoneuralization of animal caps in this medium, nor did we see significant differences between 1× LCMR and 0.4× MMR, except that the animal caps survived relatively better in 1× LCMR when cultured for longer than 48 hours. Human recombinant bFGF (Promega) and human recombinant activin (Autral Biologicals) were used at 10 ng/ml. FGF was added to the medium from stage 10.25 and activin from stage 9. All the injection experiments (both animal caps and whole embryos) were repeated at least twice and gave consistent results. For each kind of experiment, the statistical values of one representative result are shown in the text.

**In situ hybridization and histological studies**

In situ hybridization studies were performed as described previously (Sasai et al., 1994, 1995). DIG-labeled probes were synthesized from pCS2-ZCR1 or pCS2-SOX2 plasmids linearized with HindIII, using T7 RNA polymerase. For hairy 2, a partial cDNA clone was obtained from a Xenopus neurula cDNA lambda ZAP library by low-stringency hybridization screening using the HLH domain fragment of rat HES-4 as a probe (Sasai et al., 1992) (GenBank accession #AF022798). In situ hybridization with animal caps were photographed under transmission light after being colored briefly with Bouin’s fixative and cleared with Murray’s solution in order to show both shape and inside staining of the caps. Histological analyses were performed on 10 μm paraffin sections after fixing with Bouin’s fixative and complete destaining of yellow color of picric acid. Some of them were stained by the conventional hematoxylin-eosin method.

**RT-PCR analysis**

RT-PCR analysis was performed as described previously (Wilson and Hemmati-Brivanlou, 1995; Sasai et al., 1995), except that Taq Extender additive (Stratagene) was added to the PCR reaction. The new primers used here are the following: Xangnr-1 (forward, 5′-GGATGTTGCTGCTGCTACGCCAGTACC-3′; reverse, 5′-CAAGCGAGTTCAAGTTGCTGATGC-3′; 28 cycles), NeuroD (forward, 5′-CCATGGGACACGCGCATGCTGAC-3′; reverse, 5′-TGCGGATTACCTGCTCCGGAAACAG-3′; 28 cycles) and slug (forward, 5′-TCCCGCAGTGAAAATGCGACGATC-3′; reverse, 5′-CCGTCCTAAAGATGAAGGGTATCTCG-3′; 28 cycles) Two or more independent injection experiments were analyzed by RT-PCR and gave consistent results.

**RESULTS**

**Differential screening for downstream genes of neural inducers**

Chd has a functional fly homologue, Sog, which can induce neural tissues both in fly and in frog when overexpressed (Holley et al., 1995). Taking advantage of this fact, we attempted to isolate genes that are activated during early phases of neural induction by a differential cloning strategy. 1,500 animal cap explants pre-injected with sog mRNA or water (control) were prepared at a late blastula stage (stage 9), cultured until an early gastrula stage (stage 11) and then harvested for mRNA purification. We avoided using *Xenopus* Chd because the injected Chd mRNA would have caused artifacts by picking Chd cDNA as differential clones. Using sog mRNA alleviates this problem as the *Drosophila* gene should not exist in the *Xenopus* library. Using these two pools of mRNAs (sog-injected and control) as differential probes, we screened 15,000 plates of stage 11.5 *Xenopus* neuroectoderm cDNA lambda library. 78 clones showing a stronger signal with the ‘sog-injected’ probe than with the control probe were picked up and further characterized by whole-mount in situ hybridization and partial sequencing. 57 of the clones were expressed preferentially in the nervous system and 35% of the neural clones showed homology to known sequences in the database. In this study, we focused on two clones that are expressed in the neuroectoderm from very early stages of gastrulation. The first is a zinc finger factor closely related to *Drosophila* odd-paired (Benedyk et al., 1994) (Fig. 1A) and has high homology to the mammalian odd-paired-related Zic factors (Aruga et al., 1996) (90% amino acid identity to mouse Zic1, 74% to Zic2 and 69% to Zic3). In mouse, these Zic genes are expressed in early neuroectoderm in overlapping patterns (Nagai et al., 1997). Therefore, we named it *Xenopus* Zic-related-1 (Zic-r1; accession #AF022927). The second clone analyzed was the *Xenopus* counterpart of Sox-2 (91% amino acid identity to chick Sox-2; accession #AF022928) (Kamachi et al., 1995; Uwanogho et al., 1995) (Fig. 1B).

