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Developmental Biology

Use of an oocyte expression assay to reconstitute inductive signaling

(noggin/Wnt protein/*Xenopus laevis*/cDNA cloning/neural induction)

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ABSTRACT **We have developed a paracrine signaling assay capable of mimicking inductive events in the early vertebrate embryo. RNA encoding one or more secreted proteins is microinjected into a *Xenopus laevis* oocyte. After a brief incubation to allow translation, a piece of embryonic tissue competent to respond to the signaling protein is grafted onto the oocyte. The secreted protein's effect on the grafted explant is then scored by assaying expression of tissue-specific markers. Explants of ectodermal tissue from blastula or gastrula stage embryos were grafted onto oocytes that had been injected with RNA encoding activin or noggin. We found that the paracrine assay faithfully reconstitutes mesoderm induction by activin and neural induction by noggin. Blastula-stage explants grafted onto activin-expressing oocytes expressed the mesodermal marker genes brachyury, goosecoid, and muscle actin. Gastrula-stage explants grafted onto noggin-expressing oocytes expressed neural cell adhesion molecule (NCAM) and formed cement gland. By injecting pools of RNA synthesized from a cDNA expression library into the oocyte, we also used the assay to screen for secreted neural-inducing proteins. We assayed 20,000 independent transformants of a library constructed from LiCl-dorsalized *Xenopus laevis* embryos, and we identified two cDNAs that induced neural tissue in ectodermal explants from gastrula-stage embryos. Both cDNAs encode noggin. These results suggest that the paracrine assay will be useful for the cloning of novel signaling proteins as well as for the analysis of known factors.**

During vertebrate embryogenesis, extracellular signals released by certain cell populations act over short distances to alter the developmental fate of other cell populations. This form of paracrine signaling, commonly called induction, plays a key role in initiating and controlling the development of the vertebrate nervous system and most vertebrate organs (1). Identifying inductive signals and the means by which they are transduced are two of the central challenges in developmental biology.

Two general approaches have been used to identify inducing proteins that play a role in amphibian embryogenesis (reviewed in ref. 2). In one approach, explants of embryonic tissue are treated with candidate inducing substances and then analyzed for expression of markers for differentiated tissue (3–5). In a second approach, intact embryos are injected with RNAs encoding putative inducing factors and then examined for alteration of cell fate in specific tissues (6, 7). Although these approaches have been used to identify and characterize a number of mesoderm- and neural-inducing proteins, they both have important limitations. Assays based upon the first approach require purified, functional, and soluble proteins that are frequently difficult to obtain in sufficient quantities. Assays based upon the second approach usually involve expression of the putative inducing proteins in both signaling and responding tissues. This makes it difficult to determine whether observed effects are due to inductive signaling or modulation of intracellular pathways.

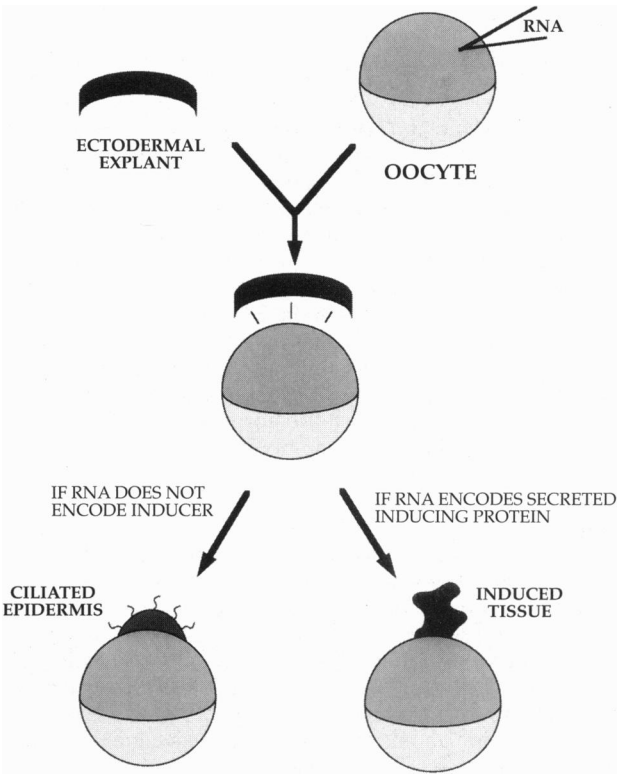

FIG. 1. Principle of the paracrine signaling assay. Synthetic RNA encoding one or more secreted signaling proteins is microinjected into a *Xenopus laevis* oocyte. After a brief incubation period to allow RNA translation, a piece of *X. laevis* embryonic tissue competent to respond to the signaling protein (e.g., an ectodermal explant) is grafted onto the oocyte. The signaling properties of the expressed protein are tested by analyzing gene expression in the grafted tissue.

