Development 121, 4293-4301 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995
DEV3226

Anti-Dorsalizing Morphogenetic Protein is a novel TGF-β homolog expressed in the Spemann organizer

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SUMMARY

We have identified a novel growth factor in *Xenopus*, which is most closely related to human Bone Morphogenetic Protein-3. Its expression peaks during gastrulation, most prominently in the Spemann organizer, and persists in the posterior neural floor plate and prechordal plate during neurulation. Injection of the corresponding mRNA into dorsal blastomeres results in dose-dependent suppression of dorsal and anterior structures, even in the presence of lithium chloride. Overexpression of the gene downregulates the dorsalizing factors noggin, goosecoid and follistatin, as well as the dorsal markers NCAM, muscle actin and MyoD; conversely, ventral markers are induced. We therefore designate this gene product Anti-Dorsalizing Morphogenetic Protein (ADMP). Though development of dorsoanterior structures is suppressed when exogenous ADMP is injected, the gene is induced by lithium chloride treatment or activin, both of which are known to produce the opposite effect. Thus, the expression of ADMP resembles that of several dorsalizing signals, but its product exerts dorsal-suppressing activity. This suggests that ADMP may moderate organizer-associated dorsalizing influences. These findings are also consistent with the recently advanced proposal of dorsally expressed inhibitory activin-like signals.

Key words: ADMP, TGF-β, BMP, *Xenopus*, organizer, mesoderm, growth factor

INTRODUCTION

Initial interest in the Bone Morphogenetic Proteins (BMPs), a group within the TGF-β superfamily, resulted from their ability to initiate endochondral bone formation in adult vertebrates (Urist, 1965). Purification and sequencing of protein with bone inductive activity led to the discovery of a family of growth factors (Wozney et al., 1988; Wang et al., 1988; Hammonds et al., 1991; Sampath et al., 1992) resembling the Drosophila *dpp* gene (Padgett et al., 1987) and Vg1 (Weeks and Melton, 1987), TGF-β homologs that had been implicated in embryonic body axis determination. Experiments with recombinant proteins indicated that single BMPs were sufficient to induce this complex cascade of events (Wozney et al., 1988). Subsequently, many related proteins were identified and found to participate in a wide variety of developmental processes (for review, see Kingsley, 1994). These findings complemented the discovery of additional dorsalizing (Smith and Harland, 1992; Niehrs et al., 1993) and mesoderm-inducing (Smith and Harland, 1991) peptide growth factors. Since noggin (Lamb et al., 1993), GDNF (Lin et al., 1993) and dorsalin (Basler et al., 1993) appear to support differentiation of nervous system components in vitro, the possibility that diffusible factors might act to instruct nearby cells to adopt a neural fate thus accorded well with the early findings of Spemann (Spemann and Mangold, 1924) and suggested a molecular link to these classic observations.

Nevertheless, the concept that neural induction requires instructive interactions has recently been challenged. Experiments with dissociated and reaggregated animal cap cells suggest that their default fate may be neural (Sato and Sargent, 1989), and overexpression of a truncated activin receptor (ΔXAR1) (Hemmati-Brivanlou and Melton, 1994) or follistatin, an antagonist of activin and perhaps related proteins (Hemmati-Brivanlou et al., 1994), have implicated one or more activin-like molecules normally present in the animal region of the embryo in suppression of dorsal structures.

Many members of the TGF-β superfamily are currently being evaluated as human therapeutic agents in various mammalian models. Since these experimental systems have limited ability to discriminate between various members of the BMP family and do not allow access to events in early vertebrate development, we explored the ability of paradigms developed in *Xenopus* to complement ongoing work in other systems. During these investigations, we identified a novel member of this family, which is one of the first factors of this group to be identified in the Spemann organizer. Because overexpression of the gene results in down regulation of known dorsalizing factors and axial suppression of affected embryos, we term the factor Anti-Dorsalizing Morphogenetic Protein

(ADMP). The fact that ADMP coexists with various dorsalizing signals present in the Spemann organizer, but suppresses the dorso-anterior axis, suggests that it may exert a moderating influence on these signals. These properties are consistent with those expected for the endogenous suppressive activity suggested by previous reports.

MATERIALS AND METHODS

Isolation of ADMP cDNA

Degenerate primers biased to detect homologs of the mammalian BMP-3 gene were used to amplify sequences from *Xenopus* genomic DNA using conditions described previously (Wharton et al., 1991). The 5′ oligonucleotide was 5′-ATHGGITGGWSNGARTG-GATHAT-3′; the 3′ oligo was 5′-ATISWYTGRATNGTRGCRT-GRTT-3′. Amplified fragments were purified by agarose gel electrophoresis, cloned into PCR II (Invitrogen, San Diego, CA) and sequenced (Sanger et al., 1977). Inserts of interest were labeled with digoxigenin by PCR and used to screen 10^6 plaques from a stage 13 λgt11 cDNA library (Richter et al., 1988). The Genius™ chemiluminescent detection protocol (Boehringer Mannheim) was modified by addition of a 3 hour wash in 3× SSC/0.1% SDS at 65°C before the prehybridization step and increasing the concentration of blocking reagent to 5%; the final stringency wash was for 15 minutes at 68°C in 0.3× SSC/0.1% SDS. Over 30 purified clones were analyzed by restriction digestion, PCR and sequencing from phage plaques (Wang et al., 1995). None of the clones contained a translation initiation site or putative transmembrane signal sequence. cDNA from stage 12 embryos was therefore prepared using Superscript II reverse transcriptase as recommended by the manufacturer (Life Technologies, Gaithersberg, MD); the 5′ region of the open reading frame was identified with the T4 RNA ligase procedure (Edwards et al., 1991). A sequence containing a complete open reading frame and translation initiation site was amplified, cloned into pCR Script (Stratagene, La Jolla, CA) and sequenced. The insert was subcloned into pSP64R1 (provided by Sergei Sokol, Harvard University) to provide a template for preparation of capped mRNA for injection experiments.

Sequence analysis

Sequence similarity was assessed with the BLAST network service of the National Center for Biotechnology Information (Bethesda, MD). Alignments were done with Geneworks (Intelligenetics, Mountain View, CA).

Embryo manipulations

Frogs were maintained and manipulated by standard methods (Gurdon, 1967; Gurdon, 1977; Peng, 1991). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). UV treatment was done with a Stratalinker (Stratagene, La Jolla, CA) (Smith and Harland, 1991) and LiCl treatment was for 1 hour in 0.1 M LiCl in 0.1× MMR plus gentamycin (Kao and Elinson, 1988).

Northern blots

Total RNA was isolated from staged embryos with TRIZOL™ (Life Technologies, Gaithersburg, MD); polyadenylated RNA was selected with Oligotex-dT (Qiagen, Chatsworth, CA). 2.5 μg from each sample (determined spectrophotometrically) was fractionated on formaldehyde-agarose gels (Farrell, 1993) and subjected to downward alkaline capillary transfer as described (Chomczynski, 1992) except that Sarkosyl was omitted from the transfer buffer. All blots were stained with methylene blue to confirm consistent loading and RNA integrity. Probes labeled to $>2\times10^9$ cts/minute/μg with [α-^{32}P]dCTP were hybridized in ExpressHyb™ (Clontech, Palo Alto, CA) as recommended by the manufacturer. Relative levels of expression were assessed with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). All reported analyses are representative of at least four separate experiments.

mRNA injection experiments

Capped mRNAs were synthesized with mMessage mMachine™ SP6 kits (Ambion, Austin, TX) from linearized plasmids, checked for integrity by denaturing agarose electrophoresis (Farrell, 1993) and prepared for injection by standard methods (Kay, 1991). The preprolactin transcription plasmid was the kind gift of Enrique Amaya (Amaya et al., 1991) and the activin plasmid was as constructed by Sergei Sokol (Sokol et al., 1991).