**Temporal and spatial expression patterns of Zic-r1 and Sox-2**

By whole-mount in situ hybridization, both Zic-r1 and Sox-2 transcripts are first detected at the beginning of gastrulation (Fig. 2A,B). During early gastrula stages when neural
induction first takes place, Zic-r1 and Sox-2 are expressed widely in the dorsal ectoderm (Fig. 2A-D). The expression pattern of Sox-2 is pan-neural throughout embryonic stages (Fig. 2E-H,J), including in the CNS, neural crest, placodes (Fig. 2I) and lateral line (not shown). By contrast, Zic-r1 expression shows two distinct phases. First, when neural induction occurs, Zic-r1 has wide and homogenous expression in the prospective neuroectoderm (also see Fig. 3). Later, at late gastrula stages, Zic-r1 expression is shut off in the dorsal midline except in the most anterior area (Fig. 2E). At the neural plate stages, Zic-r1 is expressed in the lateral part of anterior neural plate and in the flanking ectoderm (Fig. 2G). At larval stages (Fig. 2I), Zic-r1 transcripts are detected mainly in the dorsal neural tube throughout its A-P axis.

**Zic-r1 and Sox-2 are early downstream genes of the BMP-4-neural inducer signals**

As expected from the cloning strategy, both Zic-r1 and Sox-2 are upregulated by overexpression of Chd (Fig. 2K,L) and suppressed by that of BMP (Fig. 2M,N) in vivo (early gastrula stages). In animal caps, Zic-r1 and Sox-2 are induced by Chd (Fig. 2P,Q; 100%, n=20 each) in the absence of mesoderm induction (Fig. 2O; n=18) and by blocking BMP signaling with a dominant-negative BMP receptor (Graff et al., 1994; Suzuki et al., 1994) or dissociation (Wilson and Hemmati-Brivanlou, 1995) (not shown).

We next examined the time courses of expression of Chd (Fig. 3A,E,ILM), Zic-r1 (Fig. 3B,F,IN), Sox-2 (Fig. 3C,G,K,O) and the proneural gene Xngnr-1 (Fig. 3D,H,L,P) by whole-mount in situ hybridization to compare their onset of expression. Zic-r1 and Sox-2 transcripts were first detected at late blastula, about 30 minutes before the onset of gastrulation (Fig. 3F,G) (in this experiment done at 24°C, the first indication of lip formation was observed 8 hours after fertilization), following the onset of Chd (75 minutes before gastrulation started; Fig. 3A). Xngnr-1 transcript was first detected 75 minutes after the appearance of the dorsal lip (Fig. 3P). During these early stages, both Zic-r1 and Sox-2 are expressed widely in the forming neuroectoderm. Taken together with the animal cap experiment above, these results show that Zic-r1 and Sox-2 are among the genes induced at the earliest step of neural induction, regulated negatively by BMP signaling.

**Zic-r1 can promote neural differentiation in the embryonic ectoderm**

We next investigated the roles of Zic-r1 and Sox-2 in neural induction. When 200 pg of Zic-r1 mRNA was injected into each of four animal blastomeres of the 8-cell embryo, the animal cap explant from the injected embryo reproducibly differentiated into neural tissue (90%, n=30; Fig. 4A), and contained ventricle-like structures (67%) with a thin roof plate. (Fig. 4A). Zic-r1-injected animal caps expressed the pan-neural marker nrp-1 (Knecht et al., 1995) (Fig. 4C; 92%, n=22) while the control caps (C, inset) did not (7%). When injected into one blastomere of the 2-cell embryo, Zic-r1 mRNA injection increased the expression areas of Xngnr-1 (58%, n=19; Fig. 4D), neuron-specific tubulin (N-tubulin; 57%, n=21; Fig. 4E) and the neural crest marker slug (Mayor et al. 1995) (85%, n=20; Fig. 4F) in vivo on the injected side, whereas the epidermal marker keratin was suppressed (lateral views of the injected and control sides shown in Fig. 4G and H, respectively) (78%, n=23). Consistent with these findings, Zic-r1 injection expanded the neural plate area laterally on the injected side (84%, n=31), which was demarcated by hairy 2 (Turner and Weintraub, 1994) (Fig. 5I). These results show that Zic-r1 is sufficient to induce neural and neuronal differentiation in the animal cap explant and in vivo.