We have developed a signaling system using separate signaling and responding tissues that overcomes some of the drawbacks of these assays. As shown schematically in Fig. 1, embryonic explants are grafted onto *Xenopus laevis* oocytes that have been injected with RNA encoding one or more putative signaling proteins. The effect of the secreted protein on the fate of the grafted tissue is then determined by harvesting the differentiated graft and assaying expression of developmentally regulated genes. This approach is similar in principle to that taken by many embryologists who have grafted together explants from two different regions of the embryo and then used histology or molecular markers to

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Abbreviations: N/F, Nieuwkoop and Faber; RT, reverse transcriptase; NCAM, neural cell adhesion molecule; EF-1α, elongation factor 1-α.

characterize inductive signaling between the two tissues (for example, see refs. 1 and 8–10). An early attempt to examine inductive signaling between embryonic explants and oocytes injected with activin RNA did not succeed (6).

Using ectodermal explants from *X. laevis* embryos as the responding tissue, we show that the assay reconstitutes paracrine signaling by the vertebrate proteins activin (11) and noggin (12), as well as Wnt (13), which has not yet been purified in active form. We also show that the assay is sensitive enough to be used as a method for cloning neural inducers from a cDNA expression library.

EXPERIMENTAL PROCEDURES

Oocytes and Embryos. *X. laevis* ovarian tissue was surgically isolated, and stage VI oocytes were manually defolliculated (14). Oocytes were cultured at 16°C in modified Barth's solution containing penicillin at 100 units/ml, streptomycin at 100 μg/ml, and bovine serum albumin at 0.1 mg/ml (MBS) (15).

GpppG-capped RNA transcripts encoding *X. laevis* activin B, *X. laevis* noggin [a transcript lacking part of the 5′ untranslated region (nogginΔ5′)], or mouse Wnt-1 were synthesized *in vitro* (16). RNA was injected into the pigment border between the two oocyte hemispheres (17) unless otherwise indicated. Oocytes were usually injected within 1 day of surgical removal and then transferred to fresh MBS and incubated at 16°C.

X. laevis embryos were generated by *in vitro* fertilization, de-jellied, and staged according to Nieuwkoop and Faber (N/F) (18). When embryos reached the blastula (N/F stage 8) or early gastrula (N/F stage 9.5–10) stage, the vitelline membrane was removed and an eyelash scalpel (19) was used to explant presumptive ectoderm.

Grafting of Explants onto Oocytes. Six to 18 hr after RNA microinjection, oocytes were transferred to a 10-cm^2 polystyrene culture dish coated with a 1% agarose bed (prepared in MBS) that contained wells approximately 1.8 mm deep and 1.5 mm in diameter. Four to six oocytes were placed in each dish, which contained 7 ml of bovine serum albumin-free MBS. The ectodermal explant was trimmed on all sides to form an ≈350- to 450-μm square of tissue and then was immediately transferred to the dish containing oocytes. Forceps were used to gently push the explants on top of the oocytes and to orient them so that the pigmented epithelial layer was distal to the oocyte. The explant could be grafted anywhere on the oocyte surface, although in our experience grafts adhered to animal poles somewhat more avidly than to vegetal poles. Sibling embryos were cultured in 0.1× Marc's modified Ringer's solution (MMR) (15) for staging.

Harvesting and Reverse Transcriptase (RT)-PCR Analysis of Grafted Tissues. The oocyte–explant conjugates were cultured at 21°C in MBS until sibling embryos had reached N/F stage 10.5 for brachyury and goosecoid analysis or N/F stage 24–32 for muscle actin and neural cell adhesion molecule (NCAM) analysis. Then the explant was reisolated by gently pinching the oocyte just below the explant with blunt watchmaker's forceps (Dumont no. 3C). Explants were not harvested from oocytes that had become damaged during the incubation period, since dead or dying oocytes alone were occasionally capable of causing mesoderm and neural induction in grafted ectodermal explants (not shown). Oocyte tissue and debris were removed from the explant before it was stored at −80°C. Pooled sets of four to six explants were assayed to normalize for oocyte-to-oocyte variability in protein expression levels. RT-PCR analyses were carried out as described (20, 21). The PCR primer sequences used for analysis of elongation factor 1-α (EF-1α), brachyury, goosecoid, NCAM, muscle actin, and noggin were as described (22, 23).

