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opl: a zinc finger protein that regulates neural determination and patterning in *Xenopus*

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SUMMARY

In order to study the mechanism of neural patterning in *Xenopus*, we used subtractive cloning to isolate genes activated early during this process. One gene isolated was *opl*, (*odd-paired*-like) that resembles the *Drosophila* pair-rule gene *odd-paired* and encodes a zinc finger protein that is a member of the *Zic* gene family. At the onset of gastrulation, *opl* is expressed throughout the presumptive neural plate, indicating that neural determination has begun at this stage while, by neurula, *opl* expression is restricted to the dorsal neural tube and neural crest. *opl* encodes a transcriptional activator, with a carboxy terminal regulatory domain, which when removed increases *opl* activity. *opl* both sensitizes animal cap ectoderm to the neural inducer *noggin* and alters the spectrum of genes induced by *noggin*, allowing activation of the midbrain marker *engrailed*. Consistent with the later dorsal neural expression of *opl*, the activated form of *opl* is able to induce neural crest and dorsal neural tube markers both in animal caps and whole embryos. In ventral ectoderm, *opl* induces formation of loose cell aggregates that may indicate neural crest precursor cells. Aggregates do not express an epidermal marker, indicating that *opl* suppresses ventral fates. Together, these data suggest that *opl* may mediate neural competence and may be involved in activation of midbrain, dorsal neural and neural crest fates.

Key words: Subtractive cloning, Zinc finger, *odd-paired*, *Zic*, Competence, *engrailed*, Dorsal neural, Neural crest, *Xenopus*

INTRODUCTION

One of the first decisions made during neural determination in *Xenopus* is a general increase in the competence of the ectoderm to respond to subsequent neuralizing signals. Both transplantation experiments and exposure of explants to pure factors or to natural inducing tissue have shown that neural competence in *Xenopus* is maximal at early gastrula; by the end of gastrulation, ectoderm displays little competence to form neural tissue in response to mesoderm-derived signals (Nieuwkoop and Koster, 1995; Jones and Woodland, 1987; Sive et al., 1989; Servetnick and Grainger, 1991; Kintner and Dodd, 1991; Lamb et al., 1993; Knecht and Harland, 1997).

Superimposed on an increase in neural competence of the entire ectoderm is an enhanced competence of the dorsal ectoderm (the presumptive neurectoderm) for dorsal fates. The dorsal ectoderm is already biased towards dorsal fates by early blastula stages, since isolated dorsal ectoderm treated with activin goes on to form more dorsal mesoderm than does ventral ectoderm (Sokol and Melton, 1991). By early gastrula, isolated dorsal ectoderm fails to activate expression of the epidermal antigen, Epi-1, whereas this antigen is strongly specified in the ventral ectoderm, which gives rise to epidermis (Savage and Phillips, 1989). Consistently, the dorsal ectoderm is more responsive than ventral ectoderm to neural-inducing signals arising from the dorsal mesendoderm (Sharpe et al., 1987).

By midgastrula, specification assays indicate that neural determination has occurred (Sive et al., 1989; Sharpe and Gurdon, 1990). Initial neural induction is thought to be anterior in character and appears to be mediated by antagonists of Bone Morphogenetic Proteins (BMPs), such as the secreted factors *noggin* and *chordin* (Eyal-Giladi, 1954; Sive et al., 1989; reviewed in Sasai and DeRobertis, 1997). Later, the posterior part of the neural plate is converted into posterior neural tissue. Retinoids, fibroblast growth factor and wnt proteins may all be involved in this process, perhaps acting at different anteroposterior levels (reviewed in Kolm and Sive, 1997).

Initiation of dorsoventral neural tube patterning also occurs during gastrulation (reviewed in Placzek and Furley, 1996). In *Xenopus* ventral parts of the neural tube have been specified by midgastrula (Saha et al., 1997) presumably under direction of

ventral midline-derived signals, particularly sonic hedgehog (Roelink et al., 1994). Similarly, at this time, expression of *pax3* (a dorsal neural tube marker) is beginning to be restricted to the future dorsal neural plate showing that dorsal neural fates have started to be determined (Bang et al., 1997). Final dorsal neural tube fates require input from adjacent non-neural ectoderm (Liem et al., 1995; Bang et al., 1997), including members of the TGFβ gene family. The neural crest is specified later than dorsal neural tube, by the end of gastrulation, with specification increasing as the neural tube closes (Mayor et al., 1995). Neural crest induction requires signals both from the neural plate and from the juxtaposed non-neural ectoderm, and perhaps also signals from underlying paraxial mesoderm (reviewed in Bronner-Fraser, 1995).

Neural differentiation is regulated by genes such as *neurogenin*, *Xash3* and other members of the bHLH class (Turner and Weintraub, 1994; Zimmerman et al., 1993; Ma et al., 1996). Interfacing with neural differentiation is the correct patterning of the nervous system that must be controlled within the neurectoderm by a distinct set of genes. However, few genes that control early patterning decisions within the neurectoderm have been defined (for examples see Blitz and Cho, 1995; Pannese et al., 1995; Kolm and Sive, 1995a). In order to address this deficit, we have used subtractive cloning (Sagerström et al., 1997) to identify genes expressed specifically in the dorsal ectoderm during gastrula stages, after neural determination has occurred, but before neural differentiation has begun. To date, we have isolated more than 40 genes expressed preferentially in the dorsal ectoderm by midgastrula (Patel and Sive, 1996; Gamse, J., Kuo, J. S., Patel, M. and Sive, H., unpublished data). One of the earliest markers of the neurectoderm that we identified is a zinc finger gene that is similar to *Drosophila odd-paired* (*opa*) (Benedyk et al., 1994) that we called *opl* for *odd-paired*-like. *opl* is a member of the *Zic* gene family, which comprises at least four members (Aruga et al., 1996).

Here we analyse the expression and activity of the *opl* gene. We show that *opl* expression demarcates the presumptive neural plate very early, by the onset of gastrulation, and later defines the dorsal neural tube and neural crest. *opl* can sensitize the neurectoderm for induction, suggesting that *opl* may regulate neural competence. *opl* is also able to potentiate expression of a midbrain marker, as well as dorsal neural and neural crest marker genes, delineating a role in neural tube patterning for this gene.

MATERIALS AND METHODS

Growth, dissection and culture of embryos and explants

Xenopus laevis eggs were collected, fertilized and cultured as described in Sive et al. (1989). Embryos were staged according to Nieuwkoop and Faber (1967). For animal caps, late blastula (stage 9) animal hemisphere ectoderm was isolated and incubated in 0.5× MBS alone, or with other reagents as indicated. Dexamethasone (dex), and hydroxyurea and aphidicolin treatments were as described in Kolm and Sive (1995b) and Harris and Hartenstein (1991), respectively.

Subtractive cloning of *opl*

PCR-based subtractive cloning modified from Wang and Brown (1991) and described in detail by Patel and Sive (1996) was used to identify *opl*. RNA was isolated from dissected midgastrula (stage 11.5) dorsal ectoderm and blastula (stage 9-9.5) animal caps aged to midgastrula equivalent (stage 11.5) (Condie and Harland, 1987). cDNA was prepared by using the Pharmacia Timesaver cDNA synthesis kit. cDNA products were digested with *Alu*I and *Rsa*I, then ligated with oligo adaptors. Oligo 1 (5′-TAGTCCGAATTCAAGCAAGAGCACA-3′) was kinased, annealed to oligo 2 (5′-CTCTTGCTTGAATTCGGACTA-3′), and ligated to the dorsal ectoderm (D) cDNA, thereby introducing flanking *Eco*RI sites (underlined). Oligo 3 (5′-ATGCTGGATATCTTGGTACTCTTCA-3′) was kinased and annealed to oligo 4 (5′-GAGTACCAAGATATCCAGCAT-3′), and ligated to the animal cap (uninduced) ectoderm (U) cDNA, thereby introducing flanking *Eco*RV sites (underlined). Before each round of subtraction, tracer cDNA (from the D population) was PCR-amplified in the presence of ^{32}P-dCTP, and driver cDNA (from the U population) was PCR-amplified in the presence of biotinylated-11-dUTP (Enzo Diagnostics). Subtractive hybridization was carried out at 68°C, with long hybridizations (40 hours) alternating with short hybridizations (2 hours) to remove rare common sequences and abundant common sequences, respectively. Biotinylated duplexes were removed by treatment with streptavidin and phenol extraction, and enriched tracer and driver cDNAs were amplified for the next round of subtraction. 10 rounds of subtraction were performed to produce D_{10} and U_{10} cDNAs. D_{10} cDNA products with average size of 300 bp (range 150 to 1000 bp) were cut with *Eco*RI and subcloned into CS2+ vector (Rupp et al., 1994). 800 clones were randomly selected and screened by dot blot analysis with probe prepared from D_{10} and U_{10} cDNAs to identify cDNAs expressed more strongly in the dorsal pool. 194 dorsal clones were sequenced and grouped into 75 sequence classes. 44 were of these were detected by in situ hybridization in gastrula stage dorsal ectoderm but not in ventral ectoderm. One of the most abundant clones isolated encoded *opl* (Fig. 1). A full-length *opl* cDNA clone was isolated from a midgastrula (stage 11.5) λZap phage library (prepared by C. Nocente-McGrath). Two cDNA clones were identified, the larger one (TG6.11) contained a 2.6 kb insert.

opl constructs

Constructs are diagrammed in Fig. 3. All techniques are as described in Sambrook et al. (1989), unless otherwise indicated. Enzymes were obtained from Stratagene or New England Biolabs. All constructs are in pCS2+ or pCS2+MT vectors (Rupp et al., 1994; Turner and Weintraub, 1994). The *opl* plasmid contained the *opl* open reading frame (ORF) fragment (699 bp) modified to include an optimal translation initiation site (Kozak, 1987) upstream of the *opl* initiation ATG and results in two extra amino acids (M-A) at the NH2-end of translated peptide. To create *oplΔC*, a *Pst*I (end-filled)-*Xho*I (blunted) opl plasmid backbone was treated with Klenow fragment to remove 16 extra bp distal to the *Xho*I site (see Fig. 1) and then self-ligated. Myc-tagged *opl* (*MTopl*) was made by ligating an end-filled *Eco*RI-*Xba*I fragment into the *Xba*I (end-filled) site of CS2+MT. *MToplΔC* was made by ligating a *Not*I-*Not*I *oplΔC* fragment into the *Not*I-digested MT*opl* vector backbone. To make constructs encoding inducible opl proteins (*oplGR*), the ligand-binding domain of the human glucocorticoid receptor was PCR-amplified from the *MyoD*GR plasmid (Hollenberg et al., 1993) with the primers MP-GR1-*Xho*I (5′-GGCGCCGAGCTCGAGCCTCTGAAAATCCT-3′) and MP-GR2-*Xho*I (5′-GGCGGGCACTCGAGACTTTTGATGAAAC-3′). Products were then digested with *Xho*I (underlined) and inserted into an *Xho*I-digested *opl* plasmid.

