

Neural and Head Induction by Insulin-like Growth Factor Signals

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Summary

Evidence is presented for a new pathway participating in anterior neural development. It was found that IGF binding protein 5 (IGFBP-5), as well as three IGFs expressed in early embryos, promoted anterior development by increasing the head region at the expense of the trunk in mRNA-injected *Xenopus* embryos. A secreted dominant-negative type I IGF receptor (DN-IGFR) had the opposite effect. IGF mRNAs led to the induction of ectopic eyes and ectopic head-like structures containing brain tissue. In ectodermal explants, IGF signals induced anterior neural markers in the absence of mesoderm formation and DN-IGFR inhibited neural induction by the BMP antagonist Chordin. Thus, active IGF signals appear to be both required and sufficient for anterior neural induction in *Xenopus*.

Introduction

The induction of neural tissue is a fundamental event in vertebrate development. Studies in *Xenopus* have led to the important conclusion that Spemann's organizer, located in the dorsal side of the gastrula, is a source of secreted neural inducers such as Chordin, Noggin, and Follistatin. These proteins are able to induce the formation of neural tissue in *Xenopus* ectodermal explants, and act by binding to bone morphogenetic proteins (BMPs) and antagonizing their activity (reviewed by De Robertis et al., 2000). Overexpression of BMP-4 can counteract the activity of Chordin, Noggin, and Follistatin in ectodermal explants, causing them to retain epidermal fates (Sasai and De Robertis, 1997). These experiments in *Xenopus* have led to the current view that neural differentiation depends on inhibition of BMP signaling by factors secreted by the organizer (Wilson and Hemmati-Brivanlou, 1997; Sasai and De Robertis, 1997; Harland, 2000). In zebrafish, *chordin* mutants have a reduced neural plate, but a central nervous system (CNS) is still formed (Schulte-Merker et al., 1997). In the mouse, mutants that lack Hensen's node, the structure homologous to the amphibian organizer, still develop a neural plate (Klingensmith et al., 1999). Mouse mutants lacking Chordin or Noggin initially form a neural plate, although in double mutants, the anterior neural plate fails to develop at later stages (Bachiller et al., 2000). Thus, it seems likely that other factors in addition to BMP antagonists participate in the induction of CNS.

The organizer region secretes a number of other antagonists of growth factor signaling, including Wnt inhibitors (such as Frzb-1, sFRP-2, Crescent, and Dickkopf-1) and the multivalent inhibitor Cerberus (De Robertis et al., 2000). Inhibition of Wnt signaling at the gastrula stage plays an important role in the process of head induction (Glinka et al., 1997, 1998). Wnt inhibitors on their own can induce a preneural state in animal cap explants, but are unable to induce ectopic CNS in the embryo and require the simultaneous inhibition of BMP signals to generate head structures (Glinka et al., 1997, 1998; Piccolo et al., 1999). In the chick epiblast, inhibition of Wnt signaling promotes neural fates at the expense of epidermal differentiation (Wilson et al., 2001). Overexpression of Cerberus is able to induce ectopic anterior CNS in *Xenopus* embryos through the simultaneous inhibition of BMP, Wnt, and Nodal signaling (Bouwmeester et al., 1996; Piccolo et al., 1999).

Prior to these events taking place during gastrulation, earlier signals participate in neural induction (Harland, 2000; Stern, 2001). In *Xenopus*, the cortical rotation triggered by fertilization leads to an increase in nuclear β -catenin on the dorsal side, which causes inhibition of *BMP-4* transcription (Baker et al., 1999) and the early expression of BMP antagonists in the preorganizer region of the late blastula (Wessely et al., 2001), predisposing the ectoderm toward neural fates. Recent experiments in the chick embryo have shown that early FGF signals are required for CNS formation (Streit et al., 2000; Wilson et al., 2000; Stern, 2001). There is also evidence suggesting that FGF signaling participates in neural induction and patterning in *Xenopus* embryos (reviewed by Harland, 2000). In addition, the molecular nature of "heterologous" neural inducers studied in the past remains a mystery. One of the most mysterious is the archencephalic (or forebrain) inducer, which was studied for several decades (reviewed by Saxén and Toivonen, 1962). Guinea pig liver protein extracts induce forebrain structures when placed as an insoluble pellet inside a sandwich of *Triturus* animal cap explants. Other tissues, such as guinea pig bone marrow or kidney caused posterior neural inductions (called deuterencephalic and spinocaudal inductions), and were later found to contain mesoderm inducers in addition to neuralizing activity. Despite 50 years of work on the liver archencephalic inducer (Gilbert, 2001), all we know today is that it was thermostable, resistant to alcohol treatment, and probably of small molecular weight since it was dialyzable (Saxén and Toivonen, 1962). It seems unlikely that any of the presently known neural inducers would explain the guinea pig liver inductive activity. In conclusion, research in neural induction, both new and old, suggests the involvement of multiple pathways, raising the question of whether additional signals remain to be discovered.

In the present study, we introduce insulin-like growth factors (IGFs) as new players in neural and head induction. A direct screen for proteins secreted by the frog

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gastrula resulted in the isolation of the *Xenopus* homolog of IGF binding protein 5 (IGFBP-5). IGFBPs are extracellular modulators of IGF signaling that bind IGFs with very high affinity (Clemmons, 1997). (For example, human IGFBP-5 binds IGF-2 with a dissociation constant of 8×10^{-11} M; Kalus et al., 1998). We found that microinjection of *IGFBP-5* mRNA resulted in *Xenopus* embryos with enlarged head structures and cement glands. Epistatic studies using a dominant-negative type I IGF receptor (DN-IGFR) indicated that the IGFBP-5 phenotype was mediated by an increase of endogenous IGF signaling. Three IGFs were found to be expressed in the early frog embryo. IGF-1 and -2, as well as a novel molecule described here and designated IGF-3, are provided maternally in the *Xenopus* egg. Overexpression of any of these IGFs resulted in embryos with enlarged anterior structures, and microinjection of DN-IGFR led to the opposite result. Microinjected IGF mRNAs caused the formation of ectopic eyes and ectopic head-like structures. In ectodermal explants, IGF signaling was both sufficient and required for anterior neural induction. The results suggest a role for IGF signals in CNS formation.

Results

IGFBP-5 Promotes Anterior Development

A search for proteins secreted by the *Xenopus* gastrula stage embryo was carried out by transfecting pools of 16 cDNAs into human 293T cells and detecting radiolabeled proteins secreted into the culture medium (Pera and De Robertis, 2000). This method, called secretion cloning, offers the advantage of resulting in full-length clones from which synthetic mRNAs for microinjection can be readily prepared. Fifty cDNA clones encoding previously known secreted proteins were recovered, and one of them corresponded to the *Xenopus* IGFBP-5 homolog. Two unique protein bands of 38 kDa and 18 kDa were detected by SDS-PAGE in the supernatant of 293T cells transfected with *IGFBP-5* and labeled with ³⁵S-methionine and -cysteine (Figure 1A). The sequence of the cDNA clone revealed similarity to human IGFBP-5 (Figure 1C). IGFBP-5 and other members of the IGFBP family share an N-terminal IGF binding domain that contains the conserved sequence GCGCCXXC (Figure 1B). The C terminus of IGFBP-5 has homology to the thyroglobulin type I domain and is known to bind heparin. The central region is weakly conserved (Figure 1C) and is subject to cleavage by IGFBP proteases (Clemmons, 1997). IGFBP protease activity in the transfected cells may account for the occurrence of 18 kDa fragments in addition to the full-length 38 kDa xIGFBP-5 (Figure 1A).