Library Construction and Subdivision. Hyperdorsalized (dorsoanterior index 8–9) *X. laevis* embryos were generated by LiCl treatment as previously described (24) and harvested when untreated sibling embryos reached the early gastrula stage (N/F stage 10–10.5). Total RNA was isolated from the frozen tissue by using RNAzol B (Tel-Test, Friendswood, TX), and poly(A)$^+$ RNA was selected twice by using the PolyATract Kit (Promega). A directional cDNA library was constructed, following the instructions for the SuperScript Plasmid System (BRL), with slight modifications. cDNA larger than ≈0.5 kb was ligated directionally into the *Eco*RI and *Xba* I sites of the frog expression plasmid pCS2+, and ligation products were introduced into ElectroMAX DH10B *Escherichia coli* cells (BRL) by electroporation. The transformed bacteria were incubated at 37°C for 1 hr before being stored at −80°C.

Samples from the unamplified library were thawed and diluted in 2× YT medium (25). Approximately 100 independent transformants were placed in each miniprep tube and grown overnight at 37°C with shaking. The plasmid DNA isolated from these cultures was linearized with *Not* I and used as template for *in vitro* transcription reactions (16).

RESULTS

Mesoderm Induction by Activin. Explants from the animal hemisphere of blastula-stage *X. laevis* embryos can be respecified from an ectodermal fate to a mesodermal fate by treatment with purified inducing proteins such as activin (26–28), a member of the transforming growth factor β family of secreted signaling proteins (11). *X. laevis* oocytes have been shown previously to translate microinjected RNA encoding activin and to secrete an active form of the protein (22). Thus to determine whether the paracrine assay can reconstitute mesoderm induction, we grafted ectodermal explants from blastula stage embryos onto oocytes that had been injected with activin RNA. The explants were later detached and assayed by RT-PCR for expression of the mesodermal markers muscle actin (9), brachyury (29), and goosecoid (30), or the pan-neural marker NCAM (31). The expression of mesodermal or neural markers would indicate that proteins secreted by the oocyte had altered the developmental fate of the grafted explants.

Explants initially adhered tightly to the oocyte, forming an intimate contact. By the tailbud stage of sibling development, explants grafted onto control oocytes frequently became partially detached from the oocyte, whereas explants grafted onto activin RNA-injected oocytes generally remained tightly adherent. Ninety-seven percent (33/34 explants) of the blastula explants grafted onto uninjected oocytes partially rounded up (Fig. 2*A*) and produced beating cilia, characteristic behaviors of ectodermal explants cultured alone. By contrast, dramatic tissue elongation was observed in 97% (33/34 explants) of blastula stage explants grafted onto oocytes injected with 50 pg of activin RNA (Fig. 2*B*). The morphological response to activin was quite varied, with some explants forming a single elongated process and others extending multiple processes of different sizes and shapes.

RT-PCR analysis showed that mesodermal marker genes were induced in explants grafted onto activin-expressing oocytes (Fig. 2*C*). Blastula-stage explants grafted onto oocytes injected with 5 pg of activin RNA expressed brachyury, a pan-mesodermal marker. With higher doses of activin RNA, goosecoid and muscle actin were also induced and brachyury expression was submaximal. NCAM transcripts were also occasionally detected (Fig. 2*C*), probably reflecting the ability of induced dorsal mesoderm to itself induce neural tissue (32). Blastula-stage explants grafted onto control oocytes generally failed to express these marker genes, although brachyury and muscle actin were occasionally expressed at low levels. RT-PCR analysis of the ubiquitously expressed EF-1α gene (33) showed that comparable amounts of RNA were isolated from each pooled set of explants.