Microinjection

Microinjection techniques were as described (Kolm and Sive, 1995a). 200 to 500 pg of *opl*, *oplΔC* or *oplGR* RNAs were injected per embryo. 0.1 to 10 pg *noggin* RNA (derived from the CS2+*noggin* plasmid) per embryo was co-injected in the *opl* plus *noggin* synergy experiments. 100 pg *lacZ* RNA was co-injected as tracer in whole-embryo assays.

In vitro transcription

Capped sense RNAs for microinjection were synthesized as in Krieg and Melton (1984) and *lacZ* sense RNA as in Gammill and Sive (1997). All *opl*-derived sense RNAs (*opl*, *opl*ΔC, *oplGR*, MT*opl*, MT*opl*ΔC) were made by SP6 transcription of a *Nar*I-*Nar*I fragment from CS2+derived vectors. Other templates for sense RNAs are: *CAT* (p64TCAT; Kuo et al., 1996), *Sma*I linearized, SP6 transcribed; *globin* (pXβM), *Pst*I linearized, SP6 transcribed; *noggin* (pCS2+x*noggin*), *Not*I linearized, SP6 transcribed. Antisense probes for in situ hybridization were transcribed in the presence of digoxigenin-11-UTP or fluorescein-11-UTP as follows: *XCG* (Sive et al., 1989), *otx2* (Pannese et al., 1995), *en2* (Hemmati-Brivanlou and Harland, 1989), *krox20* (Bradley et al., 1993), *slug* (Mayor et al., 1995), *pax3* (Espeseth et al., 1995) and *XK81* (Jonas et al., 1985).

In situ hybridization and sectioning

Whole-mount in situ hybridization was performed on albino embryos essentially as described in Harland (1991) with modifications as in Sagerstrom et al. (1996). β-galactosidase staining was performed as in Kolm and Sive (1995a). For sections, embryos were embedded in JB4 resin (Polysciences 00226) and 5 to 10 μm sections were mounted in Crystal mount (Biomeda M03). Sections were stained with toluidine blue.

Isolation of RNA and northern analysis

RNA was prepared from explants and embryos by proteinase-K treatment and phenol extraction (Condie and Harland, 1987). RNA was analyzed by northern blotting (Sambrook et al., 1989). *opl* antisense probe was made by asymmetric PCR (Sive and Cheng, 1991) with the opl-D2 primer (5′-GTTGGCCAACTGCTCGG-3′).

Relative quantitative RT-PCR

RNA from pools of 15-30 explants or 2-4 embryos was analysed by RT-PCR as described in Sagerström et al. (1996), using RNA from 5 animal cap equivalents or 0.25 embryo equivalents per reaction. *NCAM*, *en2*, *krox20* primers are described in Hemmati-Brivanlou and Melton (1994a). *EF1α* and *XCG* primers are described in Gammill and Sive (1997). Other primers yielding product sizes as indicated are: *otx2* – 485 bp [X*otx2*.C (5′-GCAACAGCAGCAGCAGAATG-3′), X*otx2*.D (5′-TGTAATCCAGGCAGTCAGTG-3′)] (Pannese et al., 1995); *HoxD1* – 245 bp [PJK3 (5′-CAGCCCCGATTAC-GATTATTATGG-3′), PJK4 (5′-CCGGGGAGGCAGGTTTTG-3′)] (Kolm and Sive, 1995a); *HoxB9* – 201 bp [HLS51 (5′-GCCCCTGCGCAATCTGAAC-3′), HLS52 (5′-CAGCAGCGGC-TCAGACTTGAG-3′)] (Wright et al., 1990); *slug* – 220 bp [(Xslug1 (5′-GGTTCGATTTGCAGTATGG-3′), Xslug2 (5′-GAGCTGCT-TCGTAAAGCAC-3′)] (Mayor et al., 1995); *pax3* – 181 bp [pax3A (5′-CAGCCGAATTTTGAGGAGCA*CAT*-3′), pax3B (5′-GGGCAG-GTCTGGTTCGGAGTC-3′)] (Espeseth et al., 1995); *NK2* – 406 bp [*NK2*.1 (5′-AGAAACCGTCGGCTGATGAGTC-3′), *NK2*.2 (5′-TGGGCGCTATAGGCAGAGAAAG-3′)] (Saha et al., 1993); *shh* – 191 bp [shh1 (5′-GGTTGACCGCGGCCCATCTAC-3′), shh2 (5′-AGGCGCATAAGCTCCAGTGTCC-3′)] (Ekker et al., 1995); *Xash3* – 227 bp [forward (5′-AAGCGGCTCCTGTCAAACACTAT-3′), reverse (5′-CCCCCGCGCTCCTCTCC-3′)] (Zimmerman et al., 1993); *neurogenin* – 263 bp [forward (5′-CGCCGCA-ACCCGACTCACCT-3′), reverse (5′-CCTGCATCGCG-GGCTGTTCTC-3′)](Ma et al., 1996); *XK81* – 308 bp [forward (5′-T*CAT*TCCGTTCCAGCTCTTCTTAC-3′), reverse (5′-TCCAG-GGCTCTTACTTTCTCCAG-3′)] (Jonas et al., 1985); *GATA2* – 415 bp [forward (5′-CTAAACAGAGGAGCAAGAGC-3′), reverse (5′-CCTGGAAAGTTCCTCAAAAC-3′)] (Walmsley et al., 1994); *m-actin* – 222 bp [forward (5′-GCTGACAGAATGCAGAAG-3′), reverse (5′-TTGCTTGGAGGAGTGTGT-3′)] (Stutz and Spohr, 1986).

Western analysis and immunocytochemistry

Western blotting (Kolm and Sive, 1995b) and whole-mount immunostaining (Hemmati-Brivanlou and Harland, 1989) were performed using the c-myc 9E10 antibody (Santa Cruz Biotechnology) diluted 1:1000 (0.1 μg/ml) and the ECL Vector Elite kit from Amersham.

Transient transfection reporter assays

A reporter plasmid (pGL15) containing three Gli3 DNA-binding sites (Vortkamp et al., 1995) and a luciferase reporter was mixed with CS2+ or CS2+*opl* ORF or CS2+*oplΔC* plasmids for transfections at the ratio of 5 μg:1 μg. The human pancreatic tumor cell line BXPC (ATCC #CRL 1687) were transfected using the DEAE-dextran/chloroquine method (Aruffo and Seed, 1987), and cell lysates were assayed 48 hours after transfection for luciferase activity using the Promega Luciferase Assay System (E1501) on a Analytical Luminescence Laboratory Monolight 2010 machine. Duplicate transfections were done for each DNA mix and duplicate readings were taken for each lysate sample.

RESULTS

opl encodes a zinc finger protein with similarity to the Zic family

In order to isolate genes activated soon after neural determination has begun in *Xenopus*, we subtracted cDNAs from microdissected midgastrula dorsal ectoderm with those from uninduced ectoderm aged until midgastrula (Patel and Sive, 1996). One of the genes that we isolated encodes a 443 amino acid protein with five zinc fingers of the C2H2 class (GenBank accession no. AF028805). By comparison with invertebrate zinc finger genes, the zinc fingers of this gene were most similar (85%) to those of the *Drosophila odd-paired* gene (*opa*), a pair-rule gene (Benedyk et al., 1994), and we therefore named this gene *opl* for *o*dd-*p*aired-*l*ike. Among the vertebrate zinc finger genes, the zinc fingers of *opl* were similar to those of the *Gli* family genes (53% identity to the zinc fingers of human *Gli3* (Ruppert et al., 1988)), but even more similar to the family of mouse *Zic* genes. The *Zic* genes share intron/exon boundaries with *Drosophila odd-paired* (Aruga et al., 1996) indicating that these are true homologs of *opa* (Fig. 1A). Over the entire coding region, opl was very similar to mouse Zic1, with 91% overall amino acid identity (Fig. 1B, (Aruga et al., 1994)). It was much less similar to the proteins encoded by other members of the *Zic* gene family, with 63% identity to mouse Zic2, 60% identity to Zic3 and 54% identity to Zic4 (Aruga et al., 1996). Recent reports describing isolation of *Xenopus* Zic members include Zic3 (Nakata et al., 1997), which has 60% identity to *opl*, while the percentage identity of opl and Zic-r1 (Mizuseki et al., 1998) is not clear. On the basis of these data, *opl* may be the *Xenopus* homolog of *Zic1*. However, since we cannot conclude this unequivocally and in order to reflect the similarity of this gene to *Drosophila opa*, we chose the name *opl*.

Temporal and spatial characterization of *opl* expression

We next examined the time course of *opl* expression by northern analysis (Fig. 2). *opl* RNA is activated zygotically by late blastula (stage 9.5, lane 3). Expression was maximal by midgastrula (lanes 4-7), with lower expression persisting into tailbud stages (lanes 8-11).

By whole-mount in situ hybridization, *opl* expression was

biphasic. By early gastrula (Fig. 3), *opl* was expressed strongly in the dorsal ectoderm (Fig. 3A,B), with much lower levels in the ventral ectoderm, and trace levels of expression in the marginal zone. Strong expression throughout the presumptive neural plate was observed at midgastrula, with increased *opl* expression at the lateral edges of the neural plate (Fig. 3C,D). By early neurula, *opl* expression had entered a second phase, with restriction to the lateral edges of the neural plate, that is the presumptive dorsal neural tube and neural crest (Fig. 3E,F). As the neural tube closed, expression was predominant in the anterior dorsal neural tube and the premigratory presumptive cranial neural crest (Fig. 3G,H). The precise region of presumptive neural crest expressing *opl* changes during neurulation. At early neurula (stage 15), *opl* expression was in the most anterior mandibular crest (not shown) while, by stage 18 (Fig. 3G,H), *opl* was expressed predominantly in the more

