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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

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Summary

In amphibian embryos, formation of the basic body plan depends on positional differences in the mesoderm. Although peptide growth factors involved in mesoderm induction have tentatively been identified, additional signals are required to generate pattern in this tissue. We have isolated a *Xenopus* cDNA for a *Wnt*-1 related gene, designated *Xwnt*-8, which is activated in response to mesoderm-inducing growth factors. *Xwnt*-8 transcripts are transiently expressed, being most abundant during gastrulation at which time expression is confined primarily to ventral mesodermal cells. Embryos dorsoanteriorized by exposure to lithium exhibit greatly reduced levels of *Xwnt*-8 mRNA, supporting a correlation between *Xwnt*-8 expression and a ventral mesodermal cell fate. Surprisingly, ectopic expression of *Xwnt*-8 in embryos causes a dorsoanterior-enhanced phenotype. These findings suggest that *Xwnt*-8 may be a secondary signalling agent which is produced in response to mesoderm-inducing factors and is involved in the early steps of mesodermal patterning.

Key words: *Xenopus*, *Xwnt*-8, pattern formation, *Wnt*-1, mesoderm.

Introduction

During amphibian development, a unicellular zygote gives rise to an embryo, the cells of which receive and interpret positional information to generate the basic body plan. While it has been clear for almost a century that a series of tissue inductions are required for pattern formation during development, only recently have the molecules involved in embryonic cell–cell signalling begun to be identified. The major body axes (dorsal–ventral and anterior–posterior) are first laid out in the embryonic mesoderm, which is formed at the early blastula stage in response to interactions between animal and vegetal hemisphere cells (Spemann, 1938; Nieuwkoop, 1969). This process of mesoderm induction most likely involves peptide growth factors, such as basic fibroblast growth factor (bFGF) and members of the transforming growth factor-β (TGF-β) family (Smith, 1987; Slack *et al.* 1987; Kimelman and Kirschner, 1987; Rosa *et al.* 1988; Smith *et al.* 1988; Smith *et al.* 1990). These same factors may specify some positional differences within the mesoderm, possibly by regulating the expression of a series of homeobox genes (Ruiz i Altaba and Melton, 1989). While these findings provide an initial basis for understanding morphogenesis, they do not fully account for the patterning events of early development, leading to speculation that additional signalling molecules are involved in organizing the amphibian body plan. In support of this contention, the existence of a peptide inducing agent that partially mimics the action of Spemann's organizer was recently reported (Sokol *et al.* 1990).

The *int*-1/*Wnt*-1 family of genes has been hypothesized to play a role in embryonic patterning (Nusse, 1988; McMahon and Moon, 1989*a*; Gavin *et al.* 1990). Although *Wnt*-1 itself was originally discovered as an oncogene activated by proviral insertion in mouse mammary tumors (Nusse and Varmus, 1982; Nusse *et al.* 1984; van Ooyen and Nusse, 1984), the identification of its *Drosophila* homologue as the segment polarity gene *wingless* (Rijsewijk *et al.* 1987) suggested that *Wnt*-1 might function in generating embryonic pattern during normal mouse development. In keeping with its proposed role, *Wnt*-1 is expressed in a temporally and spatially restricted fashion during murine embryogenesis (Wilkinson *et al.* 1987*a*; Shackleford and Varmus, 1987). In addition, deregulation of *Wnt*-1 expression in *Xenopus laevis* embryos leads to pattern abnormalities

(McMahon and Moon, 1989*b*). Recently analysis of *Wnt*-1 null mutants, generated by homologous recombination in mouse embryo-derived stem cells, has provided direct evidence that the *Wnt*-1 gene is required for development of a large region of the fetal brain (McMahon and Bradley, 1990; Thomas and Capecchi, 1990).

The growing *Wnt* gene family now includes at least ten members in the mouse (Wainwright *et al.* 1988; McMahon and McMahon, 1989; Roelink *et al.* 1990; Gavin *et al.* 1990; Nusse *et al.* 1991) and five members in *Xenopus* (Noordermeer *et al.* 1989; Christian *et al.* 1991), each of which is expressed during embryonic development. A detailed examination of the spatial distribution of embryonic transcripts from each gene has not been completed, but initial analyses in mice (Gavin *et al.* 1990) and in *Xenopus* (Christian, McGrew and Moon, unpublished observation) suggest that individual members may be expressed in unique, tissue-restricted patterns. All of the *Wnt*-1 related genes identified to date encode polypeptides with features characteristic of secreted proteins, although secretion has been definitively demonstrated only for *Wnt*-1 (Papkoff and Schryver, 1990; Bradley and Brown, 1990). Thus, evidence supports the hypothesis that members of the *Wnt* gene family play distinct roles as cell–cell signalling molecules during embryonic development.

