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Dorsalizing and neuralizing properties of Xdsh, a maternally expressed *Xenopus* homolog of *dishevelled*

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SUMMARY

Signaling factors of the Wnt proto-oncogene family are implicated in dorsal axis formation during vertebrate development, but the molecular mechanism of this process is not known. Studies in *Drosophila* have indicated that the *dishevelled* gene product is required for *wingless* (Wnt1 homolog) signal transduction. We demonstrate that injection of mRNA encoding a *Xenopus* homolog of *dishevelled* (Xdsh) into prospective ventral mesodermal cells triggers a complete dorsal axis formation in *Xenopus* embryos. Lineage tracing experiments show that cells derived from the injected blastomere contribute to anterior and dorsal structures of the induced axis. In contrast to its effect on mesoderm, overexpression of *Xdsh* mRNA in prospective ectodermal cells triggers anterior neural tissue differentiation. These studies suggest that Wnt signal transduction pathway is conserved between *Drosophila* and vertebrates and point to a role for maternal Xdsh product in dorsal axis formation and in neural induction.

Key words: *Xenopus*, Wnt, *dishevelled*, dorsal axis formation, neuralizing activity

INTRODUCTION

An amphibian egg is laid with a clear animal-vegetal polarity, but its dorsoventral axis is not specified. Dorsoventral differences are specified quite early in *Xenopus* development as a result of a cortico-cytoplasmic rotation that occurs soon after fertilization. During this microtubule-mediated displacement of internal egg cytoplasm relative to the cell cortex (Gerhart et al., 1989; Elinson and Rowning, 1988), dorsal cytoplasm acquires an ability to trigger dorsal development upon microinjection into a ventral blastomere (Fujisue et al., 1993; Holowacz and Elinson, 1993). Two models may be proposed to explain dorsoventral patterning of mesoderm. According to the ‘permissive’ model of dorsoventral patterning, the axis-inducing activity is mediated by maternally encoded factor(s), called dorsal determinants or modifiers, which cause a local change in marginal zone cell competence to mesoderm-inducing signals produced by vegetal pole cells (Sokol and Melton, 1991; Moon and Christian, 1992). As a result of this change, not only mesoderm is induced, but it becomes polarized (or regionalized) into future dorsal (notochord, muscle) and ventral (mesenchyme, kidney, blood) tissues. According to the ‘instructive’ model, multiple inducers or different levels of a single inducer directly specify formation of mesoderm with different dorsal or ventral character (Nieuwkoop, 1973; Dale and Slack, 1987b).

Whereas soluble peptide growth factors from the TGFβ and FGF families are thought to play a role in mesoderm induction (see Smith, 1993; Dawid, 1991, for reviews), two other classes of secreted polypeptides, Wnts, related to the int-1 (Wnt1) proto-oncogene product (Sokol et al., 1991; Smith and Harland, 1991), and noggin (Smith and Harland, 1992) have been shown to affect dorsoventral patterning of embryonic mesoderm. Although low levels of noggin mRNA are detected maternally (Smith and Harland, 1992), it is mainly expressed in the Spemann organizer region after the midblastula transition and the onset of zygotic transcription (Newport and Kirschner, 1982). Noggin has been shown to possess both neuralizing and dorsalizing activities (Smith et al., 1993; Lamb et al., 1993), suggesting that it mediates some of the Spemann organizer activities.

Different members of the Wnt family are expressed in specific regions of the embryos of various species and have been implicated in *Drosophila* segmentation, murine central nervous system development and in MMTV-induced mammary gland carcinogenesis (see Dickinson and McMahon, 1992; Nusse and Varmus, 1992, for reviews). Several Wnt products have been shown to induce dorsal axis formation in *Xenopus* embryos (Moon et al., 1993; Klein and Melton, 1994). Since these Wnts are not expressed at the right time to perform this function during normal development (Moon et al., 1993), they were proposed to mimic yet unknown Wnt product(s) and to affect the same signal transduction pathway that operates in the embryo. Interestingly, a maternal Wnt, Xwnt11, has been identified which is capable of induction of a partial dorsal axis (Ku and Melton, 1993).

While the molecular mechanism by which a Wnt signal is transmitted in embryonic cells is unknown, genetic studies in *Drosophila* have implicated products of several segment polarity genes: *wingless* (*wg*, a homolog of *Wnt1* gene), *dishevelled (dsh)* and *armadillo (arm)* in this pathway. Indeed, the phenotypes of these segment polarity mutants are almost identical as judged by similar cuticular defects, lack of segmental furrows, fusion of tracheal pits and a characteristic pattern of cell death (Perrimon, 1994). In cells responding to *wg* signaling, all three genes are required for the correct expression of the target gene *engrailed* (Perrimon, 1994). The *dsh* product is required for the stimulatory effect of *wg* on *arm* protein (Riggleman et al., 1990; Noordermeer et al., 1994), which is a homolog of mammalian plakoglobin and β-catenin (McCrea et al., 1991). In addition to these gene products, *zeste-white 3*, a homolog of mammalian glycogen synthase kinase (Woodgett, 1990), has been shown to suppress the *wg* effect on *arm* and to function in the same pathway downstream of *dsh* (Siegfried et al., 1992, 1994).

The phenotype of flies overexpressing *wg* is completely suppressed in *dsh* mutants (Noordermeer et al., 1994; Siegfried et al., 1994), indicating that the *dsh* product is required for *wg* to function. Clonal analysis of *dsh* mutations during either embryonic or imaginal development has shown that cells with a mutant *dsh* product display the mutant phenotype and cannot be rescued by the *dsh* product supplied by neighboring wild-type cells (Klingensmith et al., 1994; Theisen et al., 1994). Thus, *dsh* functions cell autonomously and is likely to participate in the reception or interpretation of the *wg* signal by the responding cells. Taken together, these observations suggest that the *dsh* protein is a critical regulatory element of the Wnt signal transduction pathway.

To test for the possible involvement of *dishevelled* in dorsoventral patterning in vertebrate embryos, we isolated a *Xenopus* homolog of *dsh (Xdsh)*. The results of this study, which evaluates the role of *Xdsh* in *Xenopus* development, suggest that dorsal body axis is determined through the Wnt signaling pathway and that this pathway is evolutionarily conserved between *Drosophila* and vertebrates.

MATERIALS AND METHODS

Eggs and embryos

Eggs were obtained by injecting *Xenopus laevis* females with 800 units of human chorionic gonadotropin. Fertilization and embryo culture were done in 0.1× MMR (1× MMR =100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM Hepes (pH 7.6), 0.1 mM EDTA) as described (Newport and Kirschner, 1982). Staging was according to Nieuwkoop and Faber (1967). Ultraviolet light irradiation was performed similar to Scharf and Gerhart (1980). Embryos in a plastic chamber with a Saran Wrap bottom were placed on the surface of a UV lamp (UVG-11, 254 nm) and were irradiated for 1 minute within 30-35 minutes after fertilization. The optimal time of exposure to UV light was determined in preliminary experiments. Only experiments on embryos with an average dorsoanterior index (DAI) of less than 1 (Kao and Elinson, 1988) were taken into account.