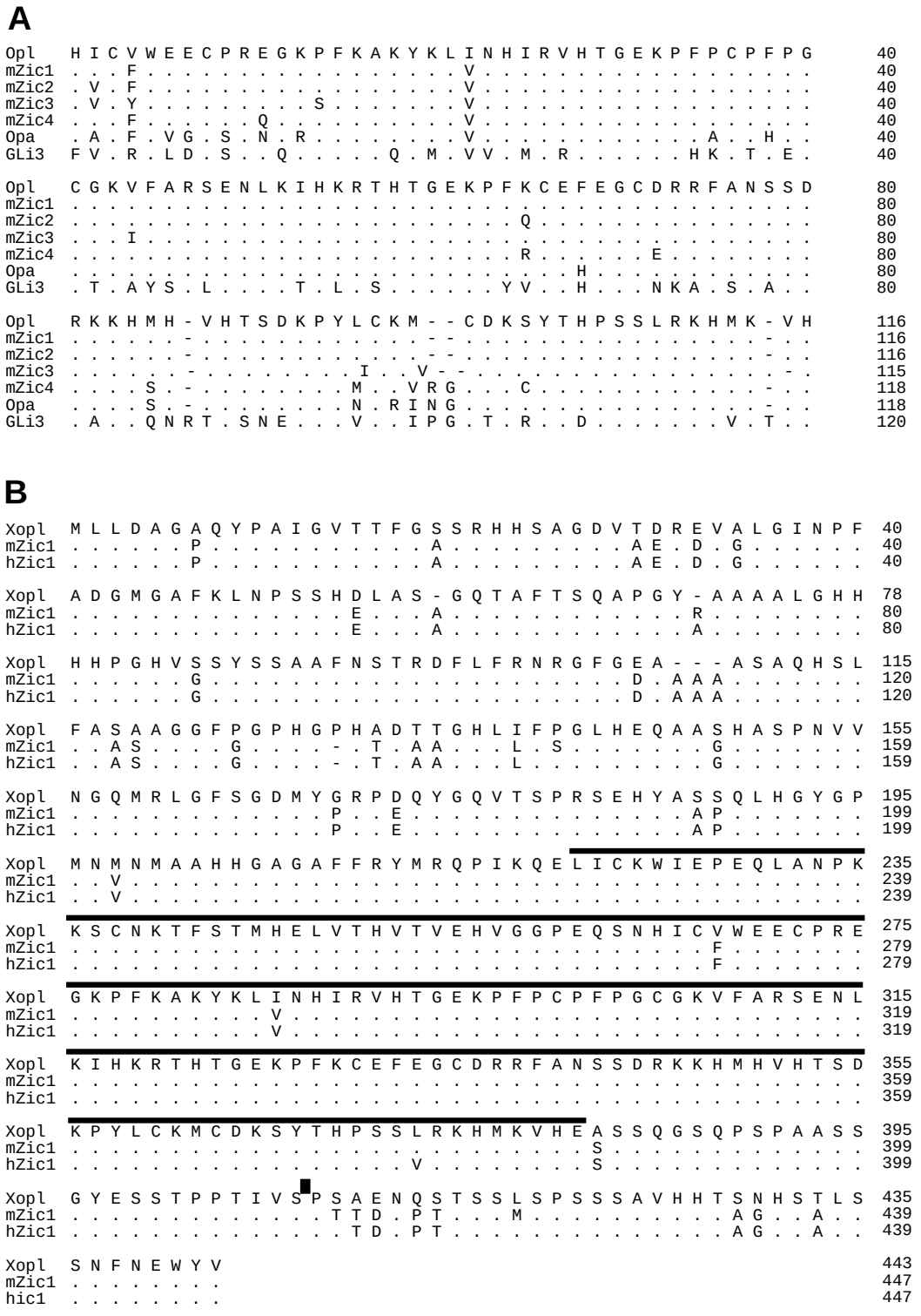

Fig. 1. opl protein sequence alignments. Amino acid residues in the compared proteins that are identical to *Xenopus* opl are indicated by periods. Sequence gaps introduced for optimal protein alignment are indicated by dashes. Numbers at right indicate amino acid position. (A) Alignment of zinc fingers II-V of opl, mouse Zic proteins (Aruga et al., 1996): mZic1, mZic2, mZic3, mZic4, *Drosophila* Opa (Benedyk et al., 1994) and human Gli3 (Ruppert et al., 1988). The first zinc finger is omitted since it is incomplete. This region of the *Xenopus* opl protein (aa 265-381) is 98% identical to the equivalent regions in mZic1, 97% identical to mZic2, 90% identical to mZic3, 85% identical to Opa and 53% identical to Gli3. (The GenBank accession number for opl is AF028805). (B) Alignment of conceptually translated *Xenopus* opl, mouse Zic1 and human Zic proteins. The predicted zinc fingers are indicated by a bar above the sequence. Block indicates start of polypeptide deleted in *opl*Δ*C* constructs. *Xenopus* opl is 92% similar to both mouse Zic1 and human Zic proteins (Yokota et al., 1996). opl is 60% overall identical to both mouse and *Xenopus* Zic3 (Nakata et al., 1997).

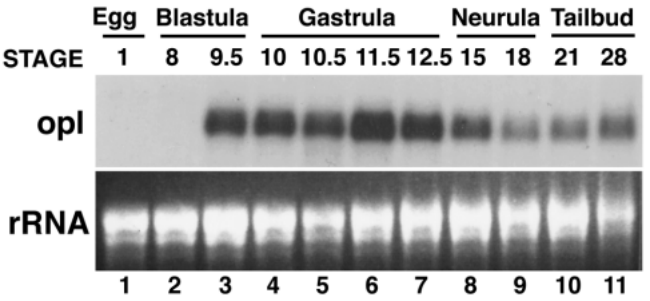

Fig. 2. (A) Northern analysis of *Xenopus* embryos. One embryo equivalent per lane was analyzed for *opl* RNA (top row) at various embryonic stages shown. Ethidium-stained 28S rRNA is a loading control (bottom row).

posterior hyoid and branchial neural crest (Sadaghiani and Thiebaud, 1987). By tailbud, expression was high in two stripes located in the telencephalon and diencephalon of the forebrain, and more posteriorly along the entire length of the neural tube (Fig. 3I,J).

In order to analyse *opl* expression in more detail, embryos stained for *opl* RNA were sectioned sagittally. At stage 10+ (the onset of gastrulation), *opl* expression was observed in both the inner and outer layers of the dorsal ectoderm (Fig. 4A,B) in a region largely without underlying mesendoderm (bracket). By midgastrula (stage 11) (Fig. 4C,D) and late gastrula (Fig. 4E), *opl* expression was excluded from the extreme posterior neurectoderm. Sequential transverse sections of a tailbud embryo showed that, within the spinal cord, *opl* expression was restricted to the roof plate of the neural tube (Fig. 4F, black arrow) and the migratory neural crest (Fig. 4F, white arrow). In the hindbrain, *opl* expression extended into the more ventral alar plate region of the neural tube (Fig. 4G,H). At the midbrain level, *opl* expression extended through the dorsal half of the neural tube (Fig. 4I), while more anteriorly, in the forebrain, *opl* expression was lost from roof plate and retained in the alar plate (Fig. 4J).

In order to delineate the expression of *opl* relative to other positions in the neural tube, we performed comparative in situ hybridization with other dorsal ectodermal markers (Fig. 5). At midneurula, expression of the forebrain and cement gland marker *otx2* (Blitz and Cho, 1995; Pannese et al., 1995) overlapped with that of *opl* anteriorly, extending to the anterior boundary of the neural plate, but *opl* is excluded from the dorsal medial region and the future cement gland (bracket) (Fig. 5A). Consistently, *opl* was expressed more posteriorly than *XCG*, a marker of the cement gland (Sive et al., 1989) that lies anterior to the neural plate (Fig. 5B). Expression of *slug* (Mayor et al., 1995) in the premigratory cranial neural crest overlapped that of *opl*, confirming that *opl* expression extended beyond the edges of the neural plate (Fig. 5C). Relative to *engrailed 2* (*en2*) at the midbrain/hindbrain junction (Hemmati-Brivanlou and Harland, 1989) and *krox20*, demarcating future rhombomeres 3 and 5 of the hindbrain (Bradley et al., 1993), *opl* expression at early neurula extends posterior to the rhombomere 5 stripe of *krox20* (Fig. 5D).

In summary, *opl* expression is an early marker of the presumptive neurectoderm with later expression marking the dorsal neural tube and neural crest. Recent analyses of the *Xenopus Zic3* (Nakata et al., 1997) and a *Zic1*-related gene (Zic-r1) (Mizuseki et al., 1998) indicate that the expression patterns of these genes and *opl* are similar.

opl is a nuclear protein whose carboxy terminal encodes a regulatory domain

We next characterized the activity and localization of the opl protein. Since mouse Zic1 is a nuclear protein that can bind the DNA target site of the related zinc finger protein Gli3 (Fig. 1A; Aruga et al., 1994), we assumed that opl was a transcription factor and asked whether it had activator or repressor functions. The carboxy terminus of opl is highly serine-rich, suggesting it may be a phosphorylation domain (see Fig. 1B). We removed this region (to create oplΔC) and asked whether it played a regulatory role in opl activity, using a reporter assay. The various constructs used are diagrammed in Fig. 6 and described in Methods. The reporter consisted of *luciferase* linked to three copies of the Gli3 consensus DNA-binding site (Fig. 7A; (Vortkamp et al., 1995). The activity of intact opl or oplΔC was assayed in transient transfection assays (see Methods). The averaged results of three experiments are shown in Fig. 7A. While opl was able to activate reporter gene expression 2.8-fold above background, oplΔC was able to activate reporter gene expression 14.6-fold above background, indicating that this mutant allele of *opl* has an enhanced transcriptional activation capacity and designating the carboxy terminus of opl as a domain that is able to suppress opl activity. This transactivation requires Gli3-binding sites, since a reporter lacking these sites (containing an SV40 promoter) was not activated above background levels by either opl or oplΔC (not shown).

We also asked whether the opl protein was localized to the nucleus and whether C-terminal removal altered its

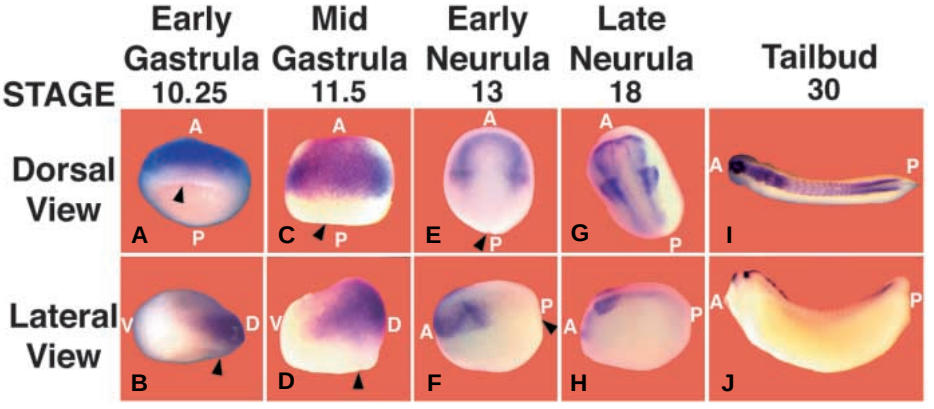

Fig. 3. Whole-mount in situ analysis of *opl* expression. Embryos were analyzed by in situ hybridization as described in Methods. Embryo orientations are indicated by A, anterior; P, posterior; D, dorsal and V, ventral; arrowhead, dorsal lip of blastopore.