For injection, embryos were maintained in 1× MMR supplemented with 5% Ficoll 400 (Molecular Biology Grade, Sigma, St. Louis) and gentamycin. Single blastomeres were injected into 2-, 4-, or 8-cell embryos at various sites as indicated. When the embryos reached stage 5-6, they were transferred to 0.1× MMR plus gentamycin. Analysis of abnormal phenotypes was based on experiments where the overall viability of injected embryos was at least 90%. Each treatment group contained over 40 viable embryos and reported results are based on at least four separate experiments. The phenotypic abnormalities displayed in Figs 4 and 5 were observed consistently in over 20 experiments in which noninjected embryos and preprolactin-injected embryos developed normally. Dorsal and ventral regions of the embryos were identified by pigment variations (Nieuwkoop and Faber, 1967).

Hybridization in situ

The procedures outlined by Harland (Harland, 1991; Smith and Harland, 1992) were followed, using Boehringer blocking reagent (Lamb et al., 1993) and BM-Purple alkaline phosphatase substrate. Sense controls were done for all stages and were negative. Sections were cut from stained embryos embedded in JB-4 resin (Polysciences, Warrington, PA).

RT-PCR assays

Separate pools of embryos or embryo pieces were prepared from at least three different fertilizations for each condition reported. Total RNA was prepared as described above and treated with DNase (Amplification Grade, Life Technologies, Gaithersburg, MD); reverse transcription was done with Superscript II (Life Technologies, Gaithersburg, MD) as described by the manufacturer with 1 μg total RNA per reaction. Assays were performed as described (Rupp and Weintraub, 1991; Niehrs et al., 1994); input cDNA for each sample was normalized to histone H4 or cSrc. Amplification was performed in 25 μl reactions containing 1× PCR buffer (Perkin Elmer, Norwalk, CT), 200 μM dNTPs, 1 μM each primer, 1 U Taq polymerase (Perkin Elmer, Norwalk, CT) and 1 μCi [α-^{32}P]dCTP. Cycling parameters were 94°C, 30 seconds; 55°C, 30 seconds; 72°C, 1 minute for each cycle; a Model PTC-100 (MJ Research, Watertown, MA) was used in all experiments. The primers used for MyoD were described in Rupp and Weintraub (1991), for Brachyury, Goosecoid, cardiac actin, and histone H4 in Niehrs et al. (1994), for follistatin in Hemmati-Brivanlou et al. (1994), and for NCAM and muscle actin in Hemmati-Brivanlou and Melton (1994). The following primers were designed using Geneworks™ from published sequences:
XTwist, F: 5′-AGTCCGATCTCAGTGAAGCG-3′,
R: 5′-AGTCGATGTATCTGGAGGCC-3′;
XHox-3, F: 5′-CTCGCATGTCTTAGTACCTCAACCAAC-3′,
R: 5′-GTCATATCTTCCCTCTG-3′;
XAR1, F: 5′-TGTTGCGAATAATCGGAGC-3′,
R: 5′-TTATGTCCAGCAACTGCAGC-3′;
XWnt-8, F: 5′-ATGGACTTCGAAGTGCAACC-3′,
R: 5′-ATGGCTCCTCTGTTGTC-AGC-3′;
ADMP, F: 5′-GAGCTGCAGCTTGATGAG-3′,
R: 5′-GCCACAGTCCAGAGGTTA-3′; and
cSrc: 5′-GAGTCGCAGTCTGGATATTGC-3′,
R: 5′-TGGAATGTAGCCAGTCTGCC-3′.

PCR products were separated on 5% acrylamide gels, dried and exposed to phosphor screens (Molecular Dynamics, Sunnyvale, CA) or Kodak XAR film for 0.5-10 hours. PCR analysis was performed at least twice for each cDNA to confirm that the amplifications were reproducible.

RESULTS

ADMP is a member of the TGF-β superfamily

Amplification of *Xenopus* genomic DNA with degenerate primers yielded homologs of BMPs 2, 4, 5 and 7. In addition, members of two novel bone morphogenetic protein subfamilies were isolated. One set, resembling the recently described Gdf-5, Gdf-6 and Gdf-7 group (Storm et al., 1994), is the subject of a separate report (Chang et al., 1994). A probe generated from a member of the other group was used to screen a neurula library. Over 30 clones were obtained, comprising two genes with greater than 95% nucleic acid identity, presumably representing the A and B alleles characteristic of *Xenopus*. The complete 2225 bp sequence has a consensus translation initiation site (Kozak, 1991) and putative signal sequence (von Heijne, 1986). A single transcript of 2.2 kb is detectable by northern analysis, corresponding closely with the size of the cDNA. The deduced amino acid sequence (Fig. 1A) contains all features characteristic of the BMP family, including a consensus RXXR proteolytic processing site (Ozkaynak et al., 1992) followed by a carboxyl terminal domain with seven highly conserved cysteine residues at the positions observed in the majority of these proteins (Reddi, 1992; Kingsley, 1994). The carboxyl terminal region following the first of the seven conserved cysteines (Fig. 1C) demonstrates 50% identity with BMP-3 and several other members of this gene family. Unlike most of these proteins, ADMP lacks a consensus glycosylation site. Most of the BMPs so far identified demonstrate at least 90% amino acid identity between mammalian and lower vertebrate homologs; ADMP therefore likely represents a novel growth factor.

ADMP is a zygotic transcript expressed in Spemann's organizer

Fig. 2A demonstrates that ADMP is undetectable by RT-PCR until stage 9, and peaks during gastrulation. Expression decreases thereafter, becoming undetectable after stage 25. At stage 9, whole mount hybridization in situ reveals nuclear staining in the organizer region (Fig. 3). The staining intensi-