Whole-mount in situ hybridization of early *Xenopus* embryos detected abundant maternal transcripts of the *xIGFBP-5* gene at the 4-cell stage (Figure 1D). During gastrulation, expression was observed on the dorsal side (data not shown), and during neurulation, it became confined to the floor plate, notochord, and dorsal endoderm (Figures 1E and 1F). At tailbud stages, additional expression domains were seen in cranial nerves, ear vesicle, dorsal fin, and somites (Figure 1G). Dorsal midline expression became restricted to the floor plate and hypochord (Figure 1H). This expression in dorsal midline signaling centers suggested the possibility that *xIGFBP-5*

might have signaling activity. We found that injection of *xIGFBP-5* mRNA into the animal pole of each blastomere at the 4-cell stage caused a striking phenotype. The injected embryos had greatly enlarged head structures and cement glands (Figures 1I and 1J). The fore- and midbrain marker *Otx-2* was expanded after a single dorsal injection of *IGFBP-5* mRNA (Figures 1K–1M). The results suggested that *xIGFBP-5* promotes anterior development.

IGFBP-5 Activates IGF Signaling

IGFBP-5 can act both as an IGF antagonist (Stewart and Rotwein, 1996; Kalus et al., 1998) and as an agent that promotes signaling by IGFs (Jones et al., 1993). To explore the mechanism by which xIGFBP-5 promoted anterior development, we first cloned *Xenopus* IGFs. xIGF-1 has been described previously (Kajimoto and Rotwein, 1990), and an EST was found for xIGF-2; both coding regions were amplified by PCR and expression constructs were prepared. In addition, we identified a new *Xenopus* IGF gene. Only two IGFs have been described in mammals; xIGF-3 is a novel and distinct member of this family (Figures 2A–2C). The mature xIGF-3 protein consists of 72 amino acids and is most similar to xIGF-2 and xIGF-1 (47% and 44% amino acid identity, respectively) and more distantly related to xinsulin (32% identity; Figure 2B). The predicted N-terminal signal peptide of xIGF-3 is unusually long (49 amino acids). An arginine residue at the C-terminal proteolytic processing site is conserved in all members of the IGF family (Figure 2C).

The expression of the *xIGF* genes was analyzed by whole-mount in situ hybridization of *Xenopus* embryos. In all cases, maternal transcripts could be detected at the 4-cell stage (Figure 2D). Zygotic expression of *IGF-1* did not occur before the tailbud stage and was restricted to the developing heart (data not shown). *IGF-2* and *IGF-3* were expressed in the dorsal midline during gastrulation and neurulation, in a pattern overlapping with *IGFBP-5* (Figures 2E and 2F). *IGF-3* was strongly expressed in the prospective ventral forebrain region of the anterior neural plate (Figure 2F). In addition, we examined the expression of the *Xenopus* type I IGF receptor (*xIGFR*) gene (Zhu et al., 1998; Figure 2G). At early stages, *xIGFR* transcripts were detected ubiquitously (data not shown) and then became stronger along the dorsal midline at neurula stage (Figure 2G). In sum, several members of the IGF signaling system were expressed as maternal mRNA and in the dorsal midline, including the anterior region of the embryo.

To investigate the activities of the IGF ligands, we injected mRNA encoding *xIGF-1*, *xIGF-2*, or *xIGF-3* into the animal pole of 4- to 8-cell stage *Xenopus* embryos. At tailbud stage, the IGF-injected embryos exhibited the same anteriorized phenotype as seen for *IGFBP-5* (Figures 2H–2J). This suggested that the IGFBP-5 phenotype could be due to an increase in endogenous levels of IGF signaling.

To test whether IGFBP-5 anteriorizes embryos through IGF signals, we needed to block IGF signaling. A construct designated *hIGF-1R-486/Stop* encodes a secreted human type I IGF receptor devoid of its transmembrane and intracellular tyrosine kinase domain that

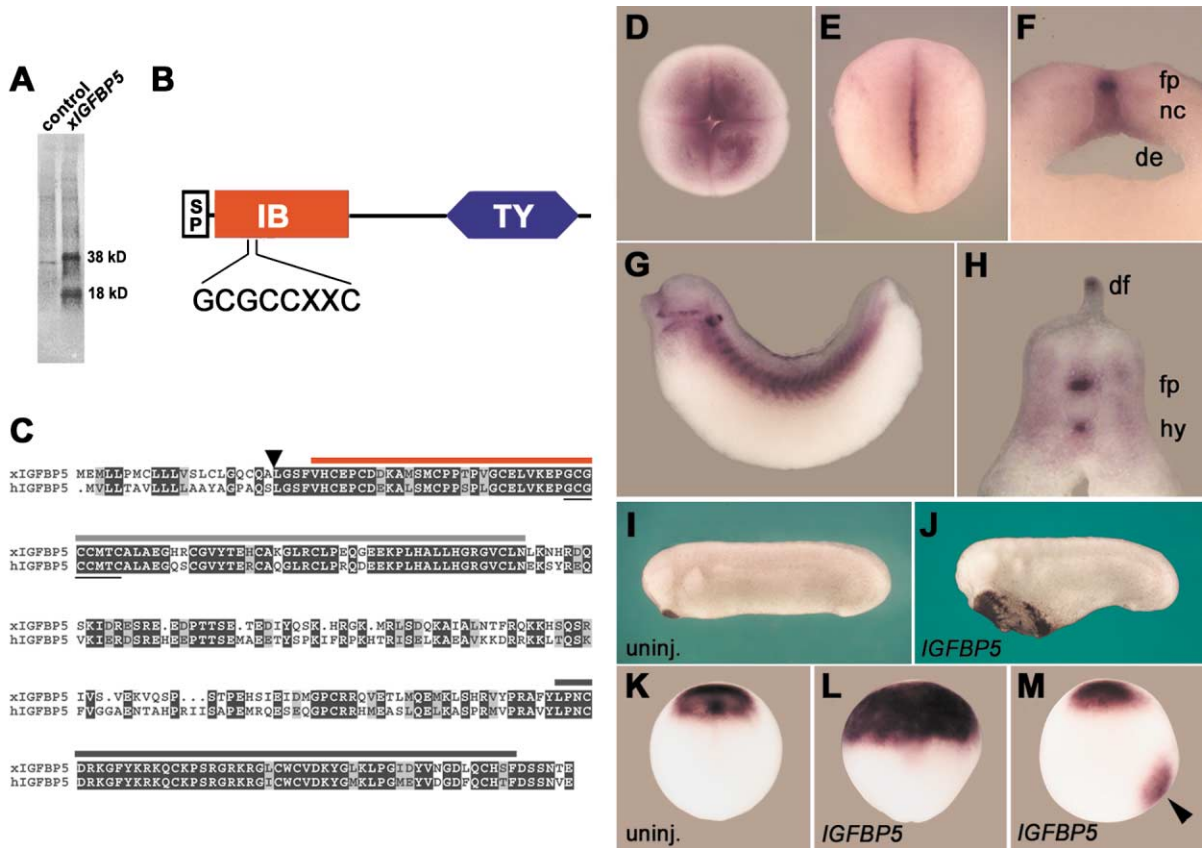


Figure 1. IGFBP-5 Is Expressed Dorsally and Has Anteriorizing Activity

(A) SDS-PAGE of conditioned medium from 293T cells labeled with ^{35}S -cysteine and -methionine and transfected with GFP (control) or *xIGFBP-5* cDNA. Protein bands at 38 kDa and 18 kDa correspond to *xIGFBP-5* full-length protein and proteolytic cleavage products, respectively.