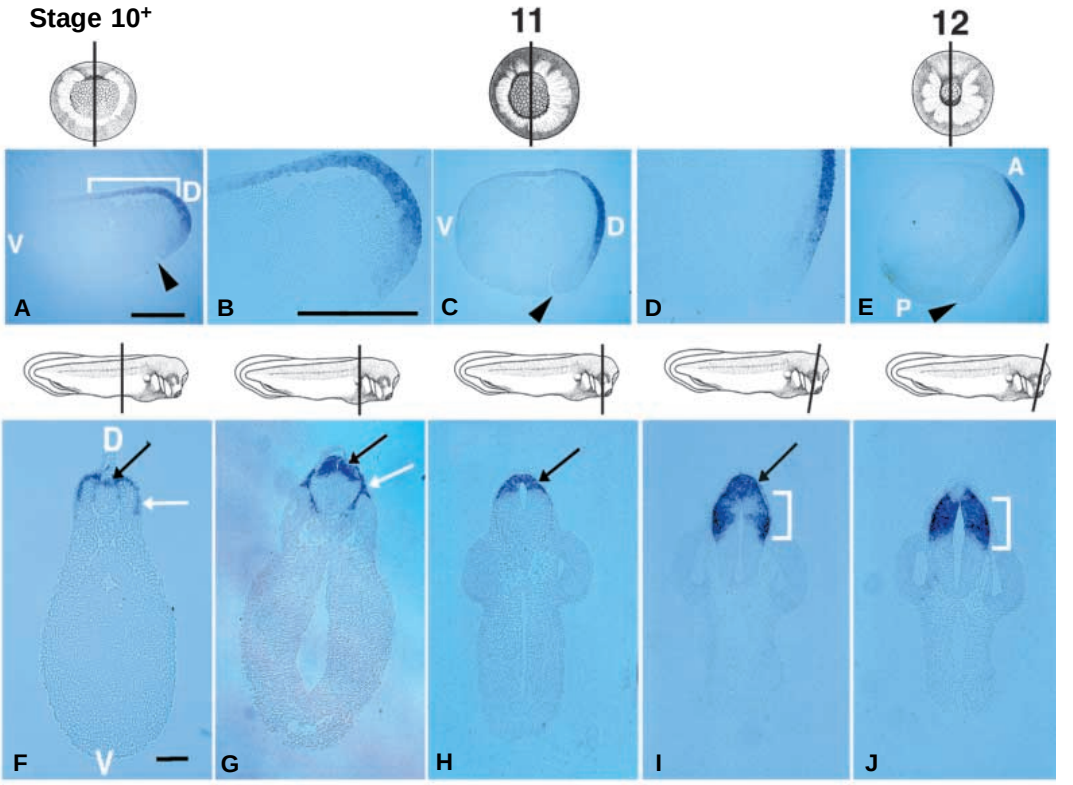

Fig. 4. Sections of embryos analyzed by whole-mount in situ hybridization for *opl* expression. Embryos were fixed, embedded and sectioned as described in Methods. Sectioning planes are shown in schematic diagrams above panels. Black arrowheads indicate position of dorsal blastopore lip. Black arrows indicate *opl* expression in roofplate. White arrows indicate spinal crest expression. Brackets indicate alar plate expression in forebrain/midbrain regions. D, dorsal; V, ventral. Scale bar, 25 μm.

nucleocytoplasmic distribution, using myc-epitope tagged versions of both opl and oplΔC, MT-opl and MT-oplΔC (see Methods). One cell of a 2-cell embryo was injected with RNA encoding either of these constructs and examined at midgastrula for distribution of the epitope-tagged opl using whole-mount immunocytochemistry. As shown in Fig. 7B, both the intact (MT-opl) and carboxy terminal truncated (MT-oplΔC) proteins were nuclear, with no obvious differences in the distribution of either construct. We were also unable to detect any differences in the amount of opl protein produced in embryos from either MT-opl or MT-oplΔC (not shown).

In summary, this analysis suggested that opl is a transcriptional activator and defined a putative regulatory domain in the carboxy region of the opl protein. This region repressed the ability of opl to act as an activator but had no effect on the nucleocytoplasmic distribution of opl.

oplΔC activates neural crest and dorsal neural tube markers

The neurectodermal restriction of *opl* expression suggested that it may play a role in neural determination. We tested this possibility in an animal cap assay. RNA encoding opl, oplΔC or CAT (as control) was injected into the animal pole region of two cell embryos. At late blastula (stage 9), animal caps were removed, cultured until tailbud (stage 22) and marker gene expression was analysed using a reverse transcriptase-polymerase chain reaction (RT-PCR) assay (Fig. 8A and Methods).

One of three representative experiments is shown in Fig. 8B. After injection of *CAT* RNA, we observed only background levels of the neural-specific markers *Xash3* (Zimmerman et al., 1993), *neurogenin* (Ma et al., 1996), *NCAM* (Kintner and Melton, 1987), the neural crest marker *slug* (Mayor et al., 1995), the dorsal neural tube marker *pax3* (Espeseth et al., 1995) and the ventral neural tube marker Sonic hedgehog (*shh*) (Ekker et al., 1995) (Fig. 5B, lane 2). In contrast, high levels of the ventral ectodermal markers *XK81* (Jonas et al., 1985) and *GATA2* (Walmsley et al., 1994) were observed after this treatment (lane 2). Injection of *opl* or *oplΔC* RNAs did not induce expression of *NCAM*, *Xash3* or *neurogenin* (lanes 3 and 4), however *oplΔC* (lane 3), but not *opl*

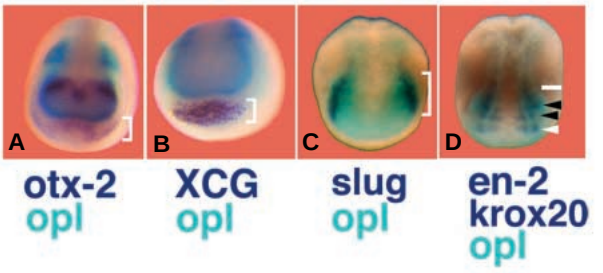

Fig. 5. Double whole-mount in situ analysis of *opl*. In situ analysis was as described in Methods with *opl* stained light blue and all other markers (*otx*-2, *XCG*, *slug*, *en*-2, *krox20*) are stained purple. (A) *opl* plus *otx*-2, a marker of forebrain and cement gland (Blitz and Cho, 1995; Pannese et al., 1995), bracket shows *otx*-2 expression in cement gland primordium; (B) *opl* plus *XCG*, a cement gland marker (Sive et al., 1989), bracket shows *XCG* expression in cement gland; (C) *opl* plus *slug*, a neural crest marker (Mayor et al., 1995), bracket shows *slug* domain contained within *opl* domain; (D) *opl* plus *en*-2, a marker of the midbrain/hindbrain regions (Hemmati-Brivanlou and Harland, 1989) and *krox20*, a marker of presumptive rhombomeres 3 and 5 in the hindbrain (Bradley et al., 1993). White arrowhead indicates *en*-2 expression, black arrowheads indicate *krox20* expression, white bar is posterior limit of early *opl* expression.

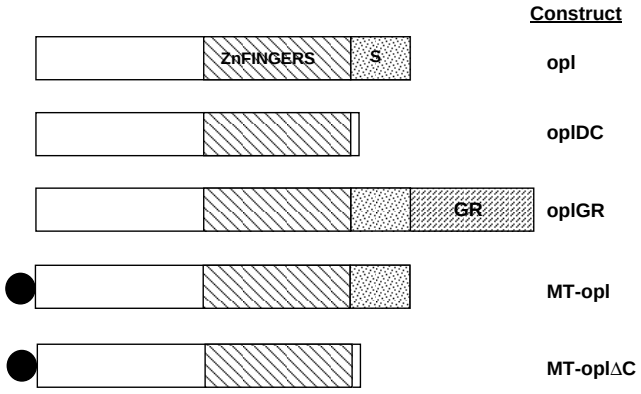

Fig. 6. *opl* constructs. The *opl* construct comprises the coding region of opl; oplΔ*C* deletes a serine-rich carboxy terminal domain (S), oplGR comprises an in-frame fusion of the opl-coding sequence with the hormone-binding domain of the glucocorticoid receptor (Kolm and Sive, 1995b). MT-opl is an in-frame fusion of the opl-coding region with a multimerized myc epitope (MT, black circle) at the amino end of *opl* (Rupp et al., 1994) and MT-oplΔC is a similar fusion of the multimerized myc epitope with the carboxy deleted opl. See Methods for details.

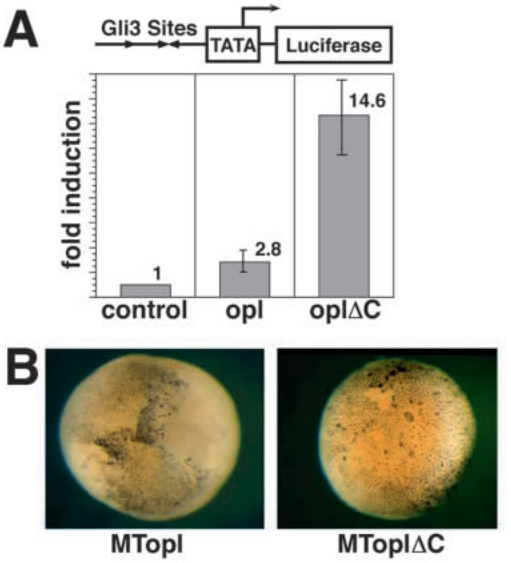

Fig. 7. opl is a nuclear protein with a regulatory domain in the COOH-terminal. (A) opl protein transactivates reporter constructs in tissue culture. Transient transfection reporter assays were performed as described in Methods, using the human pancreatic tumor BXPC cell line. The reporter construct is shown schematically: three Gli3 DNA-binding sites are located 5′ to a basal promoter (TATA box) and *luciferase* reporter gene (Vortkamp et al., 1995) Data shown are the averages of three independent experiments with corresponding error bars. In the absence of the Gli3-binding sites, neither opl nor oplΔC activated a reporter construct (not shown). DNA mixes for transient transfections are as follows, Left panel, reporter plus CS2+ vector; middle panel, reporter plus CS2+*opl* ORF right panel, reporter plus pCS2-*opl*ΔC. (B) Immunocytochemistry of injected opl and oplΔC proteins. Albino embryos were injected with 500 pg of synthetic MT*opl* or MT*opl*ΔC RNA at 2- to 4-cell stages and analyzed at stage 11 by immunostaining with anti-myc 9E10 antibody as described in Methods. Views of the animal hemispheres are shown, left panel, MT*opl*-injected embryo, right panel, MT*opl*ΔC-injected embryo.

(lane 4) was able to activate expression of *slug* (4-fold over *CAT*-injected levels, averaged over three experiments) and *pax3* (3.5-fold over *CAT*-injected levels). *shh* was not activated by injection of either construct. We also asked whether *opl* or *oplΔC* could suppress non-neural ectodermal differentiation, by assaying the expression of *XK81* and *GATA2* as ventral markers. Both *opl* and *oplΔC* were able to suppress expression of *XK81* by 2-fold relative to *CAT*-injected samples. Additionally, while *oplΔC* was unable to suppress *GATA2* expression, *opl* consistently suppressed expression of *GATA2* by 2-fold.