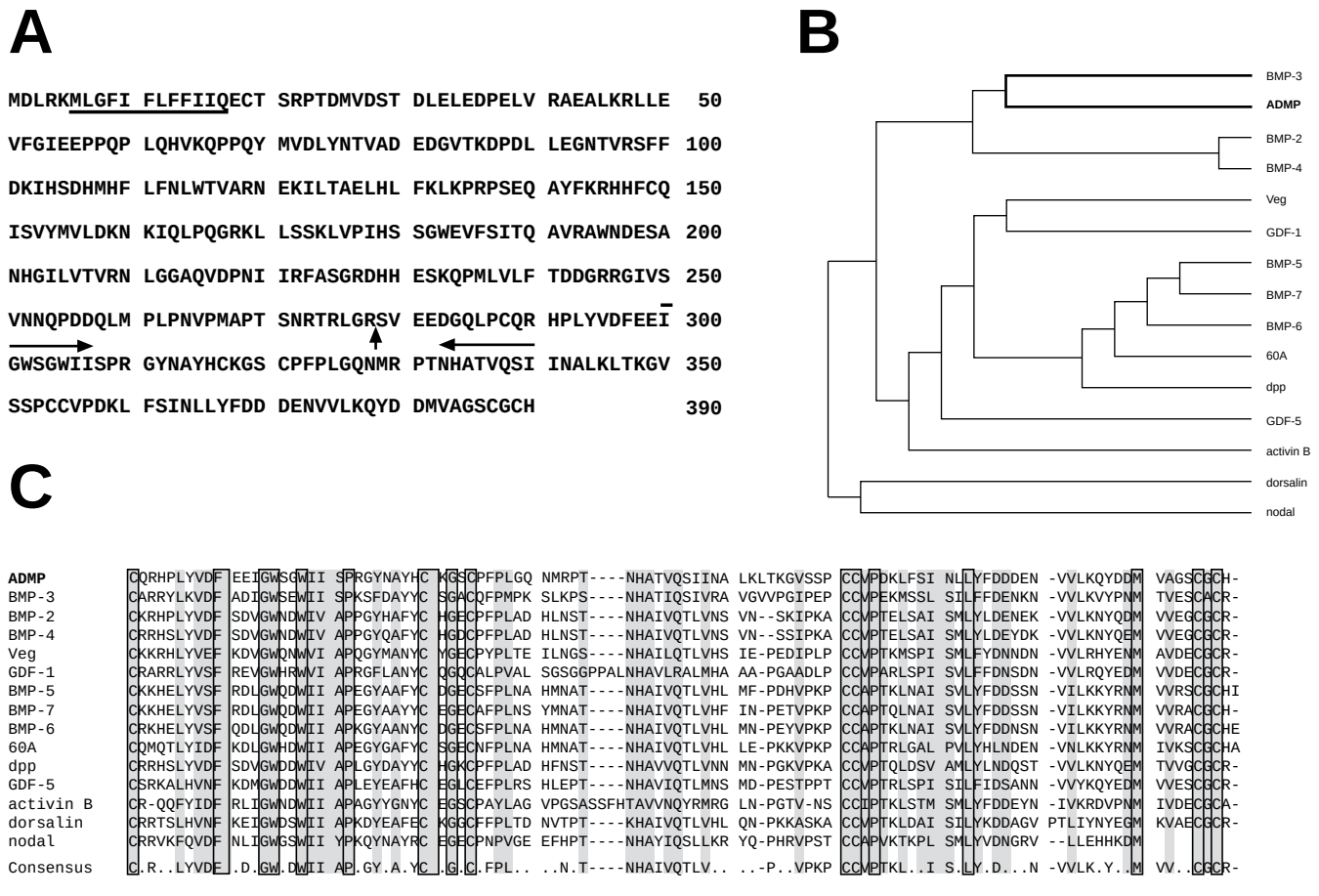

Fig. 1. ADMP is a novel member of the TGF-β superfamily. (A) Deduced amino acid sequence of ADMP. The putative signal sequence is underlined, the consensus RXXR proteolytic processing site is marked by a vertical arrow and the sequences corresponding to the PCR primers used are indicated by horizontal arrows. (B) Schematic representation of the relationship between ADMP and other members of the TGF-β superfamily. (C) Amino acid alignment of ADMP with other TGF-β proteins. The diagrams in B and C were generated using GeneWorks (Intelligenetics, Mountain View, CA) and are based on the region beginning with the first of the seven conserved cysteine residues and ending with the carboxyl terminal residue (GenBank Accession #: U22155).

fies as gastrulation proceeds, becoming more concentrated at the dorsal lip (stages 10-11) and then extending in a streak anteriorly. Between stages 11 and 15, staining is sharply graded from the blastopore region anteriorly; sections taken through whole mounts confirm this gradient (Fig. 3B; not shown) and demonstrate that expression is confined to the sensorial layer of the neuroectoderm. Involuted mesoderm appears devoid of staining. Concurrently, staining appears in anterior mesoderm (Fig. 3C) and becomes concentrated in the prechordal plate by stage 20 (Fig. 3A,D).

To evaluate the possibility of endodermal expression not detectable by hybridization histochemistry (Bolce et al., 1993), expression of ADMP in various portions of stage 10.5 embryos was compared by RT-PCR (Fig. 2B). ADMP is concentrated in the dorsal marginal zone and undetectable in the vegetal cell mass. These results support the hybridization in situ data.

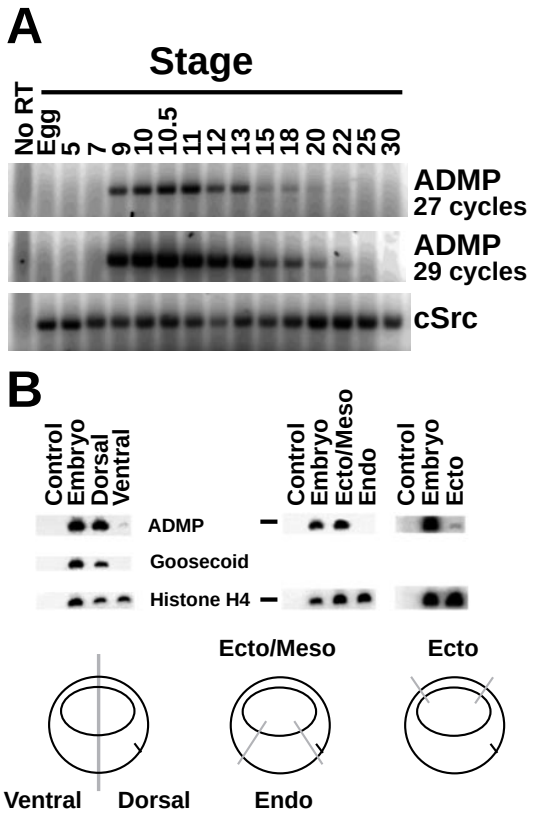

Fig. 2. Expression of ADMP during *Xenopus* development. (A) Expression levels of ADMP. RT-PCR with total RNA isolated from the indicated stages was performed for ADMP; cSrc was used to normalize input cDNA between samples. To allow visualization of high and low levels of expression within the exponential phase of the PCR, data from 27 and 29 cycles of amplification are presented. This analysis was performed twice with identical results. (B) ADMP is present in the dorsal marginal zone during gastrulation. Stage 10.5 embryos were dissected as indicated in the figure. The RNAs from each were analyzed for ADMP by RT-PCR. The template amounts were normalized to produce approximately equal amounts of histone H4 product (Niehrs et al., 1994). Goosecoid was used as a control for the dorsal versus ventral dissection and template from which reverse transcriptase was omitted was used to confirm absence of contaminating genomic DNA sequences (indicated as Control in the figure). Expression is most concentrated in the dorsal marginal zone.