(B) Schematic structure of IGFBP-5. SP, N-terminal signal peptide; IB, IGF binding domain; TY, thyroglobulin type I repeat. The GCGCCXXC sequence critical for IGF binding (Kalus et al., 1998) is indicated.

(C) Sequence alignment of *Xenopus* and human IGFBP-5 proteins. The arrowhead indicates the predicted signal peptide cleavage site and the red and blue bars indicate the IG and TY, respectively.

(D–H) Whole-mount in situ hybridization of *xIGFBP-5* transcripts.

(D) Animal view at the 4-cell stage.

(E) Dorsal view at neural plate stage showing *xIGFBP-5* expression in the dorsal midline.

(F) Transverse section of the embryo at neural plate stage; expression is seen in floor plate (fp), notochord (nc), and dorsal endoderm (de).

(G) Lateral view of tailbud tadpole.

(H) Transverse section of tailbud embryo showing *xIGFBP-5* expression in dorsal fin (df), floor plate (fp), and hypochochord (hy).

(I) Uninjected control.

(J) Embryo radially injected with a total of 400 pg *xIGFBP-5* mRNA into the animal pole of embryos at the 4- to 8-cell stage (93%, $n = 106$). Note the enlargement of the cement gland.

(K) Uninjected embryo stained for *Otx-2* at neurula stage.

(L) Sibling injected dorsally with 100 pg of *IGFBP-5* mRNA.

(M) Ectopic expression of *Otx-2* after ventral injection of 100 pg *IGFBP-5* mRNA.

was shown to efficiently and specifically block IGF-1 and IGF-2 signal transduction (D'Ambrosio et al., 1996). We constructed a *Xenopus* equivalent of this molecule, generating a secreted dominant-negative *xIGF-1R* (DN-IGFR) protein. Injection of *DN-IGFR* mRNA resulted in a reduction of anterior structures, including the cement gland (Figure 2K). As expected, *DN-IGFR* mRNA was able to block the anteriorizing effect of *IGF-1* (Figures 2H and 2L). Importantly, *DN-IGFR* also blocked the anteriorizing effects of *IGFBP-5* mRNA (Figure 2M), consistent with the view that IGFBP-5 promotes anterior development by stimulating signaling by endogenous IGFs via IGF receptors.

IGF and the Head/Trunk Boundary

We next analyzed the effects of DN-IGFR in embryos at various developmental stages. Injection of *DN-IGFR* mRNA led to a reduction or complete loss of cement glands and eyes at the swimming tadpole stage (Figure 3B). At the neurula stage, expression of the anterior markers *Otx-2* (a marker for cement gland, anterior mesendoderm, forebrain, and midbrain), *XAG-1* (cement gland), and *Rx2a* (eyes) was severely reduced by *DN-IGFR* mRNA (Figures 3C–3H).

To assess the involvement of IGF signaling in the location of the head/trunk boundary, we injected either *IGF-2* or *DN-IGFR* mRNA into a single blastomere at the

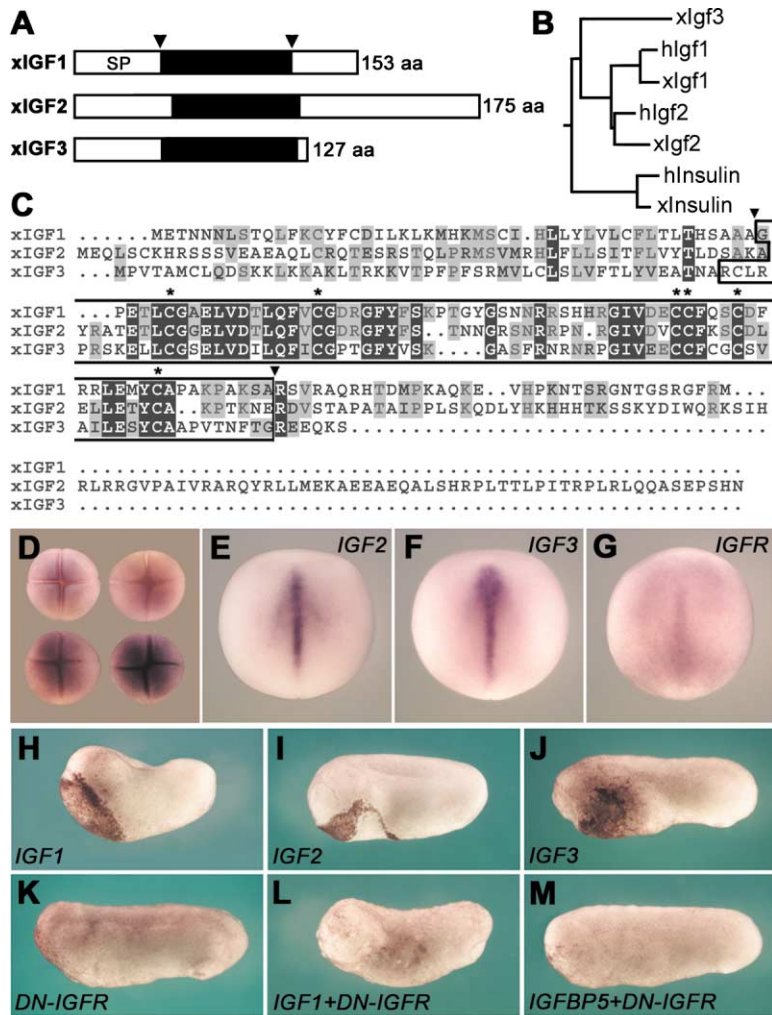


Figure 2. IGFBP-5 Enhances IGF Signaling

(A) Three IGF preproteins expressed in *Xenopus* embryos; the mature IGFs are indicated in black.

(B) Dendrogram showing sequence similarities between *Xenopus* and human members of the IGF/insulin family.

(C) Sequence alignment of xIGF-1 to -3 preproteins. The mature proteins are boxed and the conserved cysteine residues are indicated.

(D) Maternal transcripts at the 4-cell stage for *IGF-1* and *IGF-2* (top row) and *IGF-3* and *IGF type 1* receptor (bottom row).

(E-G) Expression of *IGF-2*, *IGF-3*, and *IGF type 1* receptor (*IGFR*), shown in dorsal view at late gastrula stage. Note the common expression domains in the dorsal midline.