In summary, these data showed that neither *opl* nor its activated form, *oplΔC*, could activate expression of neural-specific genes in uninduced ectoderm. However, consistent with the later expression pattern of *opl* in the dorsal neural tube, *oplΔC* efficiently induced expression of neural crest and dorsal neural tube markers. These data contrast with those obtained after injection of *Zic3* and *Zic-r1* RNAs, where multiple neural-specific markers as well as *slug* were activated (Nakata et al., 1997; Mizuseki et al., 1998). It is not clear whether this represents a difference in the experimental conditions used, or a difference in the activities of *opl* and these related genes.

oplΔC sensitizes the ectoderm to induction by noggin

The early expression domain of *opl* in the dorsal ectoderm correlated with the region of the gastrula ectoderm that is most competent to respond to neural induction. We therefore asked whether *opl* could act as a modulator of neural competence and sensitize ectoderm to the neural-inducing activity of noggin, a factor that acts by antagonizing BMP signaling (Lamb et al., 1993), using the animal cap assay diagrammed in Fig. 8A. Embryos were injected with varying amounts of *noggin* RNA alone or together with *oplΔC* RNA or with *globin* RNA as a control and caps later analysed by RT-PCR for expression of two neural-specific genes, *NCAM* and *Xash3*.

A representative experiment (of three) is shown in Fig. 9. Neither *globin* RNA (lane 1) nor *oplΔC* alone (lane 2) induced *NCAM* or *Xash3* RNA expression, consistent with results shown in Fig. 8. 0.1 pg *noggin* RNA did not induce either *NCAM* or *Xash3* (lane 3) and 1 pg *noggin* RNA did not induce *NCAM* and activated *Xash3* only weakly (lane 4). 10 pg *noggin* RNA strongly activated both *NCAM* and *Xash3* expression (lane 5). At 0.1 pg *noggin* RNA plus *oplΔC* RNA, no *NCAM* and very weak *Xash3* expression was observed (lane 6); however, at 1 pg *noggin* RNA plus *oplΔC*, strong expression of both *NCAM* and *Xash3* was observed (lane 7), to levels that were almost as strong as seen with 10 pg of *noggin* alone. At higher *noggin* concentration (10 pg) plus *oplΔC*, no further potentiation of *NCAM* and *Xash3* was observed (lane 8) and, in the experiment shown, *Xash3* RNA accumulation declined relative to 1 pg *noggin* plus *oplΔC*, perhaps indicating that saturation of the induction machinery had occurred.

In summary, these data showed that *oplΔC* could sensitize isolated ectoderm to the neural-inducing effects of *noggin*, indicating that *opl* can regulate neural induction, and consistent with a role for *opl* in mediating neural competence.

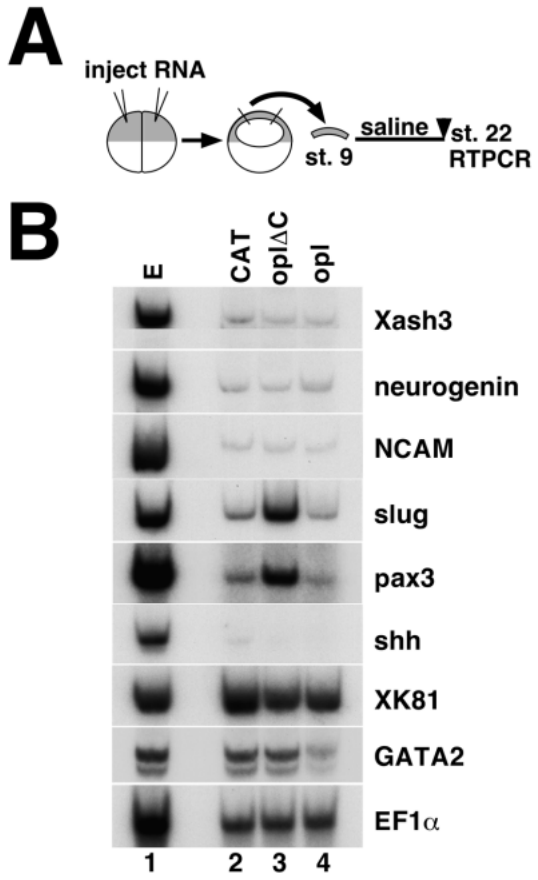

Fig. 8. oplΔC can activate neural crest and dorsal neural tube marker genes in animal caps. (A) Experimental scheme. Wild-type embryos were injected in both blastomeres with 200 pg of indicated RNAs at the 2-cell stage. Injection of *CAT* RNA served as negative control by equalizing the injected RNA dose. Pools of 15-20 animal caps were isolated from injected embryos at late blastula (stage 9) and incubated in saline until harvest at tailbud (stage 22) for RT-PCR analysis. See Methods for details. (B) Expression of marker genes in animal caps. Uninjected embryos serve as control for baseline expression level. *Xash3* (Zimmerman et al., 1993), *neurogenin* (Ma et al., 1996)and *NCAM* (Kintner and Melton, 1987) are neural-specific markers; *slug* (Mayor et al., 1995) is a neural crest marker; *pax3* (Espeseth et al., 1995) marks the dorsal neural tube, *shh* marks the floorplate (Ekker et al., 1995); *XK81* (Jonas et al., 1985) and *GATA2* (Walmsley et al., 1994) are markers of the ventral ectoderm; *EF1α* (Krieg et al., 1989) served as loading control. Samples processed without RT did not show *EF1α* signal after PCR (not shown). Data from one representative experiment is shown; similar results were obtained from three experiments. Lane 1, stage 22 embryo; lane 2, *CAT*-injected animal caps; lane 3, *oplΔC-injected* caps; lane 4, *opl*-injected caps.

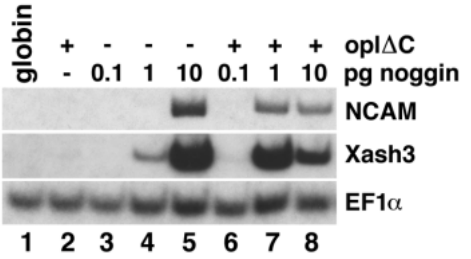

Fig. 9. *oplΔC* sensitizes the ectoderm to induction by *noggin*. Wild-type embryos were injected in both blastomeres with indicated RNAs at the two-cell stage, using 200 pg *globin* or *oplΔ*C, and 0.1 pg. 1 pg or 10 pg *noggin* (Smith and Harland, 1992) RNAs, as diagrammed in Fig. 8A, except injection of *globin* RNA served as negative control by equalizing the injected RNA dose in all lanes. Pools of 15-20 animal caps were isolated from injected embryos at late blastula (stage 9) and incubated in saline until harvest at tailbud (stage 22) for RT-PCR analysis. See Methods for details. Expression of neural markers *NCAM* (Kintner and Melton, 1987) and *Xash3* (Zimmerman et al., 1993) was analysed. *EF1α* served as loading control (Krieg et al., 1989). Samples processed without RT did not show *EF1α* signal after PCR (not shown). Data from one representative experiment is shown, similar results were obtained from three experiments. Lane 1, *globin*-injected caps; lane 2, caps injected with 200 pg *oplΔ*C RNA; lane 3, caps injected with 0.1 pg *noggin* RNA; lane 4, caps injected with 1 pg *noggin* RNA; lane 5, caps injected with 10 pg *noggin* RNA; lane 6, caps injected with 0.1 pg *noggin* RNA plus 200 pg *oplΔ*C RNA; lane 7, caps injected with 1 pg *noggin* RNA plus 200 pg *oplΔC* RNA; lane 8, caps injected with 10 pg *noggin* RNA plus 200 pg *oplΔ*C RNA.

In conjunction with noggin, opl activates posterior and dorsoventral neural markers

Having shown that *oplΔC* could sensitize the ectoderm for responsiveness to noggin, we next asked whether *opl* or *oplΔC* could alter the spectrum of neural markers induced by high levels of noggin that were characteristic of particular anteroposterior or dorsoventral axial positions, using the animal cap assay diagrammed in Fig. 8A with *CAT* RNA as control. Expression levels of the general neural marker *NCAM* and marker genes differentially expressed along the anteroposterior (A/P) or dorsoventral (D/V) axes of the neural tube were assayed by RT-PCR.

One of four representative experiments is shown in Fig. 10. Uninjected caps (or caps injected with *CAT* RNAs (not shown)) failed to express all dorsal ectodermal and mesodermal markers examined (lane 2). Injection of *noggin* plus *CAT* RNAs (lane 3) led to activation of *NCAM*, the cement gland marker *XCG* (Sive et al., 1989) and the forebrain plus cement gland marker *otx2* (Blitz and Cho, 1995; Pannese et al., 1995), but activated only low levels of markers that were more posterior. Injection of *oplΔC* (lane 3) or intact *opl* (not shown) RNAs alone led to weak activation of the hindbrain markers *krox20* (Bradley et al., 1993), *HoxD1* (Kolm and Sive, 1995a) and less reproducibly, the spinal cord marker *HoxB9* (Wright et al., 1990), but failed to activate either *NCAM* or the anterior markers *XCG*, *otx2* and *en2* (Hemmati-Brivanlou and Harland, 1989). However, expression of *oplΔC* plus *noggin* (lane 5) or *opl* plus *noggin* (lane 6), reproducibly led to very strong activation of *en2*, expressed at the midbrain/hindbrain junction (25-fold for *oplΔC*, and 7-fold for *opl*, averaged over four experiments). *oplΔC* (but not *opl*) was also able to activate expression of *krox20* and *HoxD1*, (lane 5), with the extent of activation varying between experiments, while *HoxB9* expression was not reproducibly activated.