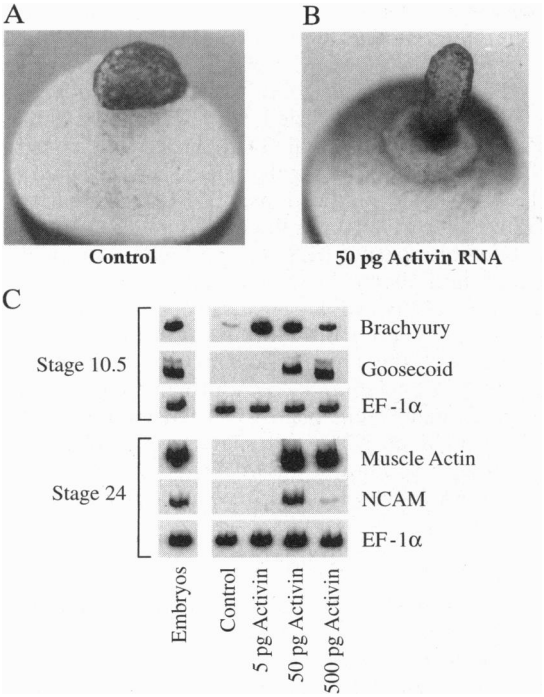

FIG. 2. Mesoderm induction by activin. Ectodermal explants from blastula stage embryos were grafted onto uninjected control oocytes (for *A*) or onto oocytes injected with 50 pg of activin RNA (for *B*) or the indicated amount of activin RNA (for *C*). For *A* and *B*, the oocyte–explant conjugates were photographed when sibling embryos reached the early tailbud stage. (×23.) For *C*, explants were detached from the oocytes when sibling embryos reached the indicated stage, and then assayed by RT-PCR for expression of the indicated genes.

Neural Induction by Noggin. Gastrula-stage ectodermal explants can be respecified from an epidermal fate to a neural fate by treatment with purified noggin protein (34). To determine whether the paracrine assay can reconstitute neural induction, we grafted ectodermal explants from early gastrula-stage embryos onto noggin RNA-injected oocytes and then assayed the grafted tissue for expression of NCAM and muscle actin. *X. laevis* oocytes have been shown previously to translate microinjected RNA encoding noggin and to secrete an active form of the protein (35). We used a mutant of the noggin gene (nogginΔ5′) that lacks most of the 5′ untranslated region but is more effective at rescuing UV-ventralized embryos than is the full-length gene (36).

Gastrula-stage explants grafted onto noggin-expressing oocytes expressed transcripts encoding NCAM (Fig. 3*A*) and, occasionally, muscle actin (not shown). NCAM expression occurred in the absence of muscle actin formation in 13 of 15 experiments (118 explants tested), indicating that neural induction by noggin was not a consequence of dorsal mesoderm induction. The lack of mesoderm induction by noggin was not simply due to a loss of competence to form mesoderm, since muscle actin was still induced by activin in early gastrula-stage explants (Fig. 3*A*). As little as 0.2 ng of nogginΔ5′ RNA was sufficient to induce NCAM. Control gastrula-stage explants failed to express NCAM or muscle actin.

Ninety-seven percent (88/91) of gastrula-stage ectodermal explants grafted onto uninjected oocytes partially rounded up (Fig. 3*B*) and produced beating cilia. By contrast, morphological changes were observed in 85% (88/103) of gastrula-stage explants grafted onto oocytes injected with 1 or 2 ng of nogginΔ5′ RNA (Fig. 3*C*). Most explants formed a single process that elongated away from the noggin-expressing oocytes. Several distinct regions were typically observed within each process: a region proximal to the oocyte that contained

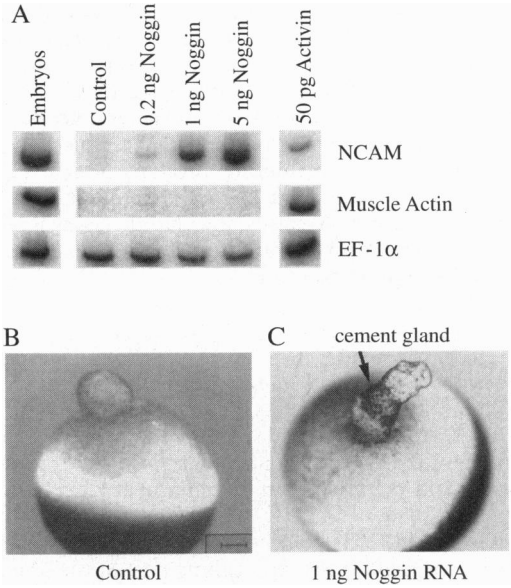

FIG. 3. Neural induction by noggin. Ectodermal explants from early gastrula-stage embryos were grafted onto uninjected control oocytes or oocytes injected with the indicated dose of nogginΔ5′ or activin RNA. For *A*, explants were detached from the oocytes when sibling embryos reached the early tailbud stage, and then assayed by RT-PCR for expression of the indicated genes. For *B* and *C*, the oocyte–explant conjugates were photographed when sibling embryos reached the early tailbud stage. (Scale bar = 200 μm.)