(H-J) Anteriorized embryos at the tailbud stage obtained by injection of *xIGF-1* mRNA (97%, n = 71) (H), *xIGF-2* mRNA (85%, n = 46) (I), and *xIGF-3* mRNA (69%, n = 13) (J) into each animal blastomere at the 4- to 8-cell stage.

(K) Loss of anterior structures by injection of *DN-IGFR* mRNA (97%, n = 74). Note the reduction of the pigmented cement gland.

(L) *DN-IGFR* mRNA abrogates the activity of *IGF-1* mRNA (90%, n = 21).

(M) *DN-IGFR* blocks the anteriorizing effect of *IGFBP-5* mRNA. *IGFBP-5* promotes anterior development through IGF receptor. Amounts of mRNA injected per blastomere were: *IGF-1*, 100 pg; *IGF-2*, 200 pg; *IGF-3*, 400 pg; *DN-IGFR*, 500 pg.

IGFBP5 → IGF1-3 → IGFR → Anterior

4- to 8-cell stage together with nuclear *lacZ* mRNA to mark the injected cells. *Six-3* (an anterior neural plate marker) was expanded by *IGF-2* and reduced by *DN-IGFR* on the injected side (Figures 3I-3K). The *HoxD1* probe marks the trunk, and its anterior border is located at the level of rhombomere 4. This border was displaced posteriorly by *IGF-2* and anteriorly by *DN-IGFR* (Figures 3L-3N). The number of injected nuclei marked by *LacZ* were comparable whether injected with *lacZ* mRNA alone or coinjected with *IGF-2* or *DN-IGFR*. Thus, despite a slight increase in apoptosis seen with *DN-IGFR* in TUNEL assays (data not shown), the observed phenotypes were due to changes in cell fates and not to variations in cell proliferation or death. We conclude that IGF signals regulate the position of the head/trunk boundary, promoting head development at the expense of trunk formation.

IGFs Induce Ectopic Eyes and Heads

IGF mRNA has a robust anteriorizing activity. When *IGF-1* or *IGF-2* was injected at the 4- to 8-cell stage into a dorsal blastomere in the region fated to become neural

plate, multiple ectopic eyes were formed (Figure 4A). Ectopic expression sites of the eye molecular markers β -*crystallin*, *Rx2a*, and *Pax-6* were observed (Figures 4B and 4C and data not shown). Histological analyses of 7 day tadpoles showed that the ectopic eyes had a typical multilayered neural retina, retinal pigment epithelium, and in some cases, a lens (Figures 4D and 4E). Ectopic eyes were observed with *IGF-1* (66%, n = 93) and *IGF-2* (29%, n = 56), but not with *IGF-3* or *IGFBP-5* mRNA microinjections. The ectopic eye phenotype is similar to that caused by microinjection of *Pax-6* mRNA (Chow et al., 1999) and indicates a significant anteriorizing activity.

An additional and striking anteriorizing activity of IGFs was revealed by ventral injections into the marginal zone. *IGF-2* mRNA caused the formation of secondary head-like structures containing a cement gland (21%, n = 296) and in some cases, ectopic eyes (7%; Figures 5B, 5E, and 5G). Coinjection of *IGF-2* and *IGFBP-5* mRNAs also induced ectopic heads (63%, n = 60), which had a higher frequency of eye structures (17%; Figures 5D and 5F). In some embryos, a second heart was seen beating close to the base of the ectopic head after 3

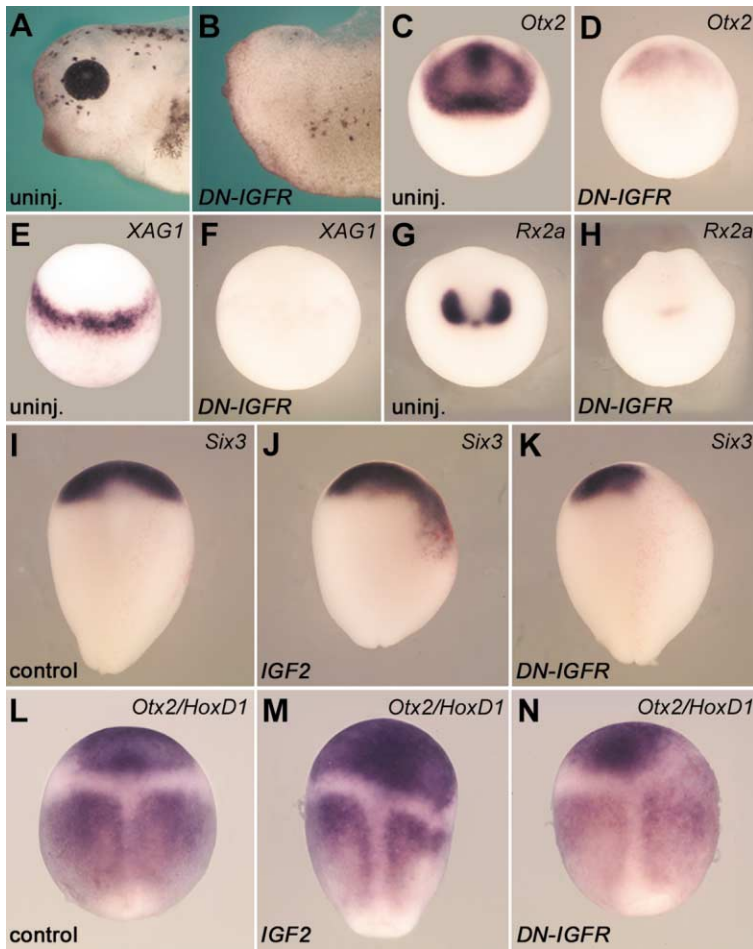


Figure 3. IGF Signaling and the Head/Trunk Boundary

(A) Uninjected 3 day tadpole in lateral view. (B) Embryo injected with 500 pg of *DN-IGFR* mRNA per animal blastomere at the 4- to 8-cell stage showing reduction of cement gland and eye structures (92%, n = 48). (C-H) The domains of expression of *Otx-2* (head), *XAG-1* (cement gland), and *Rx2a* (eye) markers are reduced by *DN-IGFR* mRNA. (I-N) Dorsal views of neurula stage embryos injected into one blastomere at the 4-cell stage with nuclear *lacZ* mRNA (note red nuclei). In situ hybridization was performed at neurula stage with probes for *Six-3* (I-K) or a combination of *Otx-2* and the trunk marker *HoxD1* (L-N). *IGF-2* (400 pg) shifts the head/trunk border caudally (n = 25) and *DN-IGFR* mRNA (500 pg) displaces it anteriorly (n = 15). *IGF-2* and *DN-IGFR* mRNA injections do not significantly change the number of nuclear LacZ-labeled nuclei.

days of development (data not shown). Microinjection of *IGFBP-5* mRNA alone induced protrusions containing cement glands and small patches of neural tissue (42%, n = 40) that lacked ectopic eyes (Figure 5C and data not shown). Histologically, the ectopic head-like structures

induced by *IGF-2* mRNA (with or without *IGFBP-5*) contained ectopic brain tissue, olfactory placodes, and eyes (Figures 5E and 5F). In embryos with the strongest phenotypes, multiple eyes containing well-differentiated neural retina (nr) and retinal pigment epithelium (rpe)