ADMP overexpression produces profound axial suppression

Injection of ADMP mRNA into dorsal blastomeres at the 4- to 8-cell stage produced a range of phenotypes demonstrating suppression of the anteroposterior axis (Fig. 4B). The most prominent abnormalities are suppression of anterior-dorsal structures including the cement gland, eyes, otic vesicles and, in many cases, the entire head, hypopigmentation and dysmotility. These effects were much less pronounced if ventral blastomeres were targeted (Fig. 4C,D). Axis suppression was dose-dependent; the frequency distribution of various Dorsoanterior Index (DAI; Kao and Elinson, 1988) scores at different mRNA doses is shown in Fig. 4E. Nearly all embryos injected with preprolactin message developed normally at all doses tested. Despite the profound phenotypic alterations observed at later stages, no gross morphologic abnormalities were observed prior to neurulation at a 100 pg dose of ADMP mRNA. In particular, no abnormalities in blastopore formation or closure, which can indicate nonspecific toxic effects, were apparent in either control or ADMP-injected embryos (not

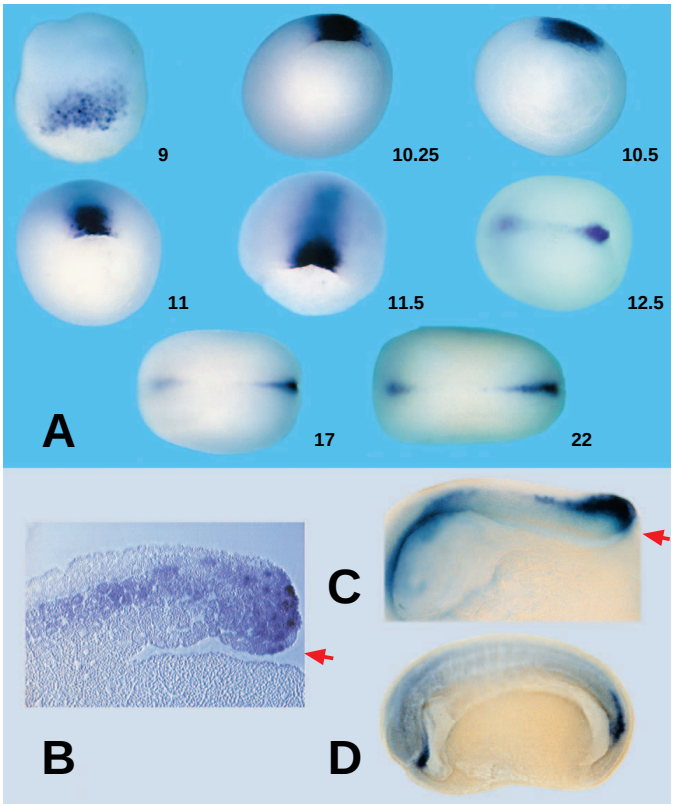

Fig. 3. ADMP expression in the Spemann organizer. (A) Series of embryos from early gastrula (top left) to neural tube (bottom right) stages demonstrating ADMP localization by hybridization in situ. (B) Sagittal section of stage 11 embryo indicating localization of ADMP expression to the dorsal blastopore lip. Staining of the epithelial layer of the neuroectoderm is not apparent. (C,D) Sagittal views of cleared stage 12.5 and 22 embryos. Expression is predominantly in the sensorial layer of the neuroectoderm in a decreasing gradient from posterior to anterior posteriorly and in the prechordal plate anteriorly. Red arrows indicate the blastopore. Hybridization in situ was performed ten times in total with similar results.

shown). Histologic sections through control (Fig. 5A), moderate (Fig. 5B) and severe (Fig. 5C) phenotypes demonstrate progressive derangement of notochord, neural tube and somites, and overall hypoplasia of the dorsal region of the embryo.

ADMP expression is increased by LiCl and activin and decreased by UV irradiation

The apparent paradox of an expression pattern similar to that of various dorsalizing signals and the ventralizing effect of ADMP overexpression prompted us to examine the effects of classical dorsalizing (LiCl, Kao and Elinson, 1988; activin,

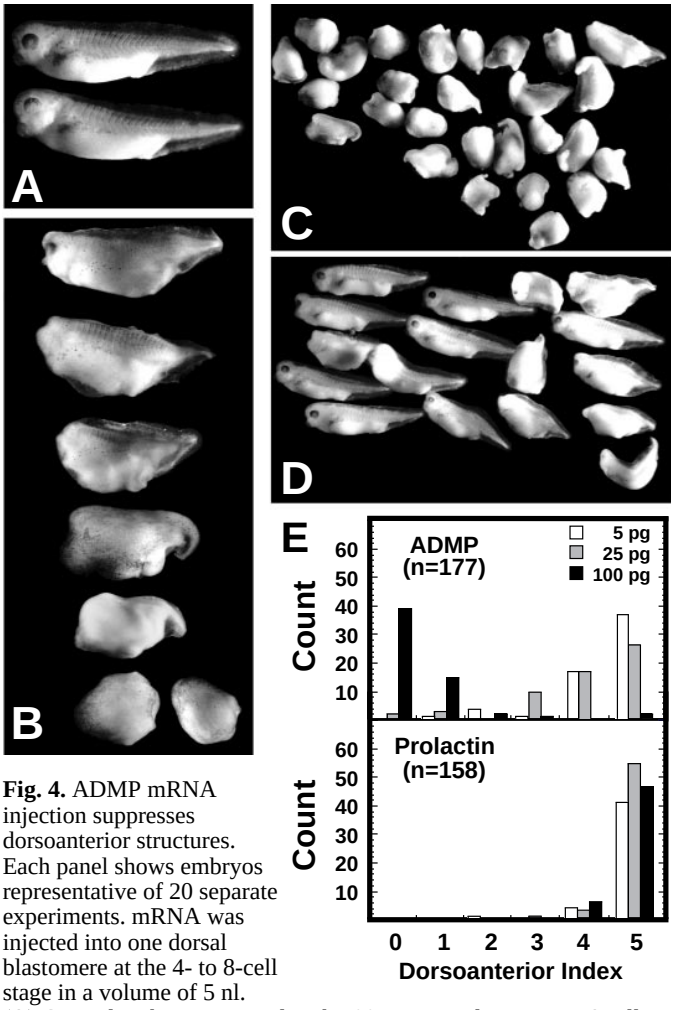

Fig. 4. ADMP mRNA injection suppresses dorsoanterior structures. Each panel shows embryos representative of 20 separate experiments. mRNA was injected into one dorsal blastomere at the 4- to 8-cell stage in a volume of 5 nl. (A) Control embryos injected with 100 pg preprolactin mRNA; all embryos are siblings at the same stage (approximately stage 35). (B) Embryos injected with ADMP mRNA. A range of phenotypes displaying varying degrees of ventralization is shown. (C) Embryos injected dorsally with 100 pg ADMP mRNA. All embryos are severely ventralized. (D) Embryos injected ventrally; the phenotypic perturbations are much less pronounced. (E) Frequency histogram of Dorsoanterior Index scores of embryos injected dorsally with 5, 25 and 100 pg of either ADMP or preprolactin mRNA demonstrating clear dose dependence. The mean DAI scores of ADMP and preprolactin-injected embryos differ significantly ($P<0.01$) at all doses. The data shown are representative of four experiments with comparable numbers of embryos and at least 20 experiments overall. Some variation in sensitivity to injected ADMP message was observed between different batches of embryos.