Cloning, in vitro transcription and microinjection of embryos with RNA

The 0.7 kb *Xho*1-*Pst*1 fragment of the *Drosophila dishevelled* cDNA (Klingensmith et al., 1994) was labeled by random hexanucleotide priming (Sambrook et al., 1989). The labeled fragment was used to probe a *Xenopus* oocyte λgt10 cDNA library (Rebagliati et al., 1985)

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      1                                                                    70
Xdsh  .......MAE TKVIYHLDEE ETPYLVKVPV PATDIRLSDF KAALG..RGH A.KYFFKAMD QDFGVVKEEI
 Dvl  .......MAE TKIIYHMDEE ETPYLVKLPV APERVTLADF KNVLSNRPVH AYKFFFKSMD QDFGVVKEEI
 Dsh  MDADRGGGQE TKVIYHIDDE TTPYLVKIPI PSAQVTLRDF KLVL.NKQNN NYKYFFKSMD ADFGVVKEEI

      71                                                                  140
Xdsh  SDDKAKRPCF NDRVVSWLAS SEGS.QPDSA PPAPATEVSP EPPPPVPPPI PPPPAESTSG IGDSRPPSFH
 Dvl  FDDNAKLPCF NGRVVSWLVL AEGA.HSDAG SQGTDSHTD. .......... LPPPLERTGG IGDSRPPSFQ
 Dsh  ADDSTILPCF NGRVVSWLVS ADGTNQSDNC SELPTSEC.. .......... .....ELGMG LTNRKLQQQQ

      141                                                                 210
Xdsh  PNVSGSTEQL DQD..NESVI SMRSDRVSRS ESSEQAGVGS GVNGRTERHL ...S.GYESS STLLTSEIE.
 Dvl  PNVASSRDGM DNETGTESMV SHRRERARRR NRDEAARTNG HPRGDRRRDL ...GLPPDSA STVLSSELES
 Dsh  QQHQQQQQQQ QQQHQQQQQQ QQQQVQPVQL AQQQQQQVLH HQKMMGNPLL QPPPLTYQSA S.VLSSDLDS

      211                                                                 280
Xdsh  TSICDSEEDD TMSSFSSSTE QSSASRLLKR HR.RRRKQSP PRLERTSSFS SVTDSTMSLN IITVTLNMEK
 Dvl  SSFIDSDEED NTSRLSSSTE QSTSSRLVRK HKCRRRKQRL RQTDRASSFS SITDSTMSLN IITVTLNMER
 Dsh  TSLFGTESEL TLDR..DMTD YSSVQRLQVR KKPQRRKKRA PSMSRTSSYS SITDSTMSLN IITVSINMEA

      281                                                                 350
Xdsh  YNFLGISIVG QSNERGDGGI YIGSMMKGGA VAADGSIEPG DMLLQVNDIN FENMSNDDAV RVLSDIVHKP
 Dvl  HHFLGISIVG QSNDRGDGGI YIGSIMKGGA VAADGRIEPG DMLLQVNDVN FENMSNDDAV RVLREIVSQT
 Dsh  VNFLGISIVG QSNRGGDGGI YVGSIMKGGA VALDGRIEPG DMILQVNDVN FENMTNDEAV RVLREVVQKP

      351                                                                 420
Xdsh  GPIVLTVAKC WDPSPQGYFT LPRNEPIHPI DPAAWVSHSA ALSGSFPVYP GSASMSSMTS STSVTETELS
 Dvl  GPISLTVAKC WDPTPRSYFT IPRADPVRPI DPAAWLSHTA ALTGALPRYG TSPCSSAITR TSSSSLTSSV
 Dsh  GPIKLVVAKC WDPNPKGYFT IPRTEPVRPI DPGAWVAHTQ AL........ ........TS HDSIIADIAE

      421                                                                 490
Xdsh  HALPPVSLFS LSVHTDLASV VKVMASPESG LEVRDRMWLK ITIPNAFLGS DVVDWLYHHV EGFQDRREAR
 Dvl  PGAPQLEEAP LTVKSDMSAI VRVMQLPDSG LEIRDRMWLK ITIANAVIGA DVVDWLYTHV EGFKERREAR
 Dsh  PIKERLD... ...QNNLEEI VKAMTKPDSG LEIRDRMWLK ITIPNAFIGA DAVNWVLENV EDVQDRREAR

      491                                                                 560
Xdsh  KFASNLLKAG FIRHTVNKIT FSEQCYYIFG DLTGCENY.. .MTNL.SLND NDGSSGASDQ DT......LA
 Dvl  KYASSMLKHG FLRHTVNKIT FSEQCYYVFG DLCS...... ...NLASLNL NSGSSGASDQ DT......LA
 Dsh  RIVSAMLRSN YIKHTVNKLT FSEQCYYVVN EERNPNLLGR GHLHPHQLPH GHGGHALSHA DTESITSDIG

      561                                                                 630
Xdsh  PLPLPGASPW PLLPTFSYQY QAPHPYSTQP PAYHELSSYS YGMGSAGSQH SEGSSSSGSN RSDGGRGMQK
 Dvl  PLPHPSV.PW PLGQGYPYQY PGPPPCF..P PAYQD.PGFS CGSGSAGSQQ SEGSKSSGST RSSHRTPGRE
 Dsh  PLPNP..... PIYMPYSATY NPSHGY..QP IQY....... ...GIAERHI SSGSSSSDVL TSKDTSASQS

      631                                                                 700
Xdsh  DDRSGVAGVG GGDSKSGSCS ESEYSTRSSM SSVGGGEAGP PSERSTSSRL PPHHPPSVHS YAAPGVPLSY
 Dvl  ERRATGAG.. ......GSGS ESDHTVPSGS GSTG...... WWERPVSQLS RGSSPRSQAS AVAPGL....
 Dsh  DITSVIHQAN QLTIAAHGSN KSSGSSNRGG GGGGGGGGNN TNDQDVSVSN YVL*...... ..........

      701                                                            765
Xdsh  NPMMLMMMPP PPLPPPGVCP PNSSVPPGAP PLVRDLASVP PELTATRQSF HMAMGNPSEF FVDVM*
 Dvl  .......... PPLHPLTKAY AVVGGPPGGP P.VRELAAVP PELTGSRQSF QKAMGNPCEF FVDIM*
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Fig. 1. The deduced Xdsh amino acid sequence and its comparison with Drosophila dsh and mouse dsh homolog (Dvl) proteins. The Xdsh ORF starts with the first available methionine and encodes a putative protein of 736 residues. Proline-rich sequences are in italics. A *discs large* homology region (DHR) is underlined.

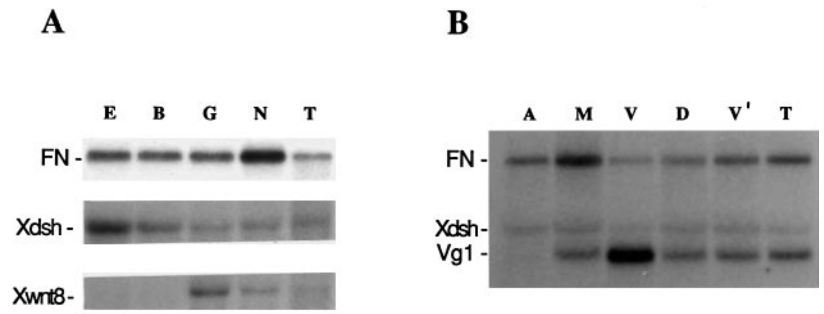

Fig. 2. Xdsh transcripts are present maternally and are equally distributed in different regions of the early blastula. Total RNA isolated from embryos at different developmental stages or from embryonic explants was analyzed by Northern blotting with specific antisense RNA probes. (A) Expression of *Xdsh* during embryogenesis: E, fertilized eggs; B, stage 7 blastulae; G, stage 11 gastrulae; N, stage 15 neurulae and T, tailbud embryos. (B) Spatial distribution of the Xdsh transcripts in stage 7 blastulae. Explants were isolated from A, animal; M, marginal; V, vegetal; D, dorsal; V′, ventral regions; T, RNA prepared from whole embryos. Two embryo equivalents of total RNA were loaded per each lane. FN, Xwnt8 and Vg1-specific probes were used as controls. Fibronectin RNA (FN) is a control for loading. Xwnt8 transcripts are known to appear only after the midblastula transition (Christian et al., 1991). Vg1 RNA is a vegetally localized maternal mRNA (Rebagliati et al., 1985).

under low stringency conditions as described (Sambrook et al., 1989). A 3.3 kb insert, containing full length *Xdsh* cDNA, was subcloned into the *Eco*RI site of the pBluescript-SK vector (Stratagene), and both DNA strands were sequenced. Alignment of the deduced Xdsh amino acid sequence with the sequences of the *Drosophila* and mouse dsh proteins was carried out using the PILEUP program of the Computer Genetics Group (Madison, WI).