We further analyzed Zic-r1-induced neural differentiation by RT-PCR. In animal cap assays, Zic-r1 injection induced the proneural gene Xngnr-1 at a gastrula stage (stage 12; Fig. 5A), and the neural marker N-CAM, the neuronal marker N-tubulin and the neural crest marker slug at a neurula stage (stage 17; Fig. 5B). Another proneural gene NeuroD (Lee et al., 1995), which has been shown to be induced by Xngnr-1 (Ma et al., 1996), was also induced (Fig. 5B). Neither the mesodermal markers (Xbra, M-actin) nor the neural inducers (Chd, Noggin, Follistatin) were induced. Thus, Zic-r1 is sufficient to induce early neuronal and neural crest markers in the ectodermal cells directly.

**Sox-2 makes the ectodermal cells responsive to the FGF-neuralizing signals**

We next tested whether Sox-2 has a neuralizing activity. When 200-600 pg of Sox-2 mRNA were injected into four blastomeres of 8-cell embryos, however, the animal cap explants from the injected embryos did not contain neural tissues (n=28; Fig. 6A). The explants were similar to the un.injected caps (except that small cement glands formed occasionally: 43%, not shown), showing that Sox-2 alone is not sufficient for neuralization. This inability of Sox-2 to induce neural tissues was not totally unexpected because previous studies had shown that the Sox factors can bind to their recognition sequence on the DNA but that they alone do not transactivate the target genes efficiently and require
additional factors to work with (Kamachi et al., 1995). Therefore, we next tested the possibility that Sox may work together with other signals that alone do not induce neuralization. An interesting candidate was bFGF, a mesoderm inducer on blastula animal caps (Smith, 1993), which had been shown to have neuralizing activity on gastrula caps under certain conditions. bFGF has little effects on intact gastrula caps (see Fig. 6B) but can induce neural tissues (preferentially posterior ones) from ‘sensitized’ animal caps that have been pretreated either by transient disaggregation (Kengaku and Okamoto, 1993) or by incubation in a very low Ca2+,Mg2+ medium (Lamb and Harland, 1995). Interestingly, these pretreatments induce cement glands in the caps, as does Sox-2 injection. When bFGF was added to Sox-injected undissociated gastrula animal caps (stage 10.25) in a standard medium (Lamb et al., 1993), neural and neuronal differentiation in the absence of dorsal mesoderm induction was observed at histological levels (78%, n=40) (Fig. 6C,D). Animal caps treated both with Sox-2 and bFGF expressed N-CAM (86%, n=22; Fig. 6G) while animal caps with Sox-2 (Fig. 6E; n=19), bFGF (Fig. 6F; n=20) alone or saline injection (n=24; not shown) did not. N-tubulin was detected in the animal caps treated with Sox-2 and bFGF.

**Fig. 2.** Spatio-temporal expression of Zic-r1 and Sox-2. Whole-mount in situ hybridization analyses of Zic-r1 (labeled as ZCR; A, C, E, G, I, K, M) and Sox-2 (labeled as SOX; B, D, F, H, J, L, N) at the early gastrula stage (stage 10.5; A-D), the early neurula stage (stage 14; E, F), the mid-neurula (stage 16; G, H) and the larva stage (stage 32; I, J). (C and D) show 10 μm paraffin sections. The signals are detected in superficial dorsal ectoderm but not in the deep mesoderm. The arrow and arrowhead in J show the branchial arch neural crest and the olfactory placode, respectively. (K-N) Radial injection of Chd mRNA (150 pg/blastomere; K, L) and of BMP-4 mRNA (75 pg/blastomere; M, N) into the 4-cell embryo. Chd injection induced Zic-r1 (P) and Sox-2 (Q) expression in the animal caps (excised at stage 9 and harvested at stage 11.5) but not the mesodermal marker Xbra (O). Activin-treated animal caps (excised at stage 9 and treated with 10 ng/ml activin until stage 11.5) for positive control for Xbra (O, inset). (P, Q, insets) Uninjected animal caps. The arrowheads show the dorsal lip in A-D and the closing blastopore in E-H.