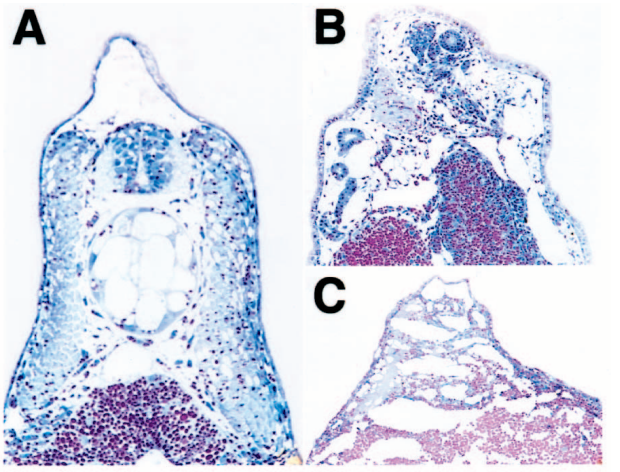

Fig. 5. Histological analysis of ADMP-injected embryos. Frontal sections of embryos injected with identical doses of (A) preprolactin or (B,C) ADMP message demonstrate progressive derangement and suppression of neural tube, notochord and somites with increasing severity of phenotype. (B) Of ten embryos with DAI 2-3, all demonstrated significant abnormalities in neural tube, notochord and somites; (C) of thirteen embryos with DAI 0-1, none had discernible notochord or neural tube.

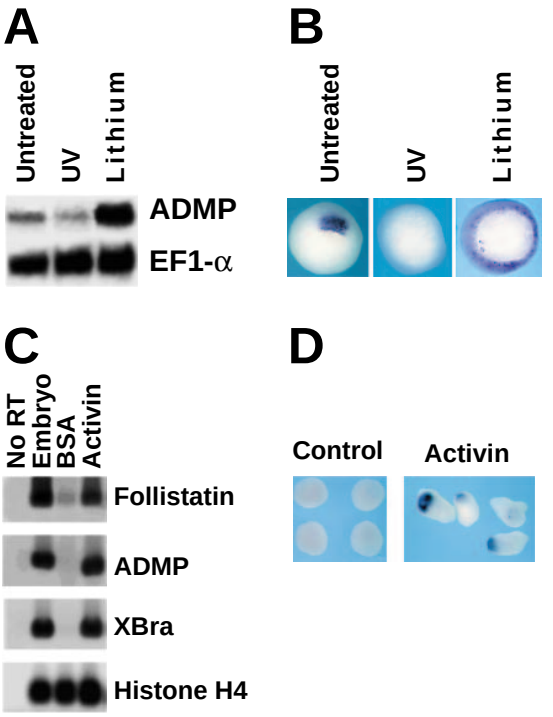

Fig. 6. Induction of ADMP by LiCl and activin. (A) Northern blots of poly(A^+) RNA from untreated, UV-irradiated or LiCl-treated embryos. UV irradiation decreased expression of ADMP; LiCl induced expression. EF1-α (Krieg et al., 1989) was used as a loading control. This experiment was performed four times. (B) Spatial expression pattern of ADMP in control, UV-irradiated and LiCl-treated embryos. Stage 11 embryos were used in A and B. (C) RT-PCR assay for ADMP in animal caps explanted at stage 8 and cultured with or without activin until sibling embryos reached stage 11. Histone H4 was used to normalize between samples (Niehrs et al., 1994). Follistatin and brachyury were assayed as positive controls. (D) Analysis of ADMP expression in caps cultured as above with and without activin by hybridization in situ. Both assays confirm induction of ADMP in response to activin.

Sokol et al., 1990 and Thomsen et al., 1990) and ventralizing (UV irradiation) perturbations on expression of the gene.

Lithium treatment produced a 4- to 8-fold increase in ADMP mRNA levels, while UV irradiation resulted in a 2- to 3-fold decrease (Fig. 6A). These changes in expression level were consistent with the results of hybridization in situ of treated embryos (Fig. 6B); the changes in expression pattern are similar to those observed for other organizer-specific genes. When embryos were injected with ADMP mRNA and subsequently treated with lithium, the dorsalizing effect of the latter was markedly reduced and several ventralized embryos resulted; control embryos injected with preprolactin mRNA before lithium treatment were all dorsalized to the same extent as noninjected embryos (not shown). When animal caps were collected at stage 8 and cultured with recombinant activin until sibling embryos reached stage 11, increased ADMP expression is readily detected with both RT-PCR and hybridization in situ (Fig. 6C,D). Similar effects on ADMP expression were observed in analogous experiments with animal cap explants from embryos injected with activin message at the 2-cell stage (not shown).

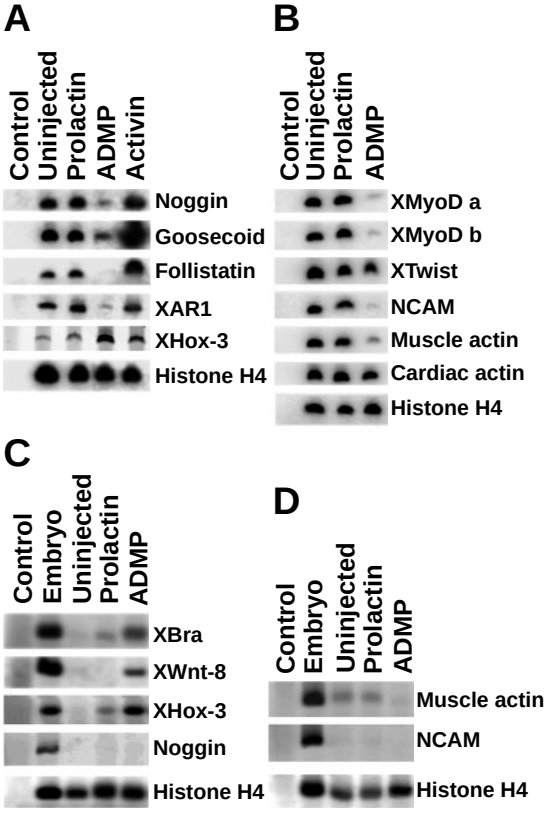

Fig. 7. Downregulation of dorsalizing signals and dorsal markers by ADMP. (A) Stage 10 or (B) stage 17 embryos were assayed for expression of the indicated genes by RT-PCR following injection of 1 ng ADMP message into one blastomere of 2-cell embryos. (C,D) Caps were explanted at stage 8 from embryos injected with ADMP message as above and cultured until sibling embryos reached stages 10.5 and 35, respectively; the indicated markers were then assayed by RT-PCR. Total RNA from pools of 10-20 embryos or caps was the template for cDNA synthesis in each case. Control indicates template from which reverse transcriptase was omitted to confirm absence of contaminating genomic DNA sequences. Separate RNA pools corresponding to the conditions displayed were prepared from each of at least three separate fertilizations; each pool was assayed at least twice for the indicated markers.

Injection of ADMP mRNA decreases expression of noggin, goosecoid, follistatin, NCAM and muscle actin

To explore the molecular consequences of ADMP overexpression, message levels for various signaling molecules and markers were analyzed by RT-PCR in stage 10.5 (Fig. 7A) or stage 17 (Fig. 7B) embryos injected with ADMP, preprolactin or activin mRNA. Injection of ADMP mRNA caused profound suppression of the organizer-specific markers noggin, goosecoid and follistatin, as well as the activin receptor. Dorsal markers, including XMyoD, NCAM and muscle actin, were also strongly downregulated. Increased expression of XHox-3, a marker for posterior ventral mesoderm (Ruiz i Altaba and Melton, 1989), was detectable in whole embryos. Increased expression of XHox-3, the ventral marker XWnt-8 (Christian et al., 1991; Lemaire and Gurdon, 1994), and the general mesoderm marker brachyury was apparent in animal caps explanted from ADMP-injected embryos at stage 8 and cultured until sibling embryos reached stage 11 (Fig. 7C); in caps cultured until siblings reached stage 35, NCAM and muscle actin were absent (Fig.7D). These results are consistent with the observed phenotype.