We also examined synergy of *opl* or *oplΔC* with *noggin* with respect to markers expressed along the D/V aspect of the neural tube. Injection of *noggin* RNA together with control *CAT* RNA (lane 3) induced low levels of the dorsal marker *pax3* (Espeseth

et al., 1995), the ventrolateral marker *NK2* (Saha et al., 1993) and the ventral marker *shh*. Consistent with the data shown in Fig. 8, *oplΔC* alone (lane 4) was very effective at activating expression of the neural crest markers *slug*, *twist* (Hopwood et al., 1989) (not shown) and *pax3*. In conjunction with *noggin*, *oplΔC* (lane 5) also activated expression of genes normally restricted to the ventrolateral and ventral neural tube including

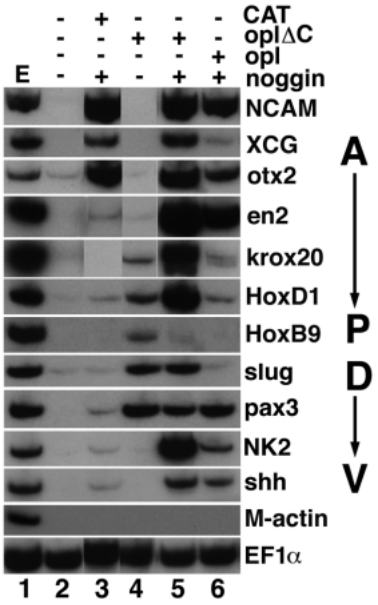

Fig. 10. *opl* synergizes with *noggin* to activate more posterior neural markers in animal caps. Wild-type embryos were injected in both blastomeres with indicated RNAs at the 2-cell stage, using 200 pg *CAT*, *opl* or *oplΔC*, and 10 pg *noggin* (Smith and Harland, 1992) RNAs, as diagrammed in Fig. 8A. Injection of *CAT* RNA served as negative control by equalizing the injected RNA dose. Pools of 15-20 animal caps were isolated from injected embryos at late blastula (stage 9) and incubated in saline until harvest at tailbud (stage 22) for RT-PCR analysis. See Methods for details. Expression of a set of neural markers expressed along the anteroposterior (A/P) or dorsoventral (D/V) neural axes was analysed. Uninjected embryos serve as control for baseline expression level. *NCAM* is a general neural marker (Kintner and Melton, 1987). Expressed in the indicated anterior (A)-to-posterior (P) series: *XCG* is a cement gland marker, the most anterior ectodermal tissue (Sive et al., 1989); *otx*-2 is a forebrain and cement gland marker (Blitz and Cho, 1995; Pannese et al., 1995); *en2* is a marker of mid/hindbrain boundary (Hemmati-Brivanlou and Harland, 1989); *krox20* marks rhombomeres 3 and 5 in the presumptive hindbrain (Bradley et al., 1993); *HoxD1* is a hindbrain (posterior to rhombomere 4) and spinal cord marker (Kolm and Sive, 1995a), and *HoxB9* is a spinal cord marker (Wright et al., 1990). Expressed in the indicated dorsal (D) to ventral (V) series of markers, *slug* (marking the neural crest, (Mayor et al., 1995)), *pax3* (marking the dorsal neural tube, (Espeseth et al., 1995)), *NK2* (marking the ventrolateral neural tube, (Saha et al., 1993)) and *shh* (marking the floorplate, (Ekker et al., 1995)). *M-actin* (Mohun et al., 1984) is a mesodermal marker; *EF1α* (Krieg et al., 1989) served as loading control. Samples processed without RT did not show *EF1α* signal after PCR (not shown). Data from one experiment are shown, comparable results were obtained in four independent experiments. Lane 1, stage 22 embryo; lane 2, uninjected animal caps; lane 3, *noggin* plus *CAT*-injected; lane 4, *oplΔC*-injected; lane 5, *noggin* plus *oplΔC*-injected; lane 6, *noggin* plus *opl*-injected.

NK2, *shh* and *F-spondin* (Ruiz i Altaba et al., 1995) (not shown). Intact *opl* protein in conjunction with *noggin* activated high levels of *pax3*, *NK2* and *shh*, but did not efficiently activate *slug* or *twist* (not shown) expression (lane 6). Expression of mesodermal genes, including muscle actin, was not activated by any of these injections.

Overall, this assay demonstrated that *opl* and *oplΔC* were able to alter the A/P and D/V spectrum of genes activated by noggin, suggesting that opl may act as a modulator of positional information along the A/P and D/V neural axes. As in the assay for transcriptional activation, oplΔC was more active than native opl protein.

opl induces cellular aggregates in the absence of DNA synthesis

Since *opl* and *oplΔC* were able to alter neural marker gene

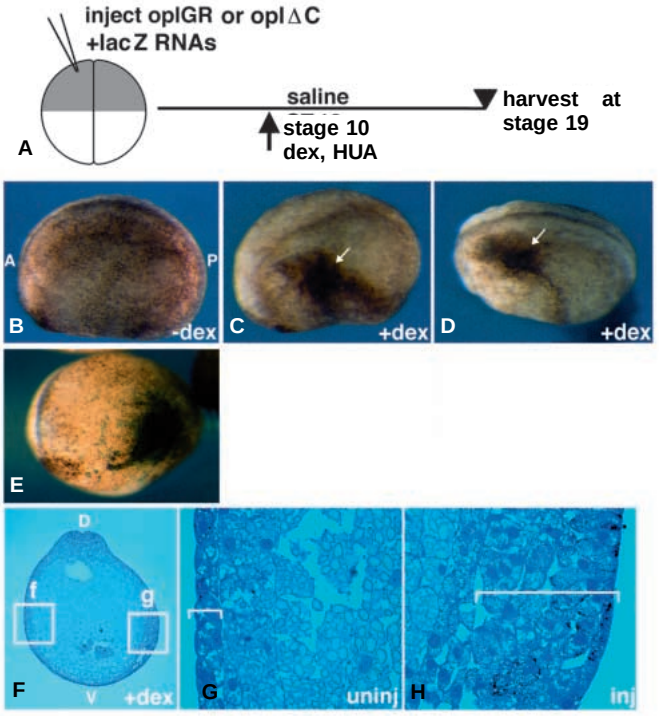

Fig. 11. *opl* induces cellular aggregates without cell division. (A) Experimental scheme. Wild-type embryos were injected in one cell at the 2-cell stage with 500 pg of *oplGR* or *oplΔC* RNA plus 100 pg *lacZ* RNA as lineage tracer. Injected embryos were incubated in saline alone or with dexamethasone (dex) and/or hydroxyurea plus aphidicolin (HUA) starting at early gastrula (stage 10) until late neurula (stage 19). See Methods for details. (B-H) Morphology and histology of *opl*-induced cellular aggregates. β-gal staining is obscured by pigment. (B-E) Anterior (A) is to the left, dorsal is to the top. (B) Embryo injected with *oplGR* RNA without dex addition; (C,D) embryos injected with *oplGR* RNA and treated with dex from stage 10 until harvest. Arrows point to cellular aggregates in ventrolateral ectoderm. (E) Embryo injected with *oplGR* RNA and treated with HUA (see Methods) plus dex from stage 10 until harvest. (F-H) Embryos sectioned and stained after *oplΔC* injection. (F) +dex, transverse section of stage 19 embryo through trunk region. Regions magnified in G and H are boxed and indicate uninjected and injected sides of the embryo respectively, as judged by β-gal staining. (G) Uninjected side; (H) injected side. The width of the ectoderm is bracketed in G and H. D, dorsal; V, ventral.

Table 1. Frequency of ectopic cellular aggregates in injected embryos

Treatment*	Ectopic aggregates (%)†	Number of embryos‡	Number of experiments§
opl	35 (15)	263	7
oplΔC	232 (64)	361	17
oplΔC+HUA	77 (57)	135	4
oplGR			
–dex	0 (0)	72	2
+dex	26 (50)	52	2
oplGR			
–HUA+dex	19 (66)	29	1
+HUA+dex	18 (58)	31	1
globin	0 (0)	244	12

Embryos were prepared as described in Fig. 11A; pigmented cellular aggregates were scored at stage 19.

*One blastomere of two-cell embryos was injected with *opl*, *oplΔC*, *oplGR* or globin RNAs. Embryos were incubated in saline or with 10 μM dexamethasone (dex) until harvest at stage 19. See Methods for details.

†Number and frequency of embryos showing pigmented cellular aggregates in the region of injected RNA (marked by βGal staining).

‡Total number of embryos examined.

§Number of independent experiments.

Table 2. Induction of cellular aggregates depends on time of addition and duration of dex treatment in *oplGR*-injected embryos

Stage of dex addition*	Stage of maximal phenotype (elapsed hours)†	Ectopic aggregates (%)‡	Number of embryos§	Number of experiments¶
8	15 (12.5)	19 (59)	32	2
10	15 (8)	28 (85)	33	2
11.5	14 (4.5)	20 (59)	34	2
12	20 (8)	22 (71)	31	2
15	24 (9)	14 (78)	18	1
18	25 (9)	3 (15)	20	2
24	36 (24)	0 (0)	10	1

Embryos were prepared as described in Fig. 8A; pigmented cellular aggregates were scored over time after addition of dexamethasone (dex).

*Stage when dex treatment was started.

†Stage and elapsed hours since start of dex treatment when maximal size and extent of pigmented cellular aggregates was observed in embryos.

‡Number and frequency of embryos with ectopic pigmented cellular aggregates.

§Total number of embryos examined.

¶Number of independent experiments.

expression in animal cap assays, we wanted to determine whether ectopic *opl* expression could alter neurogenesis in the intact embryo. To do this, we compared the effects of native opl protein, oplΔC, and a hormone inducible version of opl, oplGR, on the morphology of whole embryos. oplGR comprises the opl-coding region linked in frame to the ligand-binding domain of the glucocorticoid receptor (GR). We have previously shown that such constructs are post-translationally hormone inducible in *Xenopus* embryos, and can be activated at the time when the endogenous gene is maximally expressed (Kolm and Sive, 1995b). Embryos were injected with RNA encoding either *oplGR*, along with *lacZ* RNA as a lineage tracer (Fig. 11A). oplGR constructs were activated by dexamethasone (dex) at early gastrula, and embryos later assayed morphologically.

The results of this analysis are shown in Fig. 11 and collated in Table 1. Without dex treatment embryos appeared normal (Fig. 11B), while after activation of the oplGR protein at early gastrula, large pigmented aggregates were seen in the ventral and lateral ectoderm (Fig. 11C,D) in 50% of all embryos. These aggregates were also observed after ectopic *opl* misexpression in 15% of embryos, and after ectopic *oplΔC* expression in 64% of embryos, consistent with the greater activity of *oplΔC* (Table 1). Aggregates were observed in the ectoderm and adjacent to the neural plate, but not within the neural plate itself. The increased activity of oplGR relative to opl in this assay is similar to that of oplΔC and is likely to be due to the presence of a weak activation domain in the GR, that can overcome the inhibitory domain of intact opl protein (Mattioni et al., 1994).

We next asked whether the aggregates observed after *opl* misexpression were a result of increased proliferation or cell aggregation. Embryos injected with *opl*GR were pretreated with hydroxyurea and aphidicolin (HUA) to inhibit DNA synthesis and cell division (Harris and Hartenstein, 1991), before dexamethasone treatment (Fig. 11A). After this treatment, cells remained large, consistent with the absence of cell division (not shown). As shown in Fig. 11E, cell aggregates were observed even in the absence of DNA synthesis in 58% of embryos, similar to the percentage observed without HUA treatment (66%; Table 1). Similar results were obtained after injection of *oplΔC* in the presence of HUA (Table 1).

A section of an embryo, injected with *oplGR* RNA and treated with dex at stage 10, formed a ventral aggregate (Fig. 11F). In close up, the uninjected side shows the three germ layers, including a tightly packed ectodermal epithelium (bracket, Fig. 11G). In contrast, on the injected side of the embryos the ectoderm was organized as a loosely packed mass of cells, which included pigmented cells not arranged in the characteristic epithelium seen in control embryos (Fig. 11H). The mesodermal and endodermal layers appeared morphologically normal.