**Fig. 3.** Onset of Zic-r1 and Sox-2 expression is between those of Chd and Xngnr-1. A whole-mount in situ hybridization study comparing the spatio-temporal expression of Chd (A,E,I,M), Zic-r1 (ZCR; B,F,J,N), Sox-2 (SOX; C,G,K,O) and Xngnr-1 (Xngnr; D,H,L,P) at −75 minutes (A,B,C,D), −30 minutes (E,F,G,H), +15 minutes (I,J,K,L) and +75 minutes (M,N,O,P) relative to the onset of gastrulation, which was judged by both external and internal appearances of albino embryos and by the pigmentation pattern of pigmented embryos fertilized at the same time (Nieuwkoop and Faber, 1967). (A-O) Dorsal views (looking down on the neuroectoderm). (P) Dorso-lateral view (Tg, trigeminal ganglion; m, i and l stand for medial, intermediate and lateral primary neurons). Arrowheads show the dorsal lip.
(80%, n=20; Fig. 6H). This combination of treatment also induced pigmentation in the explants (Fig. 6I-K). In this experiment, albino eggs were fertilized with sperms from a pigmented male and therefore detected pigments were all zygotic (paternal). A combination of Sox-2 and bFGF induced zygotic pigmentation (Fig. 6K; 90%, n=20) in the caps while Sox-2 injection alone (Fig. 6I; n=16), bFGF treatment alone (Fig. 6J; n=15) or saline injection (n=18; not shown) did not. These pigments were found in scattered mesenchyme-like cells present inside the explants (Fig. 6K; not shown), indicating induction of neural crest-derived melanophores (pigments of a sibling in melanophores and retinal pigmented epithelium are shown in Fig. 6L). Similar accumulation of zygotic pigment was also observed in Zic-r1-injected animal caps (76%, n=25; not shown).

RT-PCR analysis (Fig. 7) showed that a combination of Sox-2 and bFGF, but not either alone, induced neural and neuronal markers. The induced neural tissues expressed not only anterior markers but also the posterior (spinocaudal) marker XlHbox6. This is in good agreement with the fact that bFGF has a posteriorizing effect on early neural tissues (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995). Sox-2 and bFGF or Sox-2 alone did not induce the dorsal mesodermal marker M-actin or the neural inducers Chd, Noggin or Follistatin (Fig. 7; not shown).

These data demonstrate that Sox-2 can make the gastrula ectoderm responsive to extracellular signals such as FGF, which cannot act alone on the untreated ectoderm. Sox-2 is expressed in the neuroectoderm and eFGF (which has a similar biological activity to bFGF) is expressed at high levels in the dorsal mesoderm of late gastrulae and in the posterior-dorsal mesoderm of neurulae (Isaacs et al., 1992). Therefore, both Sox-2 and FGF factors are expressed at the right time and in the right place to work together for neuralization, especially in the posterior side.

**DISCUSSION**

We carried out a differential screen for genes activated by the neural inducer Chd, which yielded a number of early neural genes, including novel ones. The strategy we used here can be applied to other developmental regulator genes that have a functional fly homologue. (For instance, neurogenin and Zic-r1 could be good candidates once fly aonal and odd-paired are shown to mimic the activity in the animal cap assay, respectively.) Two early downstream genes of the BMP/neural inducer antagonistic signals, Zic-r1 and Sox-2, were identified, and they showed distinct activities in neuralizing the ectoderm.

**Zic-r1 as a candidate regulator of neural differentiation**

Microinjection of Zic-r1 mRNA can initiate neural and neuronal differentiation in vivo and in the isolated animal cap. This activity is not via secondary induction by mesoderm since neither the mesodermal marker nor the neural inducers were induced in the explant (Fig. 5). Zic-r1 can induce the early proneural gene Xngnr-1, a candidate regulator of primary neurogenesis, as well as other neuronal marker genes such as NeuroD and N-tubulin. The onset of Zic-r1 is between those of Chd and Xngnr-1, and Zic-r1 expression is detected widely in the early gastrula neuroectoderm when neural induction occurs (Fig. 3). Thus, Zic-r1 is expressed at the right time and in the right place, and has the right direction of activity, to work in the pathway leading from the neural inducers to the proneural genes.