DISCUSSION

Anti-Dorsalizing Morphogenetic Protein is a novel member of the TGF-β family that is expressed in dorsal structures but suppresses their development

We have identified a novel gene, *ADMP*, whose product has characteristics of the Bone Morphogenetic Protein group of peptide growth factors. It is one of the first of these proteins found to be localized to the Spemann organizer. Unlike other factors normally expressed in this location during gastrulation (e.g., noggin (Smith and Harland, 1992), goosecoid (Cho et al., 1991), follistatin (Hemmati-Brivanlou et al., 1994)), ADMP causes profound axial suppression. Injection of ADMP message into dorsal blastomeres at the 4- to 8-cell stage does not produce obvious abnormalities during gastrulation, but does suppress development of dorsoanterior structures, including those of neural crest origin. This phenotype is consistent with the pronounced suppression of dorsalizing signals and markers of dorsal mesoderm and induction of ventral/posterior markers demonstrated by RT-PCR. These observations suggest diversion of mesodermal fate from dorsal/anterior to ventral/posterior.

ADMP causes axial suppression when overexpressed in its normal location; BMP-4 does not

ADMP affects phenotype and marker expression most effectively when overexpressed in its normal location. It is the only known TGF-β homolog with this behavior and the only known ventralizing factor expressed in the organizer. Another member of this family, BMP-4, has been shown to produce similar effects on the AP axis and XHox-3 when overexpressed by mRNA injection into dorsal blastomeres; this growth factor, however, is expressed ventrolaterally and is excluded from the

organizer (Fainsod et al., 1994). Recent experiments with truncated BMP-4 receptors (Graff et al., 1994; Harland, 1994; Maeno et al., 1994; Suzuki et al., 1994), which suggest that BMP-4 functions as a positive ventral inducer or competence modifier during normal development, are compatible with this expression pattern. Since significant cross-reactivity between various ligands and receptors in this family has been demonstrated, it is not surprising that two members of the family may demonstrate similar effects in mRNA injections, especially in view of the ability of many different members of the family to induce endochondral bone. Control of spatial expression domain is thus likely to prove of decisive importance in defining the roles that these growth factors play under normal physiological conditions.

Reciprocal signals expressed in the organizer

The behavior of ADMP is completely consistent with its own expression pattern as an organizer-specific gene; in particular it is induced by LiCl treatment and suppressed by UV irradiation. Unlike other genes induced by LiCl treatment, however, it demonstrates strong ventralizing activity. This is unexpected for genes expressed in the Spemann organizer in light of classical embryological evidence associating dorsalizing influences with this region of the embryo. Certain caveats apply to the interpretation of overexpression experiments; mistiming of expression or inappropriate heterodimerization with endogenous dorsalizing TGF-β-like peptides might be invoked to explain the results presented. Nevertheless, activin and BVg1, both closely related to ADMP, produce dorsalization when their mRNAs are injected and mRNA encoding Vg1 itself is without effect in this assay. The molecular activities of ADMP and BMP-4 are thus clearly distinct from those of activin and BVg1.

Another explanation for this apparent paradox is suggested by recent overexpression studies with follistatin, an antagonist of activin and presumably other members of the TGF-β family (Hemmati-Brivanlou et al., 1994), and a dominant negative activin receptor known to interfere with signaling by activin and BVg1. (Hemmati-Brivanlou and Melton, 1994; Schulte-Merker et al., 1994). These experiments suggest that interruption of TGF-β-like signaling causes exaggerated neural development and predict the presence of one or more dorsally localized members of this gene family that produce corresponding inhibitory effects. ADMP is expressed in a spatial and temporal pattern appropriate to such a signal and demonstrates biological activity consistent with this model.

Precise homeostatic regulatory mechanisms are an obvious requirement for proper execution of any complex biological process. Though attention has centered on inductive interactions since the time of Spemann, opposing roles for intracellular molecular signals have been proposed (Goldberg et al., 1973), and the possibility of negative regulation by members of the TGF-β superfamily during *Xenopus* development was advanced by Harland (Frank and Harland, 1992). The experiments with the truncated activin receptor and follistatin described earlier (Hemmati-Brivanlou and Melton, 1994; Hemmati-Brivanlou et al., 1994) provided additional evidence supporting this possibility.

Various models have depicted dorsalizing and ventralizing signals originating from different parts of the embryo to form overlapping concentration gradients (Dawid, 1994; Dawid, 1994; Cunliffe and Smith, 1994; Sive, 1993): organizer-associated proteins such as follistatin, noggin, goosecoid and Vg1 have been proposed as dorsalizing signals, and BMP-4 and Xwnt-8 (Christian and Moon, 1993), which are excluded from the organizer, are considered to act as positive ventral inducers. Indeed, it is possible that the induction of XWnt-8 observed in ADMP-loaded animal caps (Fig. 7A) may contribute to the overall ventralization seen in both histological and RT-PCR analysis of these caps. Since the expression patterns of XWnt-8 and ADMP do not overlap, it is less likely that this mechanism operates in vivo, however. Furthermore, whether induction of *Wnt-8* or the other ventrally expressed genes analyzed are primary or secondary effects remains to be determined.

Since members of the BMP family have been shown to bind with high affinity to elements of the extracellular matrix (Paralkar et al., 1990; Paralkar et al., 1991), it is likely that a number of these molecules act over very short distances. This characteristic would allow for a local feedback system that could provide precise spatial control. Thus, factors with distinct expression patterns may interact with different sets of signals.

The fact that dorsalizing and ventralizing perturbations produce opposite effects on ADMP expression suggests a possible utility for such a mechanism. If ADMP is indeed an endogenous negative influence on dorsalizing and neuralizing signals, it could be that ADMP acts as a moderating influence on such signals. The striking induction of ADMP expression produced by lithium treatment and activin, both strong dorsalizing signals, may represent such an action.

Another possible interpretation is suggested by the observation that during establishment of the neural axis, expression of ADMP is concentrated in the posterior region of the neural plate. This pattern, in view of its effect on phenotype in mRNA injection experiments, raises the possibility that ADMP is one signal that plays a role in the phenomenon of posterior dominance within the neural plate (Slack and Tannahill, 1992). It is thus possible that a signal with the activities described above may function naturally to help establish posterior structures. The significance of its later expression in the prechordal plate remains unexplained.

Conclusion

ADMP is a novel member of the TGF-β superfamily that is expressed in the Spemann organizer. It is the only known growth factor to be expressed in this location that suppresses the anteroposterior axis; other factors expressed in the organizer have dorsalizing and/or neuralizing activities. ADMP produces these effects when overexpressed in its normal location; BMP-4, the other TGF-β homolog known to be capable of suppressing the AP axis, can do so only when overexpressed ectopically. Since it produces profound suppression of neural structures, downregulates known dorsalizing signals, and is induced by dorsalizing perturbations, ADMP has properties expected for a TGF-β-like neural suppressive signal. These characteristics are in accord with existing evidence for TGF-β-like signaling molecules that interfere with specification of neural tissue (Hemmati-Brivanlou and Melton, 1994; Hemmati-Brivanlou et al., 1994) and with known properties of TGF-β proteins. We therefore suggest that ADMP may function in this capacity. Since

ADMP is induced by dorsalizing influences such as lithium chloride and activin, it may modulate the dorsalizing influences normally present in the Spemann organizer as part of a local homeostatic mechanism.