To overexpress the Xdsh product in embryos, the 3.3 kb *Xdsh* cDNA fragment was subcloned into the *Eco*RI site of the pSP64R1 vector, a modified version of pSP64T (Vize et al., 1991), which contains several convenient cloning sites and allows in vitro synthesis of efficiently translated mRNAs. A control out-of-frame Δ*Xdsh* construct was made by digesting the plasmid containing *Xdsh* cDNA with *Apa*I, followed by filling-in protruding ends with Klenow

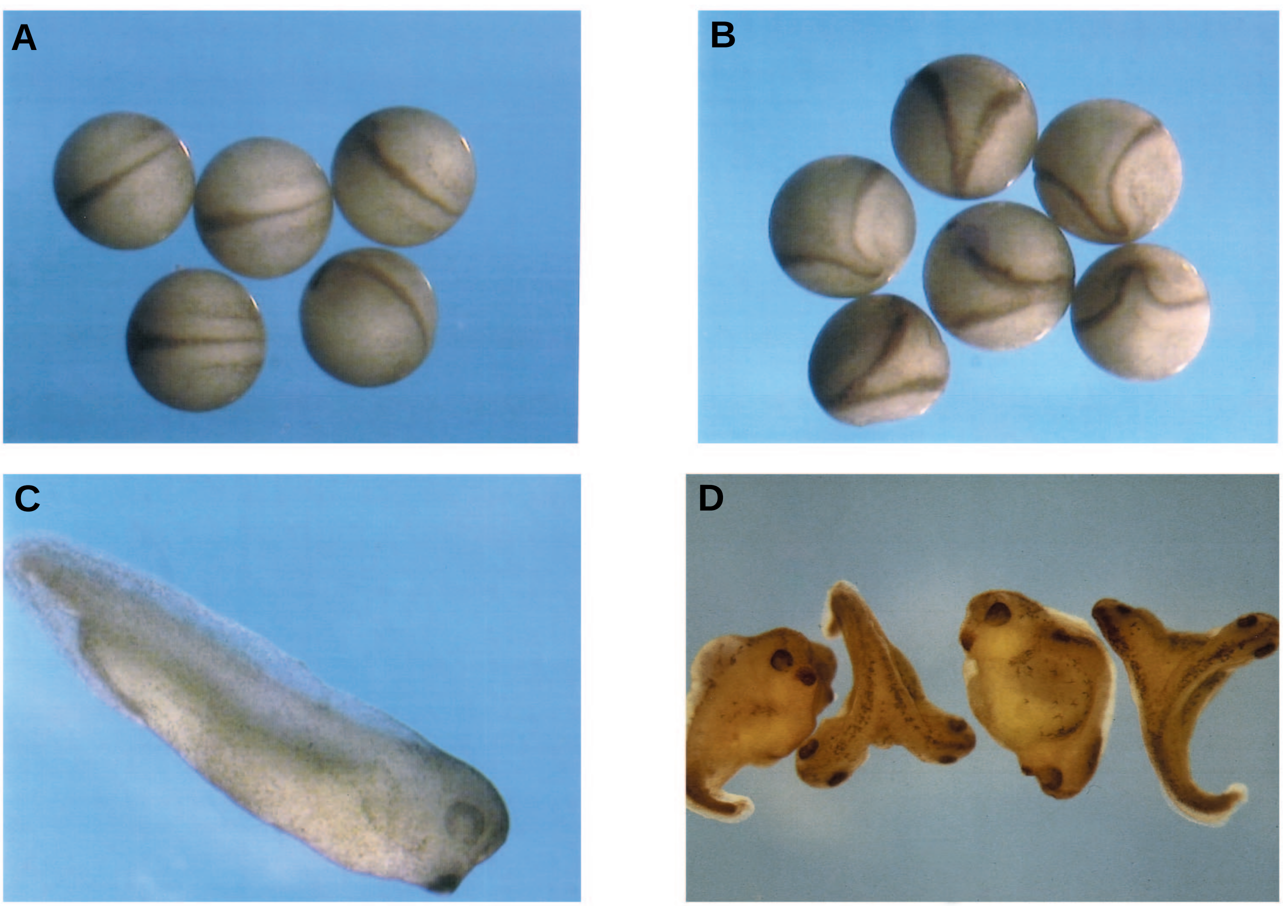

Fig. 3. The effect of *Xdsh* mRNA depends on the site of injection. A single prospective dorsal (A) or ventral (B,D) vegetal blastomere of cleavage-stage embryos (8-16 cells) was injected with 0.4 ng of *Xdsh* mRNA. Phenotypes of the injected neurulae (A,B) and of tadpoles at stages 40-42 (C,D) are presented. Axis duplications are clearly visible in embryos injected with *Xdsh* RNA in a ventral blastomere (B,D). Embryos, injected ventrally with 0.4 ng of a control Δ*Xdsh* mRNA (C), are indistinguishable from normal tadpoles or from the tadpoles, injected dorsally with *Xdsh* mRNA (as in A). Note that three embryos in D have completely duplicated body axes including most anterior and posterior structures, whereas in one embryo both dorsal axes oppose each other and posterior development is inhibited.

Table 1. Injected *Xdsh* mRNA triggers a second dorsal axis formation

mRNA injected	Total number of injected embryos	Partial axis duplications	Complete axis duplications	Other defects	No effect
Xdsh	78	28 (36%)	42 (54%)	6 (8%)	2 (2.8%)
ΔXdsh	62	0	0	7 (11%)	55 (89%)

Embryos were injected at the 4- to 8-cell stage into a single ventrovegetal blastomere with 1 ng of a mRNA and left to develop to stage 40. Partial axis duplication was scored positive when a second neural tube was visible. Complete axis duplication was scored positive when both cement glands and eyes were duplicated. Other defects included incomplete closure of the blastopore and anterior/posterior deficiencies in the primary axis. Data from three independent experiments are presented. Death rate was less than 5%.

enzyme and re-ligating the construct. A plasmid encoding β-galactosidase was a gift of R. Harland.

Capped synthetic RNAs were generated as described (Krieg and Melton, 1984) by in vitro transcription of different plasmids using SP6 RNA polymerase. Embryos, incubated in 3% Ficoll, 0.5× MMR were injected with 10 nl of RNA solution in distilled water at 8- to 16-cell stage into a single blastomere. After 1-2 hours of incubation, the medium was changed to 0.1× MMR with 50 μg/ml of gentamicin for long-term culture. Death rate for the injected embryos was usually below 5%. The prospective dorsal and ventral sides were determined by pigmentation differences in the early embryo (Nieuwkoop and Faber, 1967). Prospective ventral blastomeres are more heavily pigmented than their dorsal counterparts. The accuracy of this determination was tested in each experiment by allowing a group of control embryos to develop to determine whether the pigmentation differences correctly predicted the position of dorsal blastopore lip. The usual error of such determinations was 5-7%.