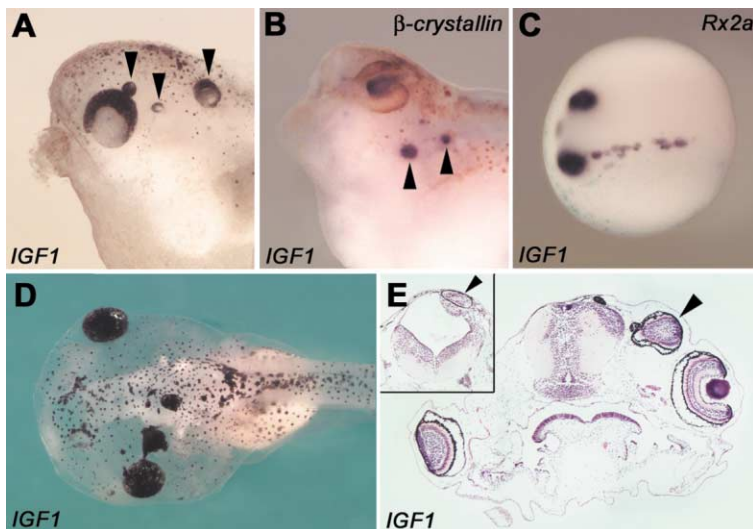


Figure 4. IGF Induces Ectopic Eyes

All embryos were injected in a single dorsal animal blastomere at the 4- to 8-cell stage with 400 pg of *IGF-1* mRNA. (A) Three ectopic eyes in the head region of a 3 day tadpole. (B) Lenses marked by β -crystallin mRNA in ectopic eyes induced by *IGF-1*. (C) Row of multiple positive structures marked by the eye-specific marker *Rx2a* at the neural plate stage. (D) Two ectopic eyes induced by *IGF-1* in the dorsal head region of a 7 day tadpole. (E) Histological section of ectopic eyes (arrowheads).

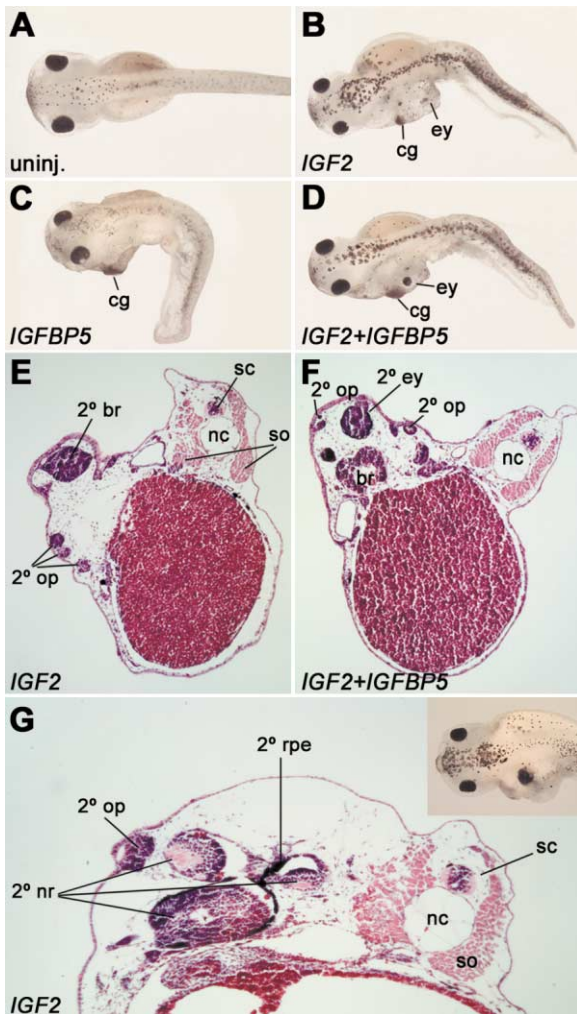


Figure 5. IGF Signaling Induces Ectopic Head-like Structures
 (A) Uninjected tadpole.
 (B) Ectopic head-like structure in an embryo injected with *IGF-2* mRNA. cg, secondary cement gland; ey, ectopic eye.
 (C) Ectopic protrusion with a cement gland induced by *IGFBP-5* mRNA.
 (D) Coinjection of *IGF-2* and *IGFBP-5* mRNA induced secondary heads containing a cement gland and a large eye.
 (E) Transverse section of the embryo in (B); ectopic brain tissue (br) and nasal placodes (op) are seen.
 (F) Cross-section of the embryo in (D) showing ectopic eye, brain, and olfactory placodes.
 (G) Section of the severely affected *IGF-2* mRNA-injected embryo shown in the inset; three ectopic eyes with well-differentiated retinal pigment epithelium (rpe) and neural retina (nr) are seen. Embryos were injected into a single ventral blastomere with 400 pg *IGF-2* or *IGFBP-5* mRNA.

were observed (Figure 5G). Secondary somites, notochord, and other trunk structures were not observed in the induced structures. The overall phenotype is most similar to that of Cerberus, which is the only other molecule known to induce ectopic heads in the absence of trunk differentiation (Bouwmeester et al., 1996). We conclude that activation of IGF signaling has anteriorizing activity, leading to the differentiation of ectopic eyes and ectopic head-like structures in the absence of trunk formation.

IGF Anteriorizes CNS

To test whether IGFs convert trunk tissue into more anterior structures, we coinjected *chordin* and *IGF-2* mRNAs. Ventral microinjection of *chordin* mRNA caused the formation of secondary axes that lacked head structures (Figure 6B), and coinjection with *IGF-2* mRNA led to the formation of complete twinned axes that contained eyes and cement glands (Figure 6D). In ventral marginal zone (VMZ) explants, which have posteriorizing activity provided by ventral mesoderm, *chordin* mRNA induced the expression of a range of markers, including the pan-neural marker *NCAM*, the anterior neural markers *Rx2a*, *Six-3*, *Pax-6*, and *Otx-2*, the posterior neural markers *En-2* and *Krox-20*, the cement gland marker *XAG-1*, and the dorsal mesoderm marker α -actin (Figure 6E, lane 4). *IGF-2* induced mostly anterior markers, and coinjection of *chordin* and *IGF-2* mRNAs promoted the expression of anterior neural markers such as *Rx2a* and *Six-3*, while the posterior neural markers *En-2* and *Krox-20* were decreased (Figure 6E, lanes 3–5). Importantly, *IGF-2* inhibited the expression of the trunk mesoderm markers α -actin, α -globin, and *Xbra* induced by *chordin* in VMZs (Figures 6E and 6F). This mesoderm-suppressing activity may help explain why IGF signals, like Cerberus, can induce ectopic heads in the absence of trunk development. Not all types of mesoderm were inhibited, as the heart marker *Nkx2.5* was increased by *IGF-2* (Figure 6E). This correlates with the formation of secondary beating hearts close to ectopic head-like structures induced by *IGF* mRNA.