Additionally, we asked when the embryo was competent to form aggregates. After injection of *oplGR* RNA followed by dex treatment at various times after injection (Fig. 11A), embryos were later scored for appearance of maximally sized aggregates. The results are collated in Table 2. After dex induction at midblastula (stage 8), embryos took 12.5 hours to form aggregates of maximal size, whereas by early gastrula (stage 10) only 8 hours elapsed before aggregate formation. By midgastrula (stage 11.5), only 4 hours elapsed after dex addition before maximal aggregate formation occurred and this was the shortest lag time observed. Older embryos (late gastrula (stage 12), early neurula (stage 15) and late neurula (stage 18) all formed aggregates, but at 8 to 9 hours after dex addition. No aggregates were observed after dex addition at tailbud (stage 24), but it was not clear whether this reflected a lack of competence to form aggregates or degradation of *opl*GR RNA.

In summary, opl, oplΔC and oplGR proteins were able to activate formation of large pigmented aggregates in the ventral and lateral ectoderm. These appeared to be loose associations of cells and were not caused by an increase in cell proliferation. Embryos were competent to form aggregates from midblastula

through late neurula, with the time of maximal competence at midgastrula.

opl activates expression of neural crest and dorsal neural tube markers and represses epidermal gene expression in whole embryos

We extended the analysis in whole embryos to ask whether *opl* constructs can activate ectopic expression of neural crest and dorsal neural tube markers and suppress ventral ectodermal (epidermal) fates. Embryos were injected with either *opl*GR or *opl*ΔC RNA, along with *lacZ* RNA as lineage tracer. For *opl*GR injections, dexamethasone (dex) was added at stage 11 when response to *opl*GR was maximal as indicated in the aggregate formation assay (Table 2, and not shown). Neural crest formation was assayed by expression of *slug*, dorsal neural tube fates were assayed by expression of *pax3* and epidermal fate was assayed by expression of *XK81*.

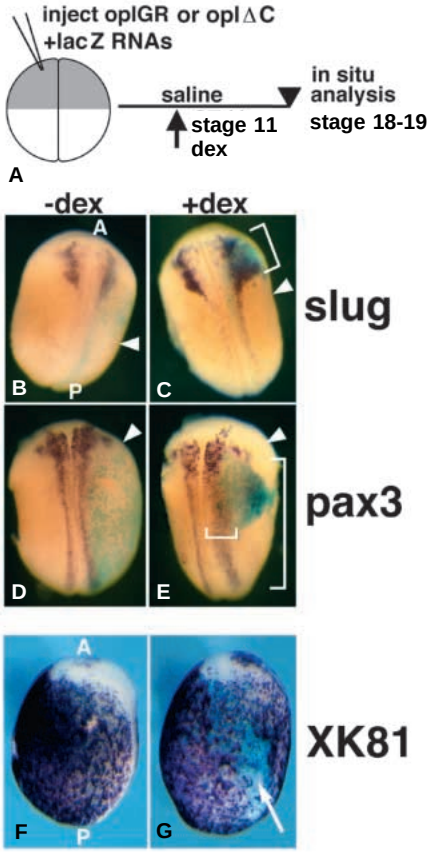

Fig. 12. *opl* induces ectopic *slug* and *pax3* and inhibits *XK81* expression in embryos. (A) Experimental scheme. Albino embryos were injected in one cell at the 2-cell stage with 500 pg *oplGR* or *oplΔC* plus 100 pg *lac*Z RNA as lineage tracer. Embryos were incubated in saline, or with addition of dexamethasone (dex) at stage 11, until embryos reached midneurula (stage 18), when they were harvested for in situ analysis. See Methods for details. (B-E) Ectopic *slug* (Mayor et al., 1995) and *pax3* (Espeseth et al., 1995) expression in whole embryos. Embryos were injected with *oplGR* plus *lacZ* RNAs and incubated without (B,D) or with dex (C,E) before histochemical staining for β-gal (light blue) and in situ hybridization for *slug* or *pax3* (purple). Anterior is to the top in all panels. Arrowheads indicate injected side. Ectopic expression of *slug* and *pax3* is indicated by brackets. (F,G) *oplΔC* suppressed expression of the epidermal cytokeratin *XK81* (Jonas et al., 1985). Embryos were injected with 500 pg *globin* (F) or *oplΔC* (G) RNAs mixed with 100 pg *lacZ* RNA, incubated in saline until stage 18, before histochemical staining for β-gal (light blue) and in situ hybridization for *XK81* (purple). Anterior is to the top in both panels. Endogenous *XK81* is expressed throughout the ventrolateral epidermis and excluded from the neural plate (white regions). White arrow indicates *oplΔC*-expressing cells (marked by β-gal staining) do not express *XK81*. A, anterior; P, posterior.

Table 3. Frequency of ectopic *slug* and *pax3* expression in *oplGR*-injected embryos

Treatment*	Marker examined†	Ectopic expression(%)‡	Number of embryos§	Number of experiments¶
oplGR	*slug*	1 (3)	35	3
–dex	*pax3*	0 (0)	38	4
+dex	*slug*	18 (42)	43	3
	pax3	58 (75)	77	4

Embryos were prepared as described in Fig. 8A; *slug* and *pax3* expression was scored by in situ hybridization on neurula (stage 18) embryos.

*One blastomere of 2-cell embryos was injected with *oplGR* RNA. Embryos were incubated in saline or with 10 mM dexamethasone (dex) starting at stage 11 until harvested at stage 18. See Methods for details.

†*slug* and *pax3* expression in embryos was examined by in situ hybridization as described in Methods.

‡Number and frequency of embryos with ectopic *slug* or *pax3* expression, of the total number with β-gal staining anywhere in the embryo. In the +dex group examined with *slug*, none showed ectopic expression in ventrolateral ectoderm not contiguous with the endogenous *slug* domain. In the +dex group examined with *pax3*, 55 (71%) embryos showed ectopic expression that was broad and contiguous with the endogenous *pax3* expression, 3 (4%) embryos showed ectopic expression in ventrolateral ectoderm that was non-contiguous with the endogenous *pax3* domain.

§Total number of embryos examined.

¶Number of independent experiments.

Representative data is shown in Fig. 12B-E and the results collated in Table 3. Without dex, uniform *slug* expression was observed on both sides of the embryo (Fig. 12B). After dex treatment, concentration of *lacZ* lineage tracer was observed reflecting the cell aggregates previously seen in pigmented embryos (see Fig. 11). In dex-treated embryos, *slug* expression expanded anteriorly on the injected side of the embryo (Fig. 12C) in 42% of injected embryos. Similarly, a large expansion of the *pax3* expression domain was observed after dex addition in 75% of injected embryos. In a few cases (4% of injected embryos), patches of *pax3* expression were not connected to the neural plate.

We also asked whether *opl* altered expression of the ventral epidermal marker, *XK81*. Since epidermis is specified early, we used the constitutively active construct *oplΔC*. Embryos injected with *globin* RNA as a control (Fig. 12F) along with *lacZ* showed *XK81* expression over the entire ventral ectoderm, obscuring the β-gal staining. After injection of *oplΔC* (Fig. 12G), however, 51% of embryos showed large patches of *lacZ* expression since *XK81* is not expressed in these regions (Table 4). Regions of ventral ectoderm that did not express *XK81* always coincided with regions of *lacZ* expression.

In summary, in whole embryos, *opl* constructs were able to expand the expression domains of a neural crest and a dorsal neural tube marker, and was able to inhibit expression of an

Table 4. Frequency of XK81 inhibition in oplΔC-injected embryos

Treatment*	*XK81* inhibition (%)†	Number of embryos‡	Number of experiments§
oplΔC	43 (51)	84	3
globin	0 (0)	85	3

Embryos were prepared as described in Fig. 8A; *XK81* expression was scored by in situ hybridization on neurula (stage 18) embryos.

*One blastomere of 2-cell embryos was injected with *oplΔC* or *globin* and embryos were incubated in saline until harvest. See Methods for details.

†Number and frequency of embryos with *XK81* expression inhibited when *opl* expression (marked by β-gal staining) was located in ventrolateral ectoderm.

‡Total number of embryos examined.

§Number of independent experiments.

epidermal cytokeratin gene, which is a marker of the ventral ectoderm. The *slug* expression domain also expands in *Zic3*- and *Zic-r1*-injected embryos, consistent with these results.

DISCUSSION

We report isolation of *opl*, a gene similar to *Drosophila odd-paired* and a new member of the *Zic* gene family in *Xenopus*. *opl* expression indicates that neural determination begins by the onset of gastrulation, and the opl protein appears to have three activities. First, *opl* can sensitize the presumptive neurectoderm for induction, suggesting that it may be a neural competence factor. Second, consistent with its later expression in the dorsal neural tube and neural crest, an activated form of *opl*, *oplΔC*, can activate neural crest and dorsal neural tube markers. Third, *opl* can synergize with noggin to induce expression of engrailed. These data indicate that *opl* may be a key regulator of *Xenopus* neurogenesis.

opl expression defines an early neurectodermal domain

opl is most similar to the mouse *Zic1* gene that is expressed in the embryonic mesoderm and presumptive neurectoderm of mice (Nagai et al., 1997), with later expression persisting in the dorsal neural tube and the dorsomedial region of the somites. *Xenopus opl* is not expressed at significant levels in the mesoderm. However, the early expression of *Xenopus opl*, mouse *Zic1* and zebrafish *opl* (Grinblat et al., 1998) during neurogenesis, and their later restriction of expression to the dorsal neural tube suggests that their function may be conserved during vertebrate neural development.