Lineage tracing and histology

Cleavage-stage embryos (8- to 32-cell stages) were injected with 10 nl of a solution containing 0.2-0.4 ng of *Xdsh* mRNA and 0.2 ng of β-gal RNA in water. After two days of culturing in 0.1× MMR, embryos were fixed in MEMFA (0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM MgSO4 and 3.7% formaldehyde; Hemmati-Brivanlou and Harland, 1989) for 30 minutes. To detect β-gal activity, embryos were rinsed in PBS and incubated with 1 mg/ml X-Gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6 \times 3H_2O$, 2 mM $MgCl_2$ in PBS. The time of staining varied from 20 minutes to several hours at room temperature depending on the desired intensity.

For histology, embryos were fixed for an additional 2 hours with MEMFA, dehydrated through ethanol-xylene series, embedded in Paraplast, and 7 μm sections were cut on a rotary microtome. Sections were stained with hematoxylin/ eosin (Sigma) according to the manufacturer's protocol.

Explant culture, RNA isolation and northern blotting

Different regions of the blastula were isolated by manual dissection. Animal, marginal and vegetal explants were about one third of the size of the embryo, while dorsal and ventral explants were half of the size of the embryo. Animal-vegetal dissections were controlled by Vg1 RNA-specific probe. Dorsal-ventral dissections were controlled by culturing five to ten explants until the gastrula stages. During gastrulation, dorsal explants underwent vigorous convergent extension movements, whereas ventral explants healed into a ball of cells and did not elongate, confirming that the dissections were done properly (data not shown).

Animal caps (approximately 1/5 of the size of the embryo) were isolated from the injected embryos at the midblastula stage (stage 8) as previously described (Sokol et al., 1990) and were cultured in 0.5× MMR until the equivalent of stage 11 and stage 31 at room temperature. At that time, explants were homogenized in a buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM EDTA, 0.5% SDS and 200 μg/ml proteinase K, and incubated for 1 hour at 37°C. Homogenates were extracted twice with phenol/chloroform (1:1) and once with chloroform, and RNA was precipitated by ethanol.

RNA samples isolated by this protocol were electrophoresed in 1% denaturing formaldehyde agarose gels and transferred to GeneScreen nylon membrane with 20× SSPE (Sambrook et al., 1989). Antisense RNA probes were generated by in vitro transcription of plasmids containing *Xenopus* fibronectin (Krieg and Melton, 1985), Vg1 (Rebagliati et al., 1985), Xwnt8 (Christian et al., 1991), XA-1 (Sive et al., 1989), Otx2 (Lamb et al., 1993; Boncinelli, personal communication), NCAM (Kintner and Melton, 1987), muscle-specific actin (Dworkin-Rastl et al., 1986), Xbra, Gsc (see Smith, 1993) and EF1α (Krieg et al., 1989) with SP6, T7 and T3 RNA polymerases.

Hybridization with different ^{32}P-labeled antisense RNA probes was carried out for 8-18 hours at 65°C in HB buffer, containing 50% formamide, 5× SSPE, 5% SDS and 125 μg/ml of denatured salmon sperm DNA. After hybridization, membranes were washed at 65°C in 0.1× SSPE, 0.1% SDS until background counts dropped significantly and were exposed to Kodak X-OMAT AR film with an intensifying screen at –70°C. When necessary, membranes were stripped by boiling for 5 minutes in distilled water followed by hybridization with a different set of probes.

RESULTS

Identification of a *Xenopus* homolog of *dishevelled*

To isolate a *Xenopus* homolog of *dsh*, a 0.7 kb *Pst*I-*Xho*I fragment of the *Drosophila dsh* cDNA (Klingensmith et al., 1994) was used to probe a *Xenopus* oocyte cDNA λgt10 library at low stringency conditions. This screen resulted in isolation of a phage containing a 2.5 kb insert, strongly hybridizing to the *Drosophila dsh* probe. Partial sequencing of the clone revealed significant similarity of its primary structure with the sequence of the deduced *Drosophila dsh* protein.

The 5′-terminal 0.6 kb fragment of the cloned partial length cDNA was used to rescreen the same cDNA library. As a result of this screen, a 3.3 kb *Xdsh* cDNA was isolated. The cDNA has been sequenced revealing an open reading frame of 2208 base pairs and encoding a protein of 736 amino acids (Fig. 1). The predicted overall amino acid sequence of the Xdsh protein is 46% identical to the sequence of the *Drosophila dsh* protein (Klingensmith et al., 1994; Theisen et al., 1994) and 60% identical to the product of the recently isolated mouse *dishevelled* cDNA (Sussman et al., 1994). The structural elements of Xdsh include two proline-rich stretches and the *discs large* homology region (DHR), a motif found in the product of the *Drosophila* tumor suppressor gene *discs large* and, in several proteins, associated with cytoskeleton and with tight junctions (Bryant et al., 1993; Anderson et al., 1993) (Fig. 1).

The first 20 amino acids of the deduced N terminus of the

Xdsh protein, starting with the first available methionine, are virtually identical (with two conservative amino acid changes) to the N terminus of mouse *dishevelled* product (Sussman et al., 1994), suggesting that the first AUG codon is the true translation start. The **A**--AUG**G** sequence surrounding the AUG codon is a good match to Kozak consensus sequences for translation initiation. According to Northern analysis, the endogenous *Xdsh* mRNA is approximately 3.5 kb. Together, these data indicate that the cloned 3.3 kb *Xdsh* cDNA is likely to encode the full-length Xdsh protein.