Formation of the head organizer requires the double inhibition of the Wnt and BMP pathways (Glinka et al., 1997, 1998). Head induction by *IGF-2* is not mediated by the activation of the secreted Wnt inhibitors Cerberus and *Frzb-1*, or of the BMP antagonist *Chordin*, since their transcript levels at the gastrula stage were not increased by *IGF-2* mRNA injection (Figure 6F). To test whether IGF signaling might interfere with the Wnt pathway, we examined the activation of the Wnt primary response genes *siamois* and *Xnr-3* in animal cap explants at the late blastula stage (Brannon et al., 1997; McKendry et al., 1997). *IGF-1* mRNA inhibited the response to *Xwnt-8* and *dnGSK-3* signals, but not the activity of β -catenin mRNA (Figure 6G). We conclude that the IGF signaling pathway is able to anteriorize posterior CNS and to inhibit the formation of trunk mesoderm. This process may involve inhibition of Wnt signals at an intracellular step acting upstream of β -catenin.

Neural Induction by IGFs

Xenopus animal cap explants provide the standard system to study neuralization. Microinjection of *IGF-2*, *IGFBP-5*, or of both mRNAs induced the expression of anterior neural markers, but not that of the more posterior (midbrain) marker *En-2* in these explants (Figure 7A). Importantly, this neural induction took place in the absence of mesoderm formation (α -actin in Figure 7A). To determine whether IGF signaling is required for neural induction, we examined whether *DN-IGFR* was able to block the activity of a BMP antagonist. We used animal caps injected with *chordin* mRNA, an agent that produced a robust induction of the neural markers *NCAM*, *Rx2a*, and *Pax-6*, and a reduced expression of the epidermal marker *Msx-1*. Coinjection of *DN-IGFR* mRNA

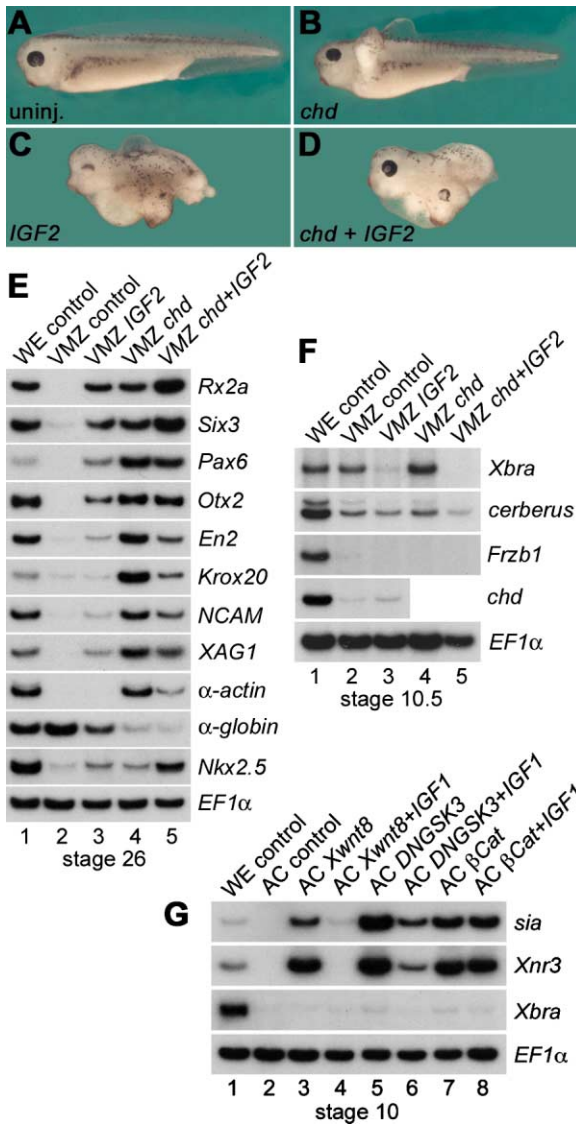


Figure 6. IGFs Anteriorize CNS and Suppress Trunk Mesoderm
(A) Uninjected 3 day tadpole stage embryo.
(B) Ventral injection of *chordin* mRNA (4 pg) induced an incomplete secondary axis containing only trunk tissue (47%, n = 83).
(C) Ectopic head with small eye induced by *IGF-2* mRNA (16%, n = 147).
(D) Complete secondary body axis after coinjection of *chordin* and *IGF-2* mRNA (23%, n = 77).
(E) Molecular analysis by RT-PCR of ventral marginal zone (VMZ) explants cultured until tailbud stage. Embryos were injected once ventrally with *IGF-2* mRNA and *chordin* mRNA either alone or in combination, together with *GFP* mRNA to identify correctly injected VMZs. *EF1 α* was used as an RNA loading control. *IGF-2* induced anterior markers, inhibited Chordin-induced posterior markers, suppressed trunk mesoderm, and induced the heart marker *Nkx2.5*.
(F) VMZs at 10.5 gastrula stage; *IGF-2* blocks the trunk mesodermal marker *Xbra* and does not affect the expression of *cerberus*, *Frzb-1*, and *chordin*.
(G) Animal cap explants at late blastula (stage 10). *Xwnt-8* mRNA (8 pg), *dnGSK-3* (50 pg), or β -catenin (50 pg) mRNAs were injected either alone or together with *IGF-1* mRNA into each animal blastomere at the 8-cell stage. Note that *Xwnt-8* and *dnGSK-3* signals were inhibited by *IGF-1*, whereas β -catenin signaling was not affected. Amounts of mRNA per injection were 400 pg for *IGF-1*, *IGF-2*, and *IGFBP-5*, and 100 pg for *GFP*.

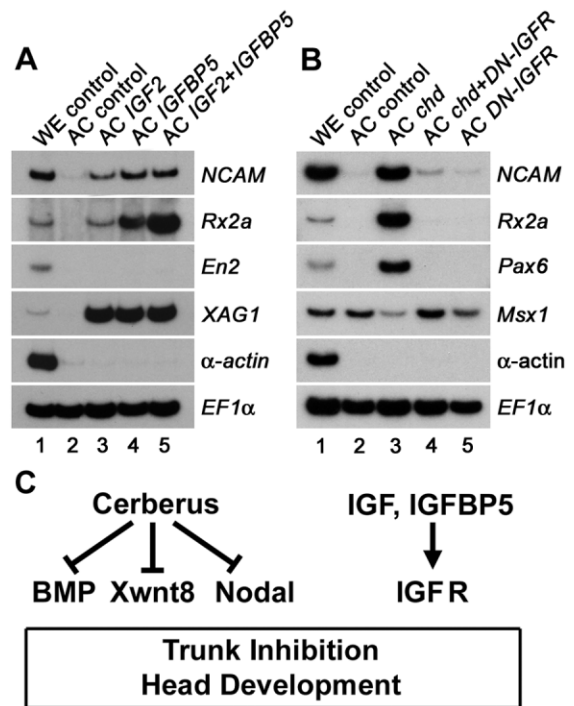


Figure 7. IGF Signaling Is Sufficient and Required for Neural Induction
(A) RT-PCR analyses of animal cap explants at tailbud stage 26. mRNA of *IGF-2*, *IGFBP-5*, or *IGF-2* and *IGFBP-5* together (400 pg each) were injected into each animal blastomere at the 4- to 8-cell stage and animal cap explants prepared at stage 9.
(B) *chordin* (4 pg), *DN-IGFR* (400 pg), and a combination of *chordin* and *DN-IGFR* mRNA were injected into each animal blastomere at the 4- to 8-cell stage. Note that *DN-IGFR* suppresses *chordin*-induced anterior neural markers and enhances the expression of the epidermal marker *Msx-1*.
(C) Model showing two parallel extracellular mechanisms implicated in head induction. Cerberus acts by inhibiting BMP, Xwnt-8, and Nodal signaling (left), while IGF recruits an active pathway (right) to inhibit trunk and promote head development.

inhibited anterior neural induction by *chordin* (Figure 7B, compare lanes 3 and 4). In whole embryos, *DN-IGFR* mRNA inhibited *Otx-2* expression and head development, but development of the trunk, including posterior CNS, was relatively unaffected (Figure 3). We conclude that IGF signals are not only sufficient but appear to be also required for anterior neural induction. The BMP antagonist Chordin, a potent neural inducer in *Xenopus*, induces neural differentiation via a pathway that requires endogenous IGF signals present in animal cap explants.