At early gastrula, when *opl* is first expressed, neural tissue is not yet specified, as judged by isolation and culture of dorsal ectoderm and later analysis for markers of neural differentiation (Sive et al., 1989). However, restricted *opl* expression in dorsal ectoderm clearly shows that the neurectoderm has begun to be set aside by the onset of gastrulation. A large part of the early expression domain of *opl* is in dorsal ectoderm not yet underlain by the involuting mesendoderm. This dorsal restriction of *opl* RNA is dependent on dorsal signals since it is abolished in UV-treated embryos (J. G. and H. S., unpublished data). These data indicate that *opl* RNA accumulation may be activated by planar signals from the

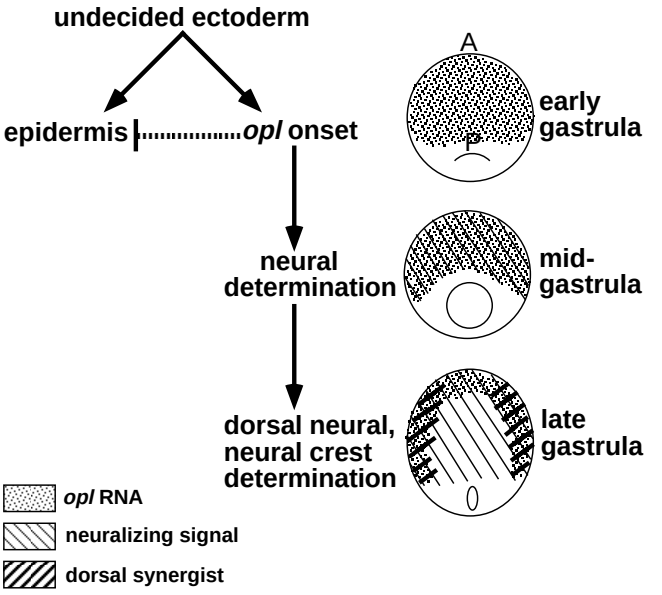

Fig. 13. Model of the roles opl plays in early neural determination. Schematics of whole embryos during gastrulation are shown (dorsal views, anterior is to the top). Arrow, dorsal lip of blastopore; A, anterior; P, posterior. High level *opl* expression (spots) is activated in the dorsal ectoderm by early gastrula. By midgastrula *opl* expression has sensitized dorsal ectodermal cells for later induction and patterning by neural inducers such as *noggin* (light stripes). *opl* also acts by suppressing ventral ectodermal fates, such as epidermis (T bar). By late gastrula, *opl* expression has become restricted to the presumptive dorsal neural tube and neural crest, where it activates expression of neural markers in these regions. A dorsally restricted synergizing activity (dark stripes) is necessary for activation of neural crest fates. See text for further discussion.

organizer, or by patterning signals present in the dorsal ectoderm prior to the onset of gastrulation (Sokol and Melton, 1991; Wylie et al., 1996). Recent reports concerning other *Xenopus* Zic family members (*Zic3* and *Zic-r1*) indicate that their expression requires removal of BMP signaling (Nakata et al., 1997; Mizuseki et al., 1998). Consistent with this, *opl* is induced by *noggin* (Kuo, J. S., Gamse, J. and Sive, H., unpublished data), however, it is not clear whether such signaling initiates *opl* expression, or is required for a later maintenance step.

opl can modulate neural competence

The early *opl* expression domain suggested that it may be a competence factor and, indeed, *opl* could increase the responsiveness of isolated ectoderm to *noggin* by 10-fold. Our data indicate that expression of *opl* may account for some of the enhanced sensitivity of the dorsal ectoderm to neural induction as noted by other investigators (Sharpe et al., 1987; Otte et al., 1991). The translation initiation factor *EIF4AII* has recently been shown to synergize with *noggin* to activate expression of some neural crest markers as well as markers of the neurectoderm proper (Morgan and Sargent, 1997). *EIF4AII* is expressed considerably later than *opl*, by midgastrula, and may be a target of *opl* activation. Protein kinase C (PKC) activity has also been implicated in conferring neural competence on the dorsal ectoderm (Otte et al., 1991), while the *Notch* signaling pathway

may also be involved in directing neural competence (Coffman et al., 1993). It is unknown whether *opl* can modulate or be modulated by either of these pathways.

opl can activate *engrailed*

In conjunction with *noggin*, *opl* and *oplΔC* strongly potentiated expression of the posterior neural marker *engrailed*, expressed in the midbrain/hindbrain region. Although *opl* is expressed in presumptive posterior neurectoderm, we did not expect this result, since *opl* RNA is present throughout the anteroposterior extent of the future brain at early stages. It is possible, however, that *opl* protein activity is modulated along the A/P neural axis. One intriguing connection between the ability of *opl* to potentiate expression of *engrailed* and *Drosophila opa* is that *opa* is necessary for the correct temporal activation of *Drosophila wingless* (Benedyk et al., 1994), while, in *Xenopus*, the wingless homologs *wnt3A* and *wnt8* can posteriorize neurectoderm in combination with *noggin* (McGrew et al., 1995, 1997; Fredieu et al., 1997) This suggests that *opl* may activate *wnt* genes or act downstream of wnt proteins. Indeed the expression patterns of *opl* and *wnt3A* are very similar from late gastrula (Wolda et al., 1993). However, unlike *wnt3A*, *opl* expression did down-regulate anterior neurectodermal markers, suggesting that the activities of these proteins are not entirely equivalent.

opl as an activator of dorsal neural tube and neural crest fates

The later expression of *opl* in the dorsal neural tube and neural crest correlated with the ability of a truncated form of *opl*, *oplΔC*, to activate genes characteristic of these fates. Like *opl*, the dorsal neural tube marker, *pax3*, is transiently expressed initially across the entire dorsoventral extent of both the *Xenopus* and chick neural plates and is later expressed solely in the future dorsal neural tube (Liem et al., 1995; Bang et al., 1997). However, *opl* is expressed earlier than *pax3*, consistent with a role for *opl* in activating *pax3*. As *pax3* expression increases in the future dorsal neural tube, *opl* expression also increases and later becomes dorsally restricted. The expression of *opl* in presumptive neural crest from late gastrula again precedes the expression of neural crest-specific markers in this region (Mayor et al., 1995), consistent with a role for *opl* in regulating neural crest formation. *opl* expression across both presumptive dorsal neural tube and neural crest regions at late gastrula suggests that these two neural domains are initially defined as a unit that later becomes subdivided into dorsal neural tube and neural crest subdomains. Since recent studies have shown that wnt proteins can also activate neural crest markers in neuralized ectoderm (Saint-Jeannet et al., 1997; Chang and Hemmati-Brivanlou, 1998), another connection between opl and wnt expression is possible.

opl activity is modulated by synergizing factors

Several lines of evidence indicate that opl protein requires synergizing factors. First, oplΔC, but not full-length opl protein could activate dorsal neural tube and neural crest markers in animal caps. In other respects, opl appears to have similar, though weaker activity to oplΔC, as both constructs could repress ventral ectodermal markers, synergize with noggin and catalyse formation of pigmented aggregates. This suggests that oplΔC is an activated form of opl, although we cannot rule out that oplΔC has a novel activity. Second, we showed that, in animal caps, intact opl in conjunction with noggin was able to activate a dorsal neural tube marker, but not neural crest markers. This suggests that factors induced by noggin modulate opl activity; however, since opl could not activate neural crest markers even in conjunction with noggin, some other factor may allow opl to activate neural crest-specific gene expression. Such a factor may act by de-repressing a carboxy terminus inhibitory function in the opl protein, perhaps by altering opl phosphorylation. Third, in whole embryos, activated opl constructs could expand the domain of *slug* and *pax3* expression but only adjacent to the normal expression domains of these genes, and never in the extreme anterior neural plate. This further suggests that *opl* activity requires cofactors that are present in the neural plate, neural crest and in early gastrula animal caps, but that are absent from or counteracted by inhibitors in the ventral ectoderm of older embryos.

These considerations raise the question of whether the activation of dorsal neural tube fates, the *engrailed*-inducing capacity and the competence-promoting activity of *opl* are distinct functions, perhaps requiring different domains of *opl* or different cofactors. In animal caps, *opl* in conjunction with *noggin* was able to promote expression of both dorsal and ventral neural tube markers, indicating that expression of ventral neural tube markers is not incompatible with *opl* activity. Thus *opl* may act by a similar mechanism throughout the neural plate, and may play a role in early gastrula to activate both dorsal and ventral neural tube markers. Since, by the end of gastrulation, *opl* is not expressed ventrally, ventral *opl* activity is only transient.

Unlike *opl*, two related genes, *Zic3* and *Zic-r1* are able to activate both general neuronal markers and a neural crest marker in animal caps without requiring *noggin* addition (Nakata et al., 1997; Mizuseki et al., 1998). The differences in our results may reflect a difference in assay conditions that were employed, or may indicate different activities for these related genes. Since the neuronal markers that were examined in these studies are expressed in restricted areas of the neural plate, whereas expression of *Zic3* and *Zic-r1* is much broader, activity of these genes may also be modulated by cofactors. Similarly, *Zic3* and *Zic-r1* increased *slug* expression only adjacent to the normal *slug* expression domain and could not induce it in ventral ectoderm.

opl modulates cell adhesion

One of the most striking phenotypes consistently produced by *opl* and also produced by *Zic3* (Nakata et al., 1997), was the appearance of cellular aggregates in the ventrolateral ectoderm, but not within the neural plate itself. Aggregates did not express neural or neural crest markers unless they directly contacted the neural plate. In both whole embryos and in animal caps injected with *opl* RNA, aggregates comprised cells that were loosely adherent (not shown). In both the whole embryo and the animal cap, the cells appeared healthy, surviving and dividing after disaggregation in the culture dish. One intriguing hypothesis is that *opl* suppresses an adhesion pathway responsible for ectodermal-epithelial integrity that is normally down-regulated as neural crest fates are determined (Bronner-Fraser, 1995). Since *opl* did not induce ectopic slug in the ventral ectoderm, but did induce aggregates there, the

aggregates are clearly not committed neural crest cells, but may represent some early step in neural crest commitment. Consistent with this hypothesis, *opl* is expressed sequentially in the cranial neural crest segments just before the cells mound up, lose adhesivity and begin migrating (Sadaghiani and Thiebaud, 1987).

opl and inhibition of epidermal fates

In *Xenopus*, formation of ventral fates including epidermis appears to be dependent on Bone Morphogenetic Protein (BMP) activity (Wilson and Hemmati-Brivanlou, 1995). Suppression of this activity by *noggin* and *chordin* proteins during gastrulation results in neural determination (Piccolo et al., 1996; Zimmerman et al., 1996). In whole embryos, *opl* can inhibit expression of the epidermal cytokeratin gene *XK81* while, in animal caps, *opl* also suppresses expression of *GATA2*, a ventral ectodermal marker that is regulated by BMP4 signaling. Since this suppression is not correlated with an ability of *opl* or *opl*ΔC alone to activate general neural markers, *opl* is able to inhibit only part of the dorsoventral ectodermal patterning pathway. In accord with this, *opl* expression overlaps that of BMP4, until midgastrula when BMP4 RNA begins to be cleared from the presumptive neural plate (J. G. and H. S., unpublished).

A model for *opl* function

Our data suggest a model for the role of *opl* in early neural determination (Fig. 13). Early during gastrulation, *opl* gene expression is induced, either by planar signals from the organizer or by pre-existing signals within the dorsal ectoderm. The early expression domain of *opl* throughout the neural plate sensitizes the neurectoderm for subsequent neuralizing signals, such as *noggin* and *chordin*, derived from the organizer. This sensitization both modulates general neural readout and is also involved in activating relatively posterior neural markers, such as *engrailed*. By the end of gastrulation, *opl* expression in the dorsal neural plate and presumptive neural crest directs fates characteristic of these regions. The activity of *opl* in the presumptive neural crest is modulated by a regulatory domain in the carboxy terminus, suggesting that a laterally, but not anteriorly, localized factor(s) may control *opl* activity. The ability of *opl* to suppress cell adhesion may be part of the commitment program for neural crest fates.

Future challenges are to analyse the consequence of *opl* ablation, to isolate the downstream direct targets of *opl*, and to further understand how *opl* fits into the hierarchy of neural regulatory genes.

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