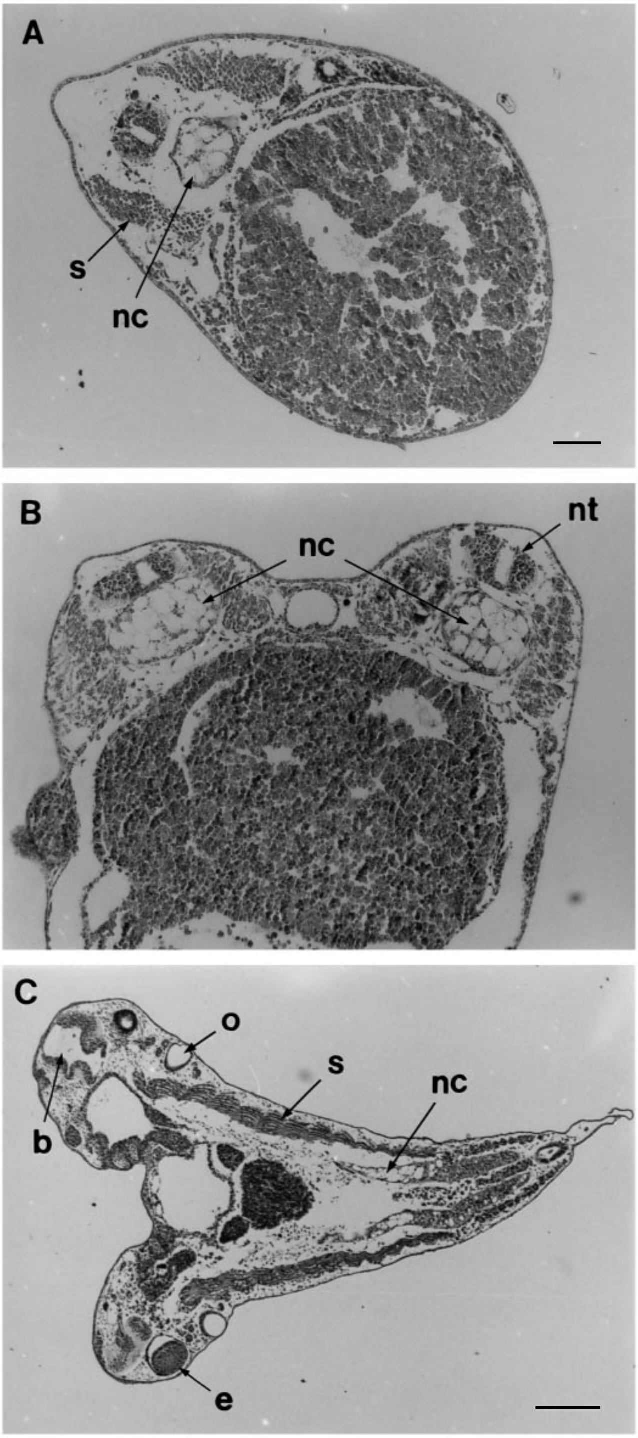

Fig. 4. Histological analysis of embryos injected with *Xdsh* mRNA. (A) Transverse section of an embryo injected ventrally with a control RNA encoding β-gal; (B,C) Embryos injected with *Xdsh* RNA; (B) transverse section and (C) horisontal section. Abbreviations are as follows: nt, neural tube; nc, notochord; s, somite; e, eye; o, otic vesicle, b, brain. The scale bar in A is 150 µm (also applies to B). The scale bar in C represents 300 µm.

Xdsh RNA is a ubiquitous maternal message

If Xdsh protein is necessary for Wnt signal transduction, it should be expressed in the blastomeres that can respond to Wnts and at the time when cells are competent to respond. Injected Xwnt8 mRNA has an effect as early as at the 32- to 64-cell stage (Olson et al., 1991), and it seems to affect any ventral or lateral blastomere within the marginal zone (Sokol et al., 1991). Thus, Xdsh is expected to be present maternally.

Northern analysis was used to study the expression pattern of *Xdsh* mRNA during *Xenopus* embryonic development (Fig. 2). A single mRNA species (approximately 3.5 kb) was detected throughout different developmental stages, being most abundant in eggs.

To determine which regions of the embryo express *Xdsh* mRNA, blastulae (stage 7-7.5) were dissected manually into animal, marginal and vegetal or into dorsal and ventral parts. It is fairly easy to distinguish different embryonic regions based on the difference in pigmentation (Nieuwkoop and Faber, 1967). A probe specific to the vegetally localized Vg1 RNA was used to control dissections along the animal-vegetal axis, while dissections along the dorsal-ventral axis were controlled by culturing five explants until the gastrula stages (see Materials and Methods). Total RNA from the dissected pieces was analyzed on Northern blots with different antisense RNA probes. While a control probe detected Vg1 mRNA mainly in the vegetal explants, *Xdsh* mRNA seems equally distributed in both animal-vegetal and dorsal-ventral directions (Fig. 2B).

Microinjection of *Xdsh* mRNA leads to induction of a complete dorsal axis

To determine whether the Xdsh product is sufficient to mimic the ability of Wnt1 mRNA to cause duplication of the body axis (McMahon and Moon, 1989), the full length *Xdsh* cDNA was subcloned into the pSP64R1 vector. In vitro synthesized *Xdsh* mRNA was microinjected into single ventral blastomeres of 8- to 16-cell *Xenopus* embryos. In several independent

Table 2. Injection of *Xdsh* mRNA induces dorsal axes in UV-treated embryos

RNA injected	Total number of injected embryos	Ventralized phenotype	Partial rescue of dorsal axis	Complete rescue
Xdsh	57	6 (11%)	23 (40%)	28 (49%)
β-gal	53	45 (90%)	7 (13%)	1 (2%)

Embryos were treated with UV light and injected at the 4- to 8-cell stage into a single blastomere with 0.4 ng of mRNA and left to develop to the equivalent of stages 40-45. Embryonic phenotypes were scored morphologically into three categories according to the DAI scale of Kao and Elinson (1988): ventralized phenotype (DAI 0-1), partial rescue (DAI 2-3) and complete rescue (DAI 4-5). Data from two independent experiments are presented.

experiments, *Xdsh* mRNA triggered the formation of a secondary dorsal axis (Fig. 3B,D) similar to the effects of Wnt1 or Xwnt8 mRNAs (Sokol et al., 1991). *Xdsh* mRNA injections into dorsal blastomeres at the same stage did not alter normal development (Fig. 3A). Embryos injected with a control *Xdsh* mRNA containing a short deletion that disrupts the open reading frame, developed normally (Fig. 3C), suggesting that the intact Xdsh protein is necessary for the observed effect.

The induced secondary axes frequently (in more than half of the injected embryos) contained a full set of dorsal structures including the most anterior structures (eyes and cement glands) (Table 1). Histological examination of the injected embryos revealed two properly organized axes with notochords, neural tubes and somites (Fig. 4). Consistent with our morphological observations, *Xdsh* mRNA injections activated the expression of *goosecoid* mRNA, a dorsal region-specific marker (Cho et al., 1991), and reduced the level of Xwnt8 mRNA, a ventrolateral marker (Christian et al., 1991), in stage 10.5 gastrulae (data not shown).

The effect of *Xdsh* mRNA was dose dependent: 0.5-1 ng of the mRNA was the optimal dose, 50-100 pg induced partial secondary axes, and injection of more than 2 ng resulted in a shortened tail and spinal cord with the head being almost normal in size. Thus, phenotypes of embryos injected with the high dose (2-4 ng) of *Xdsh* mRNA were somewhat different from the radially symmetric embryos, dorsalized by the high doses of Xwnt8 mRNA (Christian et al., 1991) or by lithium chloride (Kao and Elinson, 1988). This result may be related to the inability of the *Xdsh* mRNA and protein to spread from one cell to another, in contrast to diffusion of lithium chloride or transmission of Wnt proteins.

These observations show that over-expression of *Xdsh* mRNA alone is sufficient to trigger dorsal axis formation, and that Xdsh may, thus, transduce an endogenous signal responsible for determination of dorsal mesoderm.

Injection of *Xdsh* mRNA rescues embryos ventralized by UV light

Although ventral injections of *Xdsh* mRNA suggest that its effect does not depend on the dorsally located endogenous Spemann organizer, these observations do not exclude the possibility that the organizer synergizes with the injected mRNA. Since embryos treated with ultraviolet light (UV) are deficient in the Spemann organizer activity and fail to develop dorsal and anterior structures (Gerhart et al., 1989), they represent a useful system to study potential dorsal determinants. Several gene products including noggin, a chimeric BMP-Vg1 protein (Thomsen and Melton, 1993; Dale et al., 1993) and certain Wnt products (Moon et al., 1993) are known to restore normal development in embryos ventralized by UV light.