Discussion

The starting point for this investigation was the observation that an insulin-like growth factor binding protein (IGFBP-5) had striking anteriorizing activity in *Xenopus* embryos. Three IGFs are expressed during *Xenopus* early development, and all had similar anteriorizing effects. Microinjection of *IGF* mRNA into the prospective neural plate region resulted in the formation of ectopic eyes, and injection into prospective ventral mesoderm

led to the formation of ectopic head-like structures including brain, olfactory placodes, and eyes. Coinjection of *IGFBP-5* and *IGF* mRNAs cooperated in the formation of ectopic head structures. In contrast, a dominant-negative IGF type I receptor (DN-IGFR) blocked the activity of IGFBP-5 and of IGFs, suggesting that IGFBP-5 acts by increasing the activity of endogenous IGFs. Increased IGF signaling was sufficient to induce anterior neural markers in animal cap explants. Notably, DN-IGFR inhibited CNS induction by the BMP antagonist Chordin, implying that endogenous IGF signals are required for anterior neural development.

We propose that IGF signaling may regulate the amount of tissue allocated to the head and trunk regions of the *Xenopus* embryo. In gain-of-function experiments, IGFs expanded the head territory at the expense of trunk. In loss-of-function studies using DN-IGFR, the opposite result was obtained. The results presented here suggest the participation of an additional signaling pathway in vertebrate anterior patterning.

To date, head development has been shown to be triggered by inhibition of growth factor signals required for trunk development. Head development was considered a default state, resulting from double inhibition of Wnt and BMP signaling, or triple inhibition of Wnt, BMP, and Nodal signaling in the case of Cerberus (Glinka et al., 1997, 1998; Piccolo et al., 1999). The finding that IGF signaling is required and sufficient for head formation now introduces an active pathway into this process (Figure 7C).

The ectopic heads induced by IGF overexpression lack trunk structures, and in ventral marginal zone explants, IGFs inhibited the expression of the trunk mesodermal markers *Xbra*, α -*actin*, and α -*globin*. The overall phenotype resembles that of Cerberus, which is the only other agent known to induce ectopic heads in the absence of trunk structures (Bouwmeester et al., 1996). IGF signals share with Cerberus a strong anteriorizing activity and the ability to suppress trunk mesoderm but not heart mesoderm. There are some differences, however, as Cerberus-induced head-like structures contain a single ectopic eye, whereas those induced by IGF can have multiple ones (Figure 5G). This suggested that IGF was not inducing heads simply through an increase of *cerberus* expression, and indeed, it was found that ectopic IGF-2 mRNA did not induce the expression of *cerberus* transcripts in whole embryos at the early gastrula stage (data not shown). Similarly, IGF did not enhance the expression of the Wnt inhibitor *Frzb-1* or of the BMP antagonist *chordin* in ventral mesodermal explants (Figure 6F).

IGF signaling appears to be both sufficient and required for anterior neural induction in *Xenopus* (Figure 7). IGF signals are transduced through the type I IGF receptor, which is a receptor tyrosine kinase closely related to the insulin receptor (Stewart and Rotwein, 1996; Efstratiadis, 1998). Activation of IGF-1R by IGF-1 or -2 results in tyrosine phosphorylation of cytoplasmic insulin receptor substrates (IRS) that in turn can activate the ras-MAP kinase pathway or the PI3 kinase (phosphatidylinositol-3' kinase)-Akt pathway (Blume-Jensen and Hunter, 2001). Interestingly, these two pathways can also be activated by the FGF receptor tyrosine kinase signal (Carballada et al., 2001). Since both IGF (this work) and FGF signals (Streit et al., 2000; Stern, 2001)

are involved in neural induction, our findings raise the possibility that common downstream molecular pathways may participate in both events. However, the effects must also have important differences, since FGF is able to posteriorize anterior neural tissue, whereas the converse is true for IGF (McGrew et al., 1997; this study). It will be important to determine whether the MAPK and/or the PI3K pathway mediate anterior neural induction by IGF.

IGF signals are potent inhibitors of Wnt signaling, acting extracellularly at a step upstream of β -catenin (Figure 6G). This activity could be mediated, for example, by phosphorylation changes induced on the β -catenin degradation complex (which contains multiple phosphoproteins such as APC, Axin, GSK-3, and protein phosphatase 2A) by the MAPK or the PI3K signaling cascades. Despite the robust anti-Wnt activity of overexpressed IGF, it seems unlikely that the head-inducing activity of IGF is mediated exclusively through Wnt inhibition. This is because Wnt inhibition alone is not sufficient for ectopic head or neural induction in microinjected *Xenopus* embryos (Glinka et al., 1997, 1998; Piccolo et al., 1999; Pera and De Robertis, 2000). IGF signals presumably interact with multiple pathways to promote head development.

Experiments with a secreted DN-IGFR in *Xenopus* implicate an endogenous IGF signal in head formation. The dominant-negative IGF receptor used was a secreted *Xenopus* version of the human 486/Stop DN-IGFR. The human construct has been shown to inhibit proliferation of transfected cells, the growth of neighboring cells in transplanted tumors, and tyrosine phosphorylation of IGF1R (D'Ambrosio et al., 1996; Reiss et al., 1998). Mouse knockout studies suggest that the IGF signaling pathway is a major regulator of body growth. Animals mutated in IGF ligands, IGF type I receptor, or insulin receptor substrates are proportionate dwarfs and have smaller brains, but do not show signs of preferential loss of head or forebrain structures (Efstratiadis, 1998; D'Ercole et al., 1996). One possible explanation for the discrepancy between the *Xenopus* and mouse loss-of-function results is that multiple ligands and receptors exist (D'Ercole et al., 1996; Efstratiadis, 1998), while in the case of xDN-IGFR, the secreted protein might block all possible IGFs and their receptors, leading to more severe phenotypes.

In *Drosophila*, mutations in this pathway also affect overall body size (Brogiolo et al., 2001), but mutations in the *insulin receptor homolog (inr)* show impaired neural development. In the strongest *inr* mutants, large numbers of neurons and glia cells fail to form during neurogenesis, and in some mutants, larval head structures are entirely missing (Fernandez et al., 1995). However, the loss of neurons is not restricted to the anterior region and is observed in the entire length of the embryo.