Recent molecular analyses have revealed that the forming neuroectoderm as early as stage 11 is not homogenous but rather has regionally restricted patterns of gene markers. For instance, only a few rows of cells expressing Xngnr-1 (Ma et al., 1996) and Delta (Chitnis et al., 1995) at gastrula stages are committed to becoming primary neurons, whereas the rest of the cells in the neuroectoderm are not. As Zic-r1 is expressed

**Fig. 4. Zic-r1 injection promotes neural and neuronal differentiation in animal caps and in vivo.** After 2 days culture, when siblings reached stages 38-39, animal cap explants injected with Zic-r1 mRNA (A) contained a large neural mass. V, ventricle-like cavities; arrow, roof plate-like thin layer. The control animal cap contained atypical epidermis (B). When siblings reached stage 17, Zic-r1-injected animal caps expressed the neural marker nrp-1 (C; detected by in situ hybridization). Inset (C) shows control animal caps. Injection of 200 pg Zic-r1 mRNA into one blastomere of the 2-cell embryo expanded expression of Xngnr-1 (D; primary neurons shown by triangles; arrow, midline), N-tubulin (E; note the width of lateral neuron bands), and slug (F; arrow, ectopic expression) and suppressed that of keratin (G, H) in vivo at neural plate stages. (I) Expression of hairy 2, which marks the midline and borders of neural plate (np) (Turner and Weintraub, 1994). The width of neural plate (arrows) is significantly increased on the injected side compared to the control side. epi, epidermis. The injected side (inj; cont, control side) was determined by coinjecting a GFP (D-F; I; not shown) or β-gal (arrows in G) mRNA.
widely in the early neuroectoderm (Fig. 3), additional factors must exist to drive the proneural gene expression in the restricted position. In this sense, a similar situation is known in the case of Drosophila odd-paired (Benedyk et al., 1994). odd-paired is a pair-rule gene, mutation of which causes deletion of alternating parasegments. Unlike other pair-rule genes (such as fushi tarazu and hairy), which show zebra-like expression, both odd-paired mRNA and protein are distributed throughout all segment primodia. It has been therefore indicated that certain cofactor genes (e.g. runt) must work to confine the odd-paired activity in the spatially restricted manner (Benedyk et al., 1994).

Zic-r1 has a second-phase expression starting from late gastrula stages. Zic-r1 expression is gradually shut off from the medial area of the neural plate and ends up in the dorsal neural tube and the flanking ectoderm. Thus Zic-r1 and Sox-2 have overlapping but different expression patterns, suggesting that these two genes are controlled by more than one factor in the later phases. The late Zic-r1 expression may suggest a role for Zic-r1 in dorsoventral patterning of the nervous system, including the neural crest. Consistent with this idea, Zic-r1 can positively regulate the neural crest marker slug (Figs 4F and 5). Whether this neural crest induction is direct or not must still await further investigation, since neural crest can be also induced secondarily by interaction between epidermis and CNS tissues (Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996).

**Sox-2 changes responsiveness of the ectodermal cells**

Microinjection of Sox-2 mRNA alone cannot initiate neural induction in the animal cap, but it makes the ectoderm responsive to the FGF-neuralizing signals. This finding goes along with the previous report that Sox-2 needs additional factors to transactivate the crystalline gene (Kamachi et al., 1995). The Sry-related molecules are known to bind to and bend the promoter DNA so that an active transcription factor-DNA complex may be formed (Werner et al., 1995). With this mode of action seems reasonable that Sox factors modulate the responsiveness of early neural genes to FGF signaling.

What are possible in vivo roles of the combined neuralizing activity of Sox-2 and FGF? In chick, early Sox-2 expression overlaps but does not completely coincide with the ectodermal area of neural competence (Streit et al., 1997). In Xenopus Sox-2 is induced by the neural inducer Chd and is expressed in the forming neuroectoderm. Therefore, Sox-2 likely modulates the responsiveness of neuroectodermal cells in vivo rather than of uncommitted ectodermal cells. A possible role is that Sox-2 makes early neuroectodermal cells responsive to other neuralizing signals such as FGF, so that these cells can reinforce...
their neural fate and receive information for further specification (e.g. posteriorization). This kind of activity is important because the fate of amphibian neuroectoderm is reversible during early gastrula stages and can be changed into that of epidermis by transplanting to the ventral side (Spemann, 1918).

There is another situation where Sox factors and FGF signals may work together. A role of FGF signaling has been suggested in the differentiation of placodes (Represa et al., 1991; Grainger et al., 1992) where Sox factors are also expressed (Kamachi et al., 1995; Uwanogho et al., 1995; Collignon et al., 1996). Whether Sox factors are involved in the ectodermal competence (Jacobson, 1966; Grainger, 1992) at neurula stages and later is an interesting question for future investigation.