To test whether Xdsh is able to trigger dorsal development in the absence of the Spemann organizer, embryos were irradiated with UV light after fertilization and injected with in

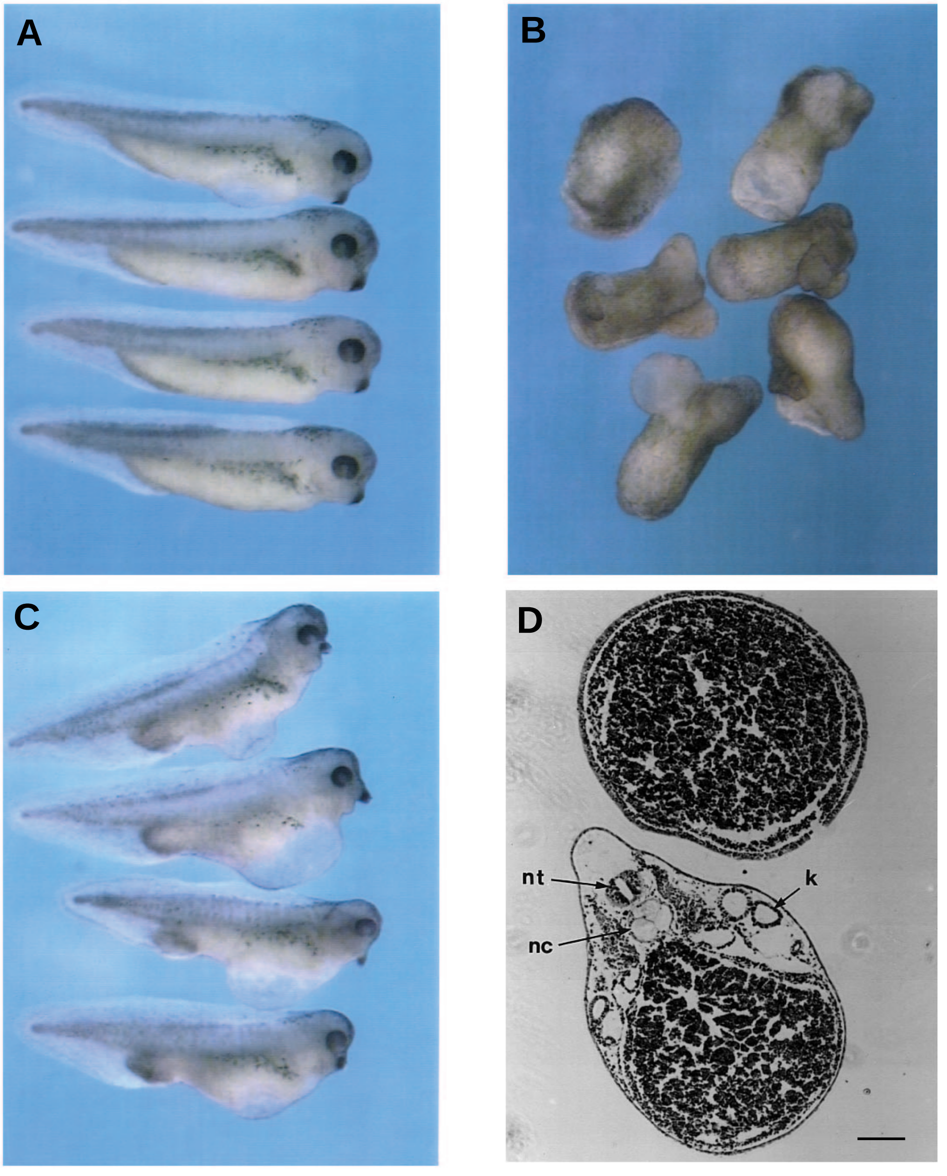

Fig. 5. *Xdsh* mRNA rescues dorsal development in axis-deficient UV-treated embryos. (A) Normal control embryos. (B) Embryos ventralized by UV treatment. (C) UV-treated embryos that were injected at the 8-cell stage in a vegetal blastomere with 0.4 ng of *Xdsh* mRNA. (D) Histological analysis of ventralized embryos (top) and embryos rescued by *Xdsh* mRNA injection (bottom). The scale bar in D represents 200 µm. Abbreviations are the same as in Fig. 4, except k, kidney tubules. Note that, in C, the most anterior morphological structures including eyes and cement glands are rescued by *Xdsh* RNA.