In *Xenopus*, zygotic expression of *IGF-2*, -3, and *IGFBP-5* is not confined to the head region and spans the entire length of the anteroposterior axis. At the neural plate stage, these genes are coexpressed in the floor plate. IGF-3, however, is also expressed strongly in the ventral forebrain (Figure 2F). In the future, it will be interesting to examine whether zygotic IGF signals contribute to the dorsoventral patterning of the CNS. The endogenous IGF signals that appear to be required for anterior neural induction by Chordin (Figure 7B) are likely

to be provided by the uniformly distributed maternal transcripts encoding *IGF-1*, *-2*, *-3*, and *IGFBP-5* in the egg. Neural induction takes place at gastrula or earlier stages (Sasai and De Robertis, 1997; Wessely et al., 2001), and therefore the zygotic IGF transcripts observed in neurula floor plate and ventral forebrain would accumulate too late to participate in this process. Although maternal IGF transcripts are uniform, IGF signals might cooperate with asymmetrically expressed molecules such as Chordin, Noggin, Cerberus, and Dickkopf to induce CNS.

Several unanswered questions remain for the future. First, could IGF correspond to the elusive archencephalic inducer discovered by Toivonen in guinea pig liver (Saxén and Toivonen, 1962)? Several interesting parallels exist. IGFs are small molecules of only 7.5 kDa that were isolated from acid alcohol fractions of human plasma and are resistant to heat treatment (Rinderknecht and Humbel, 1976). Not only are these properties intriguingly similar to those of the archencephalic inducer (Saxén and Toivonen, 1962), but in addition, the liver is the main site of IGF production in the adult (Beck et al., 1988). Second, do IGF signals cooperate with anti-BMP signals in neural development? An interesting example is provided by vertebrate cysteine-rich motor neuron protein 1 (CRIM-1). The BMP antagonist Chordin modulates BMP activity through cysteine-rich BMP binding modules called CRs (De Robertis et al., 2000). CRIM-1 is a transmembrane protein containing six CR modules and also an IGF binding domain similar to that of IGFBPs (Kolle et al., 2000). The association of BMP and IGF binding modules in an individual protein is also seen in members of the CCN family (CTGF, CYR61, and NOV-1) and may reflect the concerted function of the BMP and IGF signaling pathways during development (García-Abreu et al., 2002). Finally, the question arises of whether the anterior neural-inducing activity of IGF might be useful for the generation of forebrain tissue in culture. Studies with mammalian embryonic stem cells have demonstrated that it is possible to use them to generate an efficient source of mesencephalic dopaminergic neurons, but not forebrain cells (Lee et al., 2000; Kawasaki et al., 2000). Thus, it will be worthwhile to test whether IGF signals provide a means of directing stem cells into anterior differentiation pathways. Although many areas for future exploration remain, the results presented here implicate IGFs as active signals required for head and neural induction in vertebrate embryos.

Experimental Procedures

Secretion Cloning

An unamplified cDNA library from *Xenopus* dorsalized by treatment with lithium chloride stage 11 embryos was constructed by directionally cloning oligo dT-primed cDNA into the EcoRI and XhoI sites of the expression vector pCS2. cDNA clones were transformed into XL1-BlueMRF' (Stratagene) *E. coli* and used for secretion cloning as described by Pera and De Robertis (2000). In brief, bacterial colonies were cultured individually in 96-well plates, the culture media from 16 clones were pooled, and plasmid minipreps were prepared. 293T cells grown in 24-well plates were transfected with this mixture of cDNAs and 1.5 days later labeled with ³⁵S-methionine and -cysteine under serum-free conditions. Secreted proteins were identified by SDS-PAGE and autoradiography, and positive clones were individualized in a second transfection step by sib selection.

Expression Constructs and Synthetic RNA Synthesis

pCS2-*xIGFBP-5* was obtained as a full-length cDNA clone by secretion cloning. The complete open reading frame of *xIGF-1* (Kajimoto and Rotwein, 1990) was amplified by PCR and subcloned into the EcoRI and XhoI sites of pCS2. To prepare the *xIGF-2* expression vector, the full-length cDNA corresponding to EST BE509513 was obtained from the *Xenopus* Gene Collection (XGC) library and sequenced. Best results were obtained with an *xIGF-2* sequence encoding the mature protein and an N-terminally shortened, 24 amino acids long signal peptide that starts at an in-frame methionine. *xIGF-2* was amplified by PCR and subcloned into the EcoRI and XbaI sites of pCS2. To obtain an *xIGF-3* expression vector, the full-length cDNA corresponding to EST BE508894 from the XGC library was sequenced. The *xIGF-3* sequence encoding the mature protein and an N-terminally truncated, 18 amino acids long signal peptide starting in an in-frame methionine was amplified by PCR and subcloned into the EcoRI and XbaI sites of pCS2. To generate a secreted dominant-negative type I IGF receptor (*DN-IGFR*), cDNA encoding a C-terminal truncated version of the *Xenopus* IGF type I receptor (AF055980) with a stop codon downstream of the arginine at position 505 of the extracellular domain was amplified by PCR from gastrula cDNA and subcloned into the EcoRI and XbaI sites of pCS2.

To prepare sense mRNA, pCS2 constructs of *xIGFBP-5*, *xIGF-1*, *xIGF-2*, *xIGF-3*, *DN-IGFR*, *chordin*, *Xwnt-8*, *dnGSK-3*, and β -*catenin* were linearized with NotI and transcribed with SP6 polymerase as described (Piccolo et al., 1999). mRNA encoding nuclear β -galactosidase and green fluorescent protein (GFP) were synthesized from pXEX β gal (XbaI digestion and T7 transcription, gift of R. Harland) and p β GFP/RN3P (SfiI digestion and T3 transcription, gift of J. Gurdon).

Embryo Manipulations

Xenopus embryos were fertilized and cultured as described (Sive et al., 2000). β -Galactosidase activity in nuclear *LacZ* mRNA-injected embryos was visualized as reported (Sive et al., 2000), except that X-gal was substituted by 6-chloro-3-indolyl- β -galactoside (Red Gal, Research Organics). Animal cap explants were excised at stage 9, and ventral marginal zone explants comprising 60° of the VMZ were prepared at stage 10. Explants were cultured in 0.5 \times MMR (Sive et al., 2000) until sibling embryos reached the indicated stages. Injected VMZs were identified by coinjection of GFP mRNA.

RT-PCR and Whole-Mount In Situ Hybridization

Transcript levels in whole embryos and explants were assayed by RT-PCR. The primer sets and conditions were as reported (Sasai et al., 1995; Bouwmeester et al., 1996; <http://www.hhmi.ucla.edu/derobertis/>). Whole-mount in situ hybridization was performed as described (Sive et al., 2000; <http://www.hhmi.ucla.edu/derobertis/>), with the modification that the staining step was carried out on ice for several days to enhance the signal/noise ratio.

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Accession Numbers

The GenBank accession numbers for *xIGFBP-5*, *xIGF-2*, and *xIGF-3* cDNAs reported here are AY052629, AY050645, and AY049735, respectively.