Sox-2 is expressed in a pan-neural fashion while FGFs are expressed only in limited regions. This means that Sox-2 may also work together with factors other than FGFs. To date, we have failed to observe clear synergy of Sox-2 with Chd, Noggin and Zic-r1, which alone have strong neuralizing activity. Since many different growth factors can stimulate intracellular pathways similar to FGFs (e.g. Ras/Raf and MAP kinases) (Umbhauer et al., 1995; Gotoh et al., 1995), further investigation is required to determine whether Sox-2 shows synergy specifically with FGFs or also with those growth factors that share common signaling pathways. These are important points since the question of whether FGF signaling is essential for neural induction in vivo has not yet been answered (Kengaku and Okamoto, 1993; Lamb and Harland, 1995; Schulte-Merker and Smith, 1995; Launay et al., 1996; Sasai et al., 1996; Kroll and Amaya, 1996).

Fig. 7. RT-PCR analysis of the explants at the stage 17. Lane 1, control embryo; lane 2, control animal caps; lane 3, Sox-2-injected caps; lane 4, FGF-treated caps; lane 5, Sox-2-injected and FGF-treated caps. Otx-2 (forebrain and also weakly in cement gland), Xif3 (anterior neural plate), Krox 20 (hindbrain), XlHbox6 (spinal cord), CG13 (cement gland); other markers as in Fig. 5.

Downstream pathways of neural induction

During the last few years, substantial progress has been made in the study of downstream signaling of BMP-4 (reviewed in Mehler, 1997). Activated BMP receptors phosphorylate BMP-specific Smads, which form an active complex with Smad4. Then the signal is brought into the nucleus where various transcriptional regulation events occur. Among the target genes activated by the BMP signaling are the ventral-specific homeobox genes, GATA-1 and Msx, which can mimic the ventralizing activity of BMP-4 when overexpressed (Gawantka et al., 1995; Ladher et al., 1996; Onichtchouk et al., 1996; Papalopulu and Kintner, 1996; Schmidt et al., 1996; Tidman-Ault et al., 1996; Xu et al., 1997; Maeda et al., 1997). By contrast, Zic-r1 and Sox-2 are downstream targets regulated negatively by the BMP signaling. Although the BMP signaling can pattern both ectoderm and mesoderm, these two early neural genes are regulated dorsoventrally only in the ectoderm and not in the mesoderm. This demonstrates that germ layer-specific gene regulation is already occurring at an early transcriptional step of BMP signaling. It would therefore be intriguing to test whether Xbra and other mesodermal T-box genes (Ryan et al., 1996; Stennard et al., 1996; Lustig et al., 1996; Zhang and King, 1996) can negatively regulate Zic-r1 and Sox-2 in a direct manner.

Our working model from the present study is as follows. When BMP proteins are trapped and inactivated by the neural inducers such as Noggin (Zimmerman et al., 1996), Follistatin (Fainsod et al., 1997) and Chordin (Piccolo et al., 1996), intracellular signals after BMP receptors are attenuated. The direct consequences are transcriptional de-suppression of Zic-r1 and Sox-2 (and probably more genes) and repression of ventral-specific homeobox genes, GATA-1 and Msx. Further investigation is required to address how many of these are controlled via the smad system and how many via the TAB1-TAK1 pathway (Shibuya et al., 1996).

Once Zic-r1 is activated, it can promote neural differentiation and neurogenesis. Additional signals of positional information are required in vivo to confine expression of proneural genes in restricted regions. Activation of Sox-2 enables the gastrula cells to respond to certain extracellular signals such as FGF. Together with the downstream factors of FGF, Sox-2 helps the cell strengthen its neural fate and acquire further specification such as posteriorization. Interactions between Zic-r1, Sox-2 and the ventral-specific homeobox genes remain to be examined. As both the Zic and Sox families have multiple closely related members expressed in the early ectoderm, it is expected that they may have redundant functions within the family. Unlike proneural/neurogenic genes and Smads, whether a simple parallelism for Zic and Sox factors exists in Drosophila neurogenesis is not yet known.

Early downstream events of neural induction are already complicated and many are still to be clarified. Our screening strategy may help in the further identification of essential components of these complex pathways.

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