The authors owe a special debt to the faculty of the Cold Spring Harbor course on the early development of *Xenopus laevis*. In particular, we would like to thank Hazel Sive, Robert Grainger, Richard Harland, Richard Elinson and Enrique Amaya for instruction and reagents essential to this work. We would also like to thank Judy Kassis and Milan Jamrich for use of photomicrographic equipment and members of the Jamrich laboratory for graciously allowing access to frog facilities and microinjection equipment and for introducing us to this field. The support and advice of Frank Luyten of NIDR was essential to the execution of this work. Finally, we thank Igor Dawid of NICHD, Shannon Dixon of NIAAA, Logan Kleinwaks of CBER, Michael Kuehn of NCI and Frank Luyten of NIDR for critical review of the manuscript.

REFERENCES

Amaya, E., Musci, T. J. and Kirschner, M. W. (1991). Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in Xenopus embryos. *Cell* **66,** 257-270.

Basler, K., Edlund, T., Jessell, T. M. and Yamada, T. (1993). Control of cell pattern in the neural tube: regulation of cell differentiation by dorsalin-1, a novel TGFβ family member. *Cell* **73,** 687-702.

Bolce, M. E., Hemmati-Brivanlou, A. and Harland, R. M. (1993). XFKH2, a Xenopus HNF-3 alpha homologue, exhibits both activin-inducible and autonomous phases of expression in early embryos. *Dev. Biol.* **160,** 413-423.

Chang, S., Hoang, B., Thomas, J. T., Vukicevic, S., Luyten, F. P., Ryba, N. J. P., Filie, J. D., Kozak, C. A., Reddi, A. H. and Moos, M. (1994). Cartilage-Derived Morphogenetic Proteins: New members of the TGF-β superfamily predominantly expressed in long bones during human embryonic development. *J. Biol. Chem.* **269,** 28227-28234.

Cho, K. W., Blumberg, B., Steinbeisser, H. and De Robertis, E. M. (1991). Molecular nature of Spemann's organizer: the role of the Xenopus homeobox gene goosecoid. *Cell* **67,** 1111-1120.

Chomczynski, P. (1992). One hour downward alkaline capillary transfer for blotting of DNA and RNA. *Anal. Biochem.* **201,** 134-139.

Christian, J. L., McMahon, J. A., McMahon, A. P. and Moon, R. T. (1991). *XWnt*-8, a *Xenopus Wnt-1/int-1*-related gene responsive to mesoderm-inducing growth factors, may play a role in ventral mesodermal patterning during embryogenesis. *Development* **111,** 1045-1055.

Christian, J. L. and Moon, R. T. (1993). Interactions between Xwnt-8 and Spemann organizer signaling pathways generate dorsoventral pattern in the embryonic mesoderm of Xenopus. *Genes Dev.* **7,** 13-28.

Cunliffe, V. and Smith, J. C. (1994). Specification of mesodermal pattern in Xenopus laevis by interactions between Brachyury, noggin and Xwnt-8. *EMBO J.* **13,** 349-359.

Dawid, I. B. (1994). Intercellular signaling and gene regulation during early embryogenesis of Xenopus laevis. *J. Biol. Chem.* **269,** 6259-6262.

Dawid, I. B. (1994). Embryonic induction and axis formation in *Xenopus laevis*: growth factor action and early response genes. In *Growth Factors and Signal Transduction in Development.* New York: Wiley-Liss.

Edwards, J. B., Delort, J. and Mallet, J. (1991). Oligodeoxyribonucleotide ligation to single-stranded cDNAs: a new tool for cloning 5′ ends of mRNAs and for constructing cDNA libraries by in vitro amplification. *Nucleic Acids Res.* **19,** 5227-5232.

Fainsod, C., Steinbeisser, H. and De Robertis, E. M. (1994). On the function of BMP-4 in patterning the marginal zone of the *Xenopus* embryo. *EMBO J.* **13,** 5015-5025.

Farrell, R. E. (1993). *RNA Methodologies.* San Diego: Academic Press.

Frank, D. and Harland, R. M. (1992). Localized expression of a Xenopus POU gene depends on cell-autonomous transcriptional activation and induction-dependent inactivation. *Development* **115,** 439-448.

Goldberg, N. D., O'Dea, R. F. and Haddox, M. K. (1973). Cyclic GMP. *Adv Cyclic Nucleotides Res* **3,** 155-223.

Graff, J. M., Thies, R. S., Song, J. J., Celeste, A. J. and Melton, D. A. (1994). Studies with a Xenopus BMP receptor suggest that ventral mesoderm inducing signals override dorsal signals in vivo. *Cell* **79,** 169-179.

Gurdon, J. B. (1967). In *Methods in Developmental Biology*. New York: Crowell.

Gurdon, J. B. (1977). Methods for nuclear transplantation in amphibia. *Methods Cell Biol* **16,** 125-139.

Hammonds, R. G. J., Schwall, R., Dudley, A., Berkemeier, L., Lai, C., Lee, J., Cunningham, N., Reddi, A. H., Wood, W. I. and Mason, A. J. (1991). Bone-inducing activity of mature BMP-2b produced from a hybrid BMP-2a/2b precursor. *Mol Endocrinol.* **5,** 149-155.

Harland, R. M. (1991). In situ hybridization: An improved whole-mount method for Xenopus embryos. *Methods Cell Biol.* **36,** 685-695.

Harland, R. M. (1994). The transforming growth factor β family and induction of the vertebrate mesoderm: Bone morphogenetic proteins are ventral inducers. *Proc. Natl Acad. Sci. USA* **91,** 10243-10246.

Hemmati-Brivanlou, A., Kelly, O. G. and Melton, D. A. (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell* **77,** 283-295.

Hemmati-Brivanlou, A. and Melton, D. A. (1994). Inhibition of activin receptor signaling promotes neuralization in Xenopus. *Cell* **77,** 273-281.

Kao, K. R. and Elinson, R. P. (1988). The entire mesodermal mantle behaves as Spemann's organizer in dorsoanterior enhanced Xenopus laevis embryos. *Dev. Biol.* **127,** 64-77.

Kay, B. K. (1991). Injection of oocytes and embryos. *Methods Cell Biol.* **36,** 663-669.

Kingsley, D. M. (1994). The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev.* **8,** 133-146.

Kozak, M. (1991). Structural features in eukaryotic mRNAs that modulate the initiation of translation. *J Biol. Chem.* **266,** 19867-19870.

Krieg, P. A., Varnum, S. M., Wormington, W. M. and Melton, D. A. (1989). The mRNA encoding elongation factor 1-alpha (EF-1 alpha) is a major transcript at the midblastula transition in *Xenopus*. *Dev. Biol.* **133,** 93-100.

Lamb, T. M., Knecht, A. K., Smith, W. C., Stachel, S. E., Economides, A. N., Stahl, N., Yancopolous, G. D. and Harland, R. M. (1993). Neural induction by the secreted polypeptide noggin. *Science* **262,** 713-718.

Lemaire, P. and Gurdon, J. B. (1994). A role for cytoplasmic determinants in mesoderm patterning: cell-autonomous activation of the goosecoid and Xwnt-8 genes along the dorsoventral axis of early Xenopus embryos. *Development* **120,** 1191-1199.

Lin, L.-F. H., Doherty, D. H., Lile, J. D., Bektesh, S. and Collins, F. (1993). GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* **260,** 1130-1132.