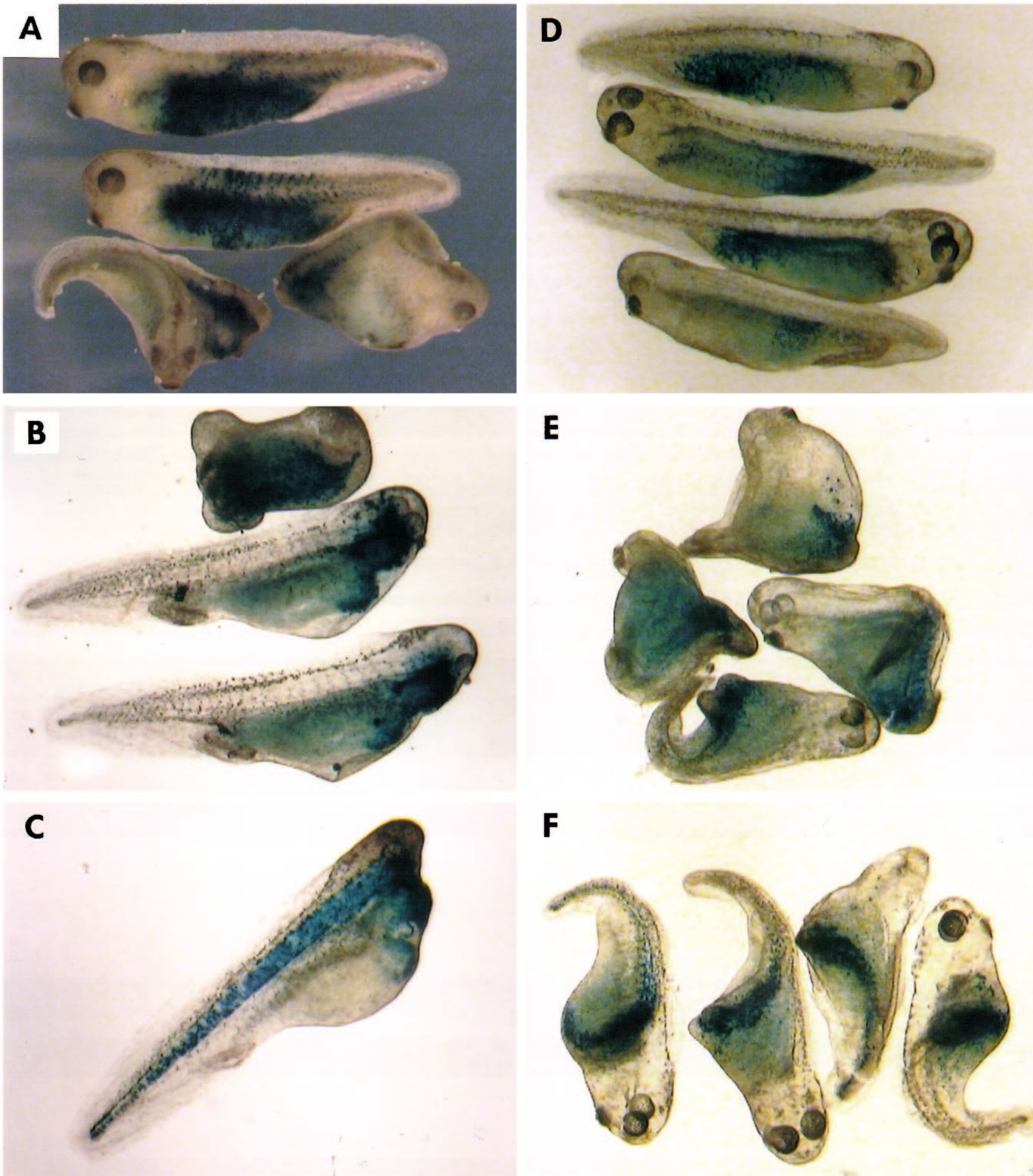

Fig. 6. Lineage tracing reveals the formation of dorsal and anterior structures by the progeny of a single blastomere injected with *Xdsh* mRNA. Cleavage-stage (8-32 cells) embryos were coinjected with 0.4 ng of *Xdsh* mRNA and 0.2 ng of β-gal RNA or with β-gal RNA alone. After 2 days of development embryos were fixed, and stained for β-gal activity. (A) Normal embryos were injected at the 8- to 16-cell stage into a ventrovegetal blastomere with β-gal RNA (two embryos on the top) or with β-gal and *Xdsh* mRNAs (bottom). (B,C) UV-treated embryos rescued by *Xdsh* RNA and coinjected with β-gal RNA. (B) Staining is mainly in the pharyngeal endoderm and head mesenchyme of the fully rescued embryos. A control embryo injected with β-gal RNA only is shown at the top. (C) In the partially rescued embryos (0.05-0.1 ng of *Xdsh* RNA injected), the staining is in the notochord and pharyngeal endoderm. (D-F) Lineage tracing at the 32-cell stage. Embryos were injected into D4 (tier 4) vegetal blastomere with β-gal RNA (D) or with β-gal and *Xdsh* mRNAs (E). (F) *Xdsh* and β-gal RNAs were microinjected into C4 (tier 3) subequatorial blastomere. Staining is mainly in the notochord and anterior mesoderm.

vitro synthesized *Xdsh* mRNA in a single vegetal blastomere of the 8- to 16-cell-stage embryos. While UV-treated embryos injected with water or with an unrelated RNA (β-gal RNA) did not have visible dorsal structures, in embryos that received *Xdsh* mRNA, the dorsal axis was rescued (Fig. 5; Table 2). In four independent experiments, we consistently observed a complete rescue of axial development of the ventralized embryos from dorsoanterior index (DAI) of less than 1 to DAI 5 (Kao and Elinson, 1988).

Together, these findings demonstrate that the *Xdsh* mRNA

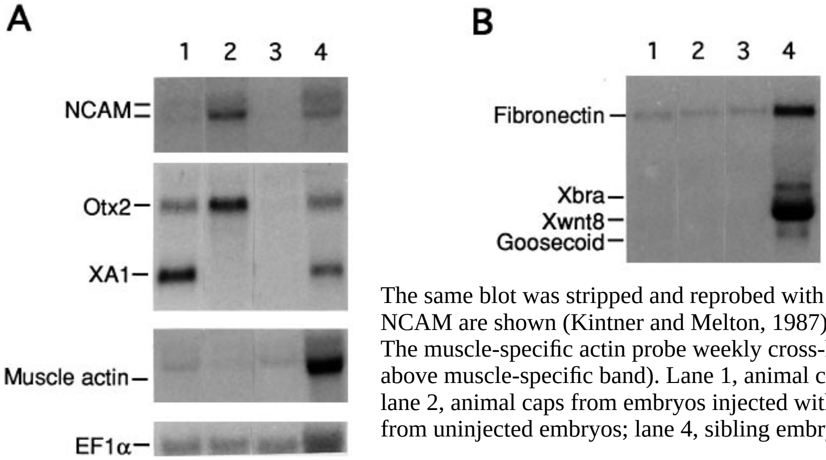

Fig. 7. Overexpression of *Xdsh* mRNA leads to neuralization of animal cap explants. Animal caps from embryos injected with 1 ng of *Xdsh* RNA were explanted from the injected embryos at stage 8 and cultured in isolation until the equivalent of stage 31 (A) or stage 11 (B). After culture, the explant RNA was extracted and analyzed by northern blotting using different ^{32}P-labeled antisense RNA probes. RNA from ten animal cap equivalents or from two embryos is loaded per lane. The same blot was stripped and reprobed with different probes. Two major specific transcripts of NCAM are shown (Kintner and Melton, 1987). EF1α and fibronectin probes are controls for loading. The muscle-specific actin probe weekly cross-hybridizes with cytoskeletal actin RNAs (the two bands above muscle-specific band). Lane 1, animal caps from embryos injected with 1 ng of *Xdsh* mRNA; lane 2, animal caps from embryos injected with 0.12 ng of *noggin* mRNA; lane 3, control animal caps from uninjected embryos; lane 4, sibling embryos, stage 31 (A) or 11 (B).

can trigger dorsal axis formation both in normal embryos and in UV-treated embryos, deficient in the endogenous Spemann organizer.

Cells overexpressing *Xdsh* mRNA directly contribute to the most anterior and dorsal axial structures

To determine which tissues in the induced axes are formed by the progeny of blastomeres, injected with *Xdsh* mRNA, lineage tracing was carried out by coinjecting *Xdsh* mRNA and β-gal mRNA into a single ventrovegetal blastomere of 8- to 16-cell embryos (Dale and Slack, 1987a). When the injected embryos reached tadpole stages (stage 40-42), they were fixed and stained for β-gal activity. In embryos injected with β-gal RNA alone (total number of 28), staining was found in ventrolateral tissues (Fig. 6A), consistent with the normal fate of the injected cells (Moody, 1987; Dale and Slack, 1987a). In contrast, all embryos injected with β-gal and *Xdsh* mRNAs (32 out of 32) were stained in dorsal and anterior tissues, e. g. in notochord, head and branchial mesenchyme and in pharyngeal endoderm (Fig. 6A). Only one out of two axes in each embryo was stained. These findings suggest that *Xdsh* mRNA functions cell autonomously: cells that received *Xdsh* mRNA change their ventral fate and, instead, may form an ectopic organizing center.

Staining of anterior and dorsal structures was also observed in the rescue experiments, where β-gal RNA was coinjected with *Xdsh* RNA into embryos ventralized by UV irradiation (Fig. 6B,C). These results are similar to what was observed in studies with *Xwnt8* RNA injections (Sokol et al., 1991; Smith and Harland, 1991), in which the majority of injected blastomeres formed a Spemann organizer and only a small percentage of them contributed exclusively to endoderm, thus, mimicking the vegetal organizing center (Gimlich and Gerhart, 1984). Interestingly, there was a correlation between the degree of rescue and the fate of the injected cells. While in completely rescued embryos (0.4 ng *Xdsh* RNA per embryo; n=37), the injected cells were found exclusively in the head mesenchyme/pharyngeal endoderm region (Fig. 6B), in partially rescued embryos (0.05 ng of *Xdsh* mRNA per embryo; n=28), cells injected with *Xdsh* mRNA populated mostly notochord and anterior mesoderm (Fig. 6C).

To extend lineage tracing analysis to the 32-cell-stage embryos, *Xdsh* mRNA (0.5 ng) was injected into C4 or D4 blastomere (according to nomenclature of Dale and Slack, 1987a). Progeny of D4 ventral blastomere injected with the same dose of *Xdsh* mRNA contributed to head mesoderm and anterior endoderm (Fig. 6E), while injected C4-derived cells were found mainly in the axial mesoderm (Fig. 6F). Thus, fates of injected cells depend on the site of injection, which is consistent with the idea that Xdsh dorsalizes prospective ventral mesoderm creating a new Spemann organizer on the ventral side. Solely endodermal staining was not observed in any of the injected embryos (n=35), arguing that the effects of *Xdsh* mRNA on the organizer formation are cell autonomous. Although *Xdsh* mRNA clearly causes changes in cell fate and, therefore, affects cell behavior during gastrulation, we cannot exclude the possibility that Xdsh directly influences cell migration, as was proposed for goosecoid (Niehrs et al., 1993).