various amounts of unpigmented cells; a ring of darkly pigmented tissue located more distally at various positions along the process (for example, see arrow in Fig. 3*C*); and, furthest from the oocyte, a region of pigmented tissue indistinguishable from control ectoderm. When sibling embryos reached the late tailbud stage, the region of darkly pigmented tissue began secreting cement, indicating that noggin had induced the formation of the cement gland, an organ derived from anterior ectoderm (18).

Contact between the explant and the oocyte was required for mesoderm induction by activin and neural induction by noggin; explants placed ≈3 mm away from activin- or noggin-expressing oocytes rounded up, formed beating cilia, and did not elongate or extend processes. Morphogenetic movements occurred whether explants were grafted onto the animal pole or vegetal pole of the oocyte, and whether activin RNA or noggin RNA was injected into the animal pole, vegetal pole, or pigment boundary of the oocytes (not shown).

Paracrine Signaling by Wnt. The lack of a soluble preparation of vertebrate Wnt protein has hampered efforts to determine the role of members of the Wnt family of secreted proteins in neural induction and patterning (13). To determine whether the paracrine assay can be used to study signaling by Wnt, we grafted blastula- or gastrula-stage ectodermal explants onto oocytes injected with RNA encoding mouse Wnt-1 and then assayed the grafted tissue for expression of neural markers. Mouse Wnt-1 has been shown to be functionally indistinguishable from a variety of *X. laevis* Wnt family members (13).

Neither NCAM nor cement gland was induced in explants grafted onto oocytes injected with 2 ng of Wnt RNA (Table 1; data not shown). Gastrula-stage explants grafted onto oocytes coinjected with noggin and Wnt RNA, however, formed fewer cement glands than did explants grafted onto oocytes injected with noggin RNA alone (Table 1). This inhibitory effect of Wnt was more pronounced in ectoderm explanted from gastrula-stage embryos than in ectoderm explanted from blastula stage embryos (Table 1). The effect of Wnt on NCAM induc-

Table 1. Wnt inhibits cement gland induction by noggin

RNA injected	% with cement gland (no. positive/no. assayed)	
	N/F stage 8 explants	N/F stage 10 explants
None	4 (1/26)	4 (2/52)
Wnt	0 (0/25)	0 (0/45)
Noggin	100 (25/25)	79 (41/52)
Noggin and Wnt	68 (17/25)	16 (10/62)

Ectodermal explants from blastula- or gastrula-stage embryos were grafted onto uninjected oocytes or onto oocytes injected with 1 ng of nogginΔ5′ RNA, 2 ng of Wnt-1 RNA, or both. When sibling embryos reached the early tailbud stage, the explants were visually scored for the presence of cement gland.

tion by noggin was quite variable; in some experiments, Wnt had little or no effect, in others Wnt partially or completely inhibited NCAM induction (not shown).

Expression Cloning. We used the assay in a screen for secreted proteins capable of causing neural induction in gastrula-stage ectodermal explants. We constructed a plasmid expression library from *X. laevis* embryos treated with LiCl, which induces excessive anterodorsal development (37, 38). The expression library, maintained in bacteria, was subdivided into pools of ≈100 plasmids, which were used as template for RNA synthesis *in vitro*. Gastrula-stage ectodermal explants were grafted onto oocytes injected with ≈100 ng of these pooled transcripts and later assayed by RT-PCR for expression of NCAM and muscle actin. Of the 200 pools screened (i.e., 20,000 independent transformants), we identified two pools that induced morphogenetic movements, cement gland formation, and NCAM expression, but not muscle actin expression (not shown). Since these effects were identical to those obtained by injection of nogginΔ5′ RNA, and since LiCl-dorsalized embryos are known to overexpress noggin (36), we used PCR analysis to determine whether the active cDNA pools contained the noggin gene. A PCR product corresponding to an ≈0.3-kb region of the noggin gene [position 402–679 (36)] was amplified from both active pools but not from 10 other randomly selected library pools (not shown).