Maeno, M., Ong, R. C., Suzuki, A., Ueno, N. and Kung, H.-F. (1994). A truncated bone morphogenetic protein 4 receptor alters the fate of ventral mesoderm: Roles of animal pole tissue in the development of ventral mesoderm. *Proc. Natl Acad. Sci. USA* **91,** 10260-10264.

Niehrs, C., Keller, R., Cho, K. W. and De Robertis, E. M. (1993). The homeobox gene goosecoid controls cell migration in Xenopus embryos. *Cell* **72,** 491-503.

Niehrs, C., Steinbeisser, H. and De Robertis, E. M. (1994). Mesodermal patterning by a gradient of the vertebrate homeobox gene goosecoid. *Science* **263,** 817-820.

Nieuwkoop, P. D. and Faber, J. (1967). *Normal table of Xenopus laevis (Daudin).* Amsterdam: North Holland.

Ozkaynak, E., Schnegelsberg, P. N., Jin, D. F., Clifford, G. M., Warren, F. D., Drier, E. A. and Oppermann, H. (1992). Osteogenic protein-2. A new member of the transforming growth factor-beta superfamily expressed early in embryogenesis. *J. Biol. Chem.* **267,** 25220-25227.

Padgett, R. W., St. Johnston, R. D. and Gelbart, W. M. (1987). A transcript from a Drosophila pattern gene predicts a protein homologous to the transforming growth factor-beta family. *Nature* **325,** 81-84.

Paralkar, V. M., Nandedkar, A. K. N., Pointer, R. H., Kleinman, H. K. and Reddi, A. H. (1990). Interaction of Osteogenin, a heparin binding bone morphogenetic protein, with type IV collagen. *J. Biol. Chem.* **265,** 17281-17284.

Paralkar, V. M., Vukicevic, S. and Reddi, A. H. (1991). Transforming growth factor beta type 1 binds to collagen IV of basement membrane matrix: implications for development. *Dev. Biol.* **143,** 303-308.

Peng, H. B. (1991). Solutions and Protocols. *Methods Cell Biol.* **36,** 657-662.

Reddi, A. H. (1992). Regulation of cartilage and bone differentiation by bone morphogenetic proteins. *Curr. Opin. Cell Biol.* **4,** 850-855.

Richter, K., Grunz, H. and Dawid, I. B. (1988). Gene expression in the

embryonic nervous system of *Xenopus laevis*. *Proc. Natl Acad. Sci. USA* **85,** 8086-8090.

Ruiz i Altaba, A. and Melton, D. A. (1989). Bimodal and graded expression of the Xenopus homeobox gene Xhox3 during embryonic development. *Development* **106,** 173-183.

Rupp, R. A. and Weintraub, H. (1991). Ubiquitous MyoD transcription at the midblastula transition precedes induction-dependent MyoD expression in presumptive mesoderm of X. laevis. *Cell* **65,** 927-937.

Sampath, T. K., Maliakal, J. C., Hauschka, P. V., Jones, W. K., Sasak, H., Tucker, R. F., White, K. H., Coughlin, J. E., Tucker, M. M. and Pang, R. H. (1992). Recombinant human osteogenic protein-1 (hOP-1) induces new bone formation in vivo with a specific activity comparable with natural bovine osteogenic protein and stimulates osteoblast proliferation and differentiation in vitro. *J. Biol. Chem.* **267,** 20352-20362.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl Acad. Sci. USA* **74,** 5463-5467.

Sato, S. M. and Sargent, T. D. (1989). Development of neural inducing capacity in dissociated Xenopus embryos. *Dev. Biol.* **134,** 263-266.

Schulte-Merker, S., Smith, J. C. and Dale, L. (1994). Effects of truncated activin and FGF receptors and of follistatin on the inducing activities of BVg1 and activin: does activin play a role in mesoderm induction? *EMBO J.* **13,** 3533-3541.

Sive, H. L. (1993). The frog prince-ss: a molecular formula for dorsoventral patterning in Xenopus. *Genes Dev.* **7,** 1-12.

Slack, J. M. and Tannahill, D. (1992). Mechanism of anteroposterior axis specification in vertebrates. Lessons from the amphibians. *Development* **114,** 285-302.

Smith, W. C. and Harland, R. M. (1991). Injected Xwnt-8 RNA acts early in Xenopus embryos to promote formation of a vegetal dorsalizing center. *Cell* **67,** 753-765.

Smith, W. C. and Harland, R. M. (1992). Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in Xenopus embryos. *Cell* **70,** 829-840.

Sokol, S., Wong, G. G. and Melton, D. A. (1990). A mouse macrophage factor induces head structures and organizes a body axis in *Xenopus*. *Science* **249,** 561-564.

Sokol, S., Christian, J. L., Moon, R. T. and Melton, D. A. (1991). Injected Wnt RNA induces a complete body axis in Xenopus embryos. *Cell* **67,** 741-752.

Spemann, H. and Mangold, H. (1924). Über Induktion von Embryonanlangen durch Implantation artfremder Organisatoren. *Wilhelm Roux's Arch. EntwMech. Org.* **100,** 599-638.

Storm, E. E., Huynh, T. V., Copeland, N. G., Jenkins, N. A., Kingsley, D. M. and Lee, S. J. (1994). Limb alterations in brachypodism mice due to mutations in a new member of the TGF beta-superfamily. *Nature* **368,** 639-643.

Suzuki, A., Thies, R. S., Yamaji, N., Song, J. J., Wozney, J. M., Murakami, K. and Ueno, N. (1994). A truncated bone morphogenetic protein receptor affects the dorsal-ventral patterning in the early *Xenopus* embryo. *Proc. Natl Acad. Sci. USA* **91,** 10255-10259.

Thomsen, G., Woolf, T., Whitman, M., Sokol, S., Vaughan, J., Vale, W. and Melton, D. A. (1990). Activins are expressed early in Xenopus embryogenesis and can induce axial mesoderm and anterior structures. *Cell* **63,** 485-493.

Urist, M. R. (1965). Bone formation by autoinduction. *Science* **150,** 893-899.

von Heijne, G. (1986). A new method for predicting signal sequence cleavage sites. *Nucl. Acids Res.* **14,** 4683-4690.

Wang, E. A., Rosen, V., Cordes, P., Hewick, R. M., Kriz, M. J., Luxenberg, D. P., Sibley, B. S. and Wozney, J. M. (1988). Purification and characterization of other distinct bone-inducing factors. *Proc. Natl Acad. Sci. USA* **85,** 9484-9488.

Wang, S., Krinks, M. and Moos, M. (1995). DNA Sequencing from single phage plaques using solid-phase magnetic capture. *Biotechniques* **18,** 130-135.

Weeks, D. L. and Melton, D. A. (1987). A maternal mRNA localized to the vegetal hemisphere in Xenopus eggs codes for a growth factor related to TGF-beta. *Cell* **51,** 861-867.

Wharton, K. A., Thomsen, G. H. and Gelbart, W. M. (1991). Drosophila 60A gene, another transforming growth factor beta family member, is closely related to human bone morphogenetic proteins. *Proc. Natl Acad. Sci USA* **88,** 9214-9218.

Wozney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M. and Wang, E. A. (1988). Novel regulators of bone formation: molecular clones and activities. *Science* **242,** 1528-1534.

(Accepted 20 August 1995)