Neuralizing activity of Xdsh in presumptive ectodermal cells

Xdsh mRNA may influence dorsal axis formation either by inducing mesoderm de novo (similar to members of TGFβ and FGF families) or by altering polarity of mesodermal cells, similar to the competence modifiers, such as some Wnts and noggin.

To discriminate between these two possibilities, differentiation of animal pole cells overexpressing *Xdsh* RNA was studied. At later stages, cultured animal caps formed prominent cement glands which are normally induced during neural induction (data not shown). Subsequent analysis revealed activation of XA-1, an anterior ectodermal marker (Sive et al., 1989), and Otx2, a forebrain-specific marker (E. Boncinelli, personal communication, also called OtxA, Lamb et al., 1993), but not muscle-specific actin transcripts (Mohun et al., 1984) (Fig. 7A). Whereas NCAM, a pan-neural marker (Kintner and Melton, 1987) is only marginally visible in Fig. 7, it was well induced in other experiments (data not shown). Northern analysis of mesoderm-specific gene expression at the midgastrula stage failed to detect significant amounts of mRNAs for Xbra, Xwnt8 and goosecoid, early mesoderm-specific markers (Fig. 7B; Smith, 1993). These findings suggest that *Xdsh* mRNA can induce neural tissue formation directly, in the absence of mesoderm.

Taken together, these observations indicate that the Xdsh properties are very similar to the effects of noggin, a factor possessing both dorsalizing and neuralizing activities (Smith et al., 1993; Lamb et al., 1993). Similar to the effects of Wnts and noggin (Sokol, 1993; Lamb et al., 1993), injection of high doses of *Xdsh* mRNA occasionally led to muscle actin activation, which may be a result of interaction with a small amount of mesoderm-inducing signals spreading into the animal pole region (Sokol, 1993). Taken together, our observations suggest that Xdsh may function during specification of dorsal-ventral polarity of mesoderm and during nervous system development.

DISCUSSION

In this paper, we report cloning of a cDNA encoding *Xdsh*, a novel *Xenopus* gene product, homologous to *Drosophila dishevelled* (Klingensmith et al., 1994). It is shown that *Xdsh* mRNA is an abundant maternal transcript which is equally distributed in different regions of *Xenopus* blastulae. Small amounts of *Xdsh* mRNA are present throughout embryogenesis. We also demonstrate that *Xdsh* mRNA, encoding the full-length Xdsh product, induces a complete body axis when injected into normal or ventralized *Xenopus* embryos and causes neuralization when overexpressed in the prospective ectodermal cells.

Genetic analysis in *Drosophila* indicates that *dsh* is an essential component of the *wg* signal transduction system (Perrimon, 1994). Our results suggest that this signaling pathway is evolutionarily conserved and may be operating during vertebrate development. Since overexpression of *Xdsh* mRNA is sufficient to trigger dorsal axis formation in the apparent absence of an exogenous Wnt signal, Xdsh may be a limiting component of the signal transduction machinery. When *Xdsh* mRNA is supplied in excess to embryonic cells by microinjection, the mechanism controlling the Xdsh function in ventral blastomeres may be overloaded. Under these circumstances, Xdsh may be activated inappropriately, resulting in the conversion of ventral cells to dorsal fates.

How does Xdsh operate? The deduced Xdsh protein is similar to its *Drosophila* and mouse counterparts (Klingensmith et al., 1994; Sussman et al., 1994) and does not appear to contain a signal sequence for secretion or a transmembrane domain. A small region of amino acid similarity (DHR) has been found between all three dsh homologs and the *Drosophila* tumor suppressor *discs large* (Bryant et al., 1993). Interestingly, the same DHR motif is present in several other proteins, including the ZO1 and ZO2 proteins of tight junctions, the erythrocyte membrane protein p55, the phosphatase PTP-BAS and the protein from the brain synapses PSD95, which have little in common, except most of them may interact with tight junctions and/or with the cytoskeleton (Anderson et al., 1993; Maekawa et al., 1994). Moreover, some of these proteins have additional enzymatic activities (guanylate kinase, phosphatase). Thus, it is conceivable that DHR is involved in protein-protein interactions.

Since the *wingless* signaling pathway is thought to include *armadillo*, a fly homolog of β-catenin and plakoglobin (McCrea et al., 1991), and β-catenin is known to regulate cadherin function in cell adhesion, it is possible that Wnts and Xdsh act by regulating cell adhesion (Moon et al., 1993). Interestingly, both antibodies to β-catenin and β-catenin-specific antisense oligonucleotides were reported to affect *Xenopus* dorsal axis formation (McCrea et al., 1993; Heasman et al., 1994), which is consistent with the idea that *Wg* signaling pathway described in *Drosophila* may be conserved in vertebrate development. Our data provide a reliable biological assay for Xdsh that should be useful in dissecting function of Xdsh at the molecular level.

Two classes of signaling factors influence formation of axial mesoderm. Mesoderm-inducing factors, such as TGFβ- and FGF-related growth factors (Smith, 1993; Klein and Melton, 1994) directly induce dorsal mesoderm in animal caps. Dorsal modifiers, in contrast, such as several Wnts and noggin, do not induce mesoderm on their own, but synergize with the endogenous mesoderm inducers and change the character of mesoderm from ventral to dorsal (Christian et al., 1992; Sokol and Melton, 1992). Dorsal modifiers trigger mesoderm formation in the animal caps only when animal caps are isolated from the embryo after stage 9 (Sokol, 1993; Lamb et al., 1993). Xdsh does not induce mesoderm in blastula animal caps, but expression of muscle-specific actin is activated if animal caps injected with Xdsh are isolated at stage 10 (data not shown). Thus, axis-inducing activity of Xdsh is very similar to the modifying effects of Wnts or noggin. It is important to point out, that Xdsh transcripts are mostly maternal, whereas noggin expression peaks after mid-blastula transition in the Spemann organizer, and the known Wnts that are capable of inducing dorsal axis (Moon et al., 1993) are also expressed zygotically. Studies of interactions between different dorsal modifiers may lead to identification of molecular pathways of vertebrate dorsal axis determination.

Neuralization of ectodermal cells overexpressing *Xdsh* mRNA indicates that Xdsh may have a second important function connected with neural tissue formation. Dorsalizing and neuralizing activities of Xdsh are similar to those of noggin (Lamb et al., 1993). Experiments are in progress to establish potential connections between Xdsh and other neuralizing factors, including noggin, follistatin (Hemmati-Brivanlou et al., 1994), vertebrate hedgehog (Roelink et al., 1994; Echelard et al., 1993) and protein kinase C (Otte et al., 1988).

The requirement for *wingless* in *Drosophila* segmentation, in the imaginal discs and at the wing margin (Perrimon, 1994) suggests that both Wnt and dsh homologs may play multiple roles in vertebrate morphogenesis as well. Since several Wnts have been implicated in CNS development (Dickinson and McMahon, 1992; Nusse and Varmus, 1992), the neuralizing activity of Xdsh is consistent with Xdsh playing a role in the transmission of Wnt signals during CNS patterning. Alternatively, the maternal Xdsh protein could be directly activated by the cortical rotation on the prospective dorsal side of the embryo and may participate in modification of the cell responses both to mesoderm induction and to neural induction. Experiments aimed at inactivation of the Xdsh function should clarify its role in Wnt signal transduction and in embryogenesis.

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