The two active cDNA pools were subdivided into smaller pools, which were then reassayed by PCR for noggin; this process was repeated until a single clone was isolated from each pool. We sequenced an ≈0.23-kb region of each active 1.8-kb cDNA and found the nucleotide sequence to be 100% identical to the published noggin sequence. From these results we conclude that both positive pools contained the noggin gene previously cloned by Smith and Harland (36).

DISCUSSION

We have developed a paracrine signaling assay that involves injection of RNA encoding a signaling protein into an *X. laevis* oocyte, which is then placed in direct contact with an explanted responsive tissue. The signaling properties of the expressed protein are tested by analyzing gene expression in the grafted tissue. This method has several advantages over existing assays that are used to examine inductive signaling. First, the choice of signaling protein is extremely wide. *X. laevis* oocytes contain large reserves of cellular components needed for protein synthesis and processing, and they have been shown to translate RNA encoding many plasma membrane-associated and secreted proteins. Second, the choice of responding tissue is wide. Manually defolliculated *X. laevis* oocytes are quite adherent and can be grafted to tissues explanted from many different regions of an *X. laevis* embryo at various stages of development (unpublished results). Third, changes in a tissue's competence to respond to a signaling protein can be measured simply by explanting the responding tissue at different stages of embryonic development. Fourth, signaling by vertebrate proteins such as Wnt that have not yet been purified in active form can be studied. Finally, only non-cell-autonomous effects of a signaling protein are measured, since the responding tissue is distinct from the signaling tissue, and no cell mixing occurs.

The assay faithfully reconstitutes mesoderm induction by activin and neural induction by noggin. Ectodermal explants grafted onto activin- or noggin-expressing oocytes elongate and express the same molecular markers (Figs. 2 and 3) as explants treated with purified activin (29, 30, 39) or noggin (34). Contact between oocyte and explant was required for induction, suggesting that induction is not simply a result of an elevated concentration of activin and noggin protein in the medium. Besides reducing the distance a secreted protein must diffuse to activate its signaling system in the responding tissue, intimate contact may also elevate the concentration of secreted protein in a microenvironment formed between the oocyte and the explant. By serving as a continuous source of secreted protein, the oocyte may even be able to generate a gradient of protein in the responding tissue, although we have no direct evidence to support this idea. The tight adherence between explant and oocyte may also keep the explant from rounding up, forming an impermeable epidermis, and thereby becoming unresponsive to soluble inducing factors.

Our findings also show that the assay can be used to characterize signaling by Wnt, which inhibits cement gland induction by noggin (Table 1) but by itself has no effect on cement gland or neural marker expression. Since noggin may be the endogenous inducer of cement gland and anterior neural tissue (12, 34), these results may explain why ectopic expression of Wnt after the mid-blastula transition (by DNA injection) inhibits cement gland formation and forebrain development in intact *X. laevis* embryos (40). By correlating the extent of anterior deficiency with the site of Wnt DNA injection in the embryo, it was concluded that Wnt did not act directly on neurectoderm but instead altered the signaling properties of underlying head mesoderm (40). Our results suggest that Wnt can act directly on presumptive neurectoderm to attenuate its response to neuralizing signals, although we have not ruled out the possibility that Wnt affects intracellular pathways in the oocyte that, for example, might inhibit noggin secretion or lead to the release of endogenous bioactive proteins.

We have demonstrated that the paracrine assay can be used in an expression cloning approach to isolate cDNAs encoding neural inducers. Although we cloned noggin, which had previously been cloned (36), our results demonstrate the feasibility of using the paracrine assay to identify novel neural inducers on the basis of functional activity. To our knowledge, no other approach has previously been used successfully to directly isolate a neural inducer (12). Noggin was originally cloned on the basis of its ability to rescue axial structures in UV-ventralized embryos (36); it was only subsequently shown to have neural-inducing properties as well (34). In principle the expression cloning approach described here is not limited to identifying proteins that induce neural tissue in *X. laevis*, but it can be used to identify secreted proteins that alter gene expression in any species whose tissues can be grafted onto the oocyte and cultured under compatible conditions.

The paracrine signaling assay we have developed should be a valuable tool both in the characterization of known signaling proteins and in the identification of novel signaling proteins. By obviating the need for protein purification, the assay represents a generally applicable approach for isolating cDNAs encoding secreted signaling proteins that is based solely on their biological activity.

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