We report here the characterization of *Xwnt*-8, a novel *Xenopus Wnt*-1-related gene, which is transiently expressed in a spatially restricted fashion during early development. Significantly, we demonstrate that its expression is an early response to mesoderm induction by peptide growth factors. Our data are consistent with the hypothesis that expression of *Xwnt*-8 occurs in response to mesoderm induction, following which *Xwnt*-8 is secreted and participates in patterning events during amphibian development.

Materials and methods

Isolation of cDNA clones and plasmid construction

A partial length *Xwnt*-8 cDNA clone (Christian *et al.* 1991) was used to screen a *X. laevis* neurula stage (stage 17) library constructed in λgt10 (gift of Dr D. Melton). Library screening and cloning procedures are described in Maniatis *et al.* (1982). One full-length cDNA was sequenced on both strands using the dideoxy chain termination method (Sanger *et al.* 1977) and Klenow enzyme. In addition, the region of the clone encompassing approximately amino acids 260 to 380 (Fig. 1) was sequenced with multiple independent primers using a Sequenase kit (United States Biochemical) which employs T7 DNA polymerase rather than Klenow. In duplicate reactions, 7-deaza-GTP or dITP were substituted for dGTP to resolve possible errors due to compressions. This same region of three independent phage clones was also sequenced. The full sequence of the *Xwnt*-8 cDNA has been submitted to GenBank.

An *Eco*RI fragment of the *Xwnt*-8 cDNA, which contains the full-length cDNA except for approximately 150 nucleotides of 3′ noncoding sequence, was subcloned into the transcription vector pSP64T (Krieg and Melton, 1984) to generate pXwnt-8. A second plasmid, pXwnt-8myc, was constructed as follows. A portion of the *Xwnt*-8 cDNA was amplified in a polymerase chain reaction primed with two synthetic oligonucleotides. The first, 5′ TTTTGCGCAG-GAGCAAAAGCTGATTTCTGAGGAGGATCTGAATG-GAATTGAGGAGTGT 3′, encodes a *Hha*I site followed by a sequence encoding a 10 amino acid epitope of human c-*myc* (EQKLISEEDL, Evan *et al.* 1985) followed by sequence encoding amino acids 50 to 55 of *Xwnt*-8. The second primer includes sequence complementary to nucleotides 748 to 764 in the coding strand of *Xwnt*-8. Reaction products were digested with *Hha*I and *Nco*I and the resulting fragment of approximately 400 bp was gel purified. This fragment was ligated along with the 5′ terminal *Hin*dIII–*Hha*I fragment of p*Xwnt*-8, and the 3′ terminal *Nco*I–*Eco*RI fragment of p*Xwnt*-8 into the vector pSP64T which had been digested with *Hin*dIII and *Eco*RI. The result is a 30 bp in-frame insertion (verified by dideoxy sequencing across this region; Sanger *et al.* 1977) such that pXwnt-8myc encodes a protein with 10 extra amino acids inserted between residues 49 and 50 of *Xwnt*-8.

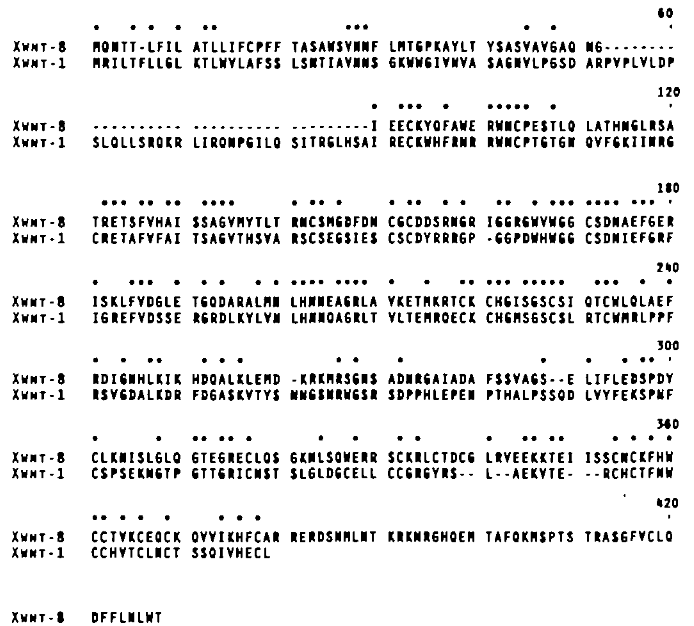

Fig. 1. Comparison of predicted *Xwnt*-8 and *Xwnt*-1 protein sequences. Gaps introduced to align the sequences are shown as dashes and identical residues are marked with asterisks.

Embryo culture and manipulations

Xenopus laevis eggs were obtained and embryos cultured in modified Barth solution (MBS) as described by Moon and Christian (1989). All embryonic stages are according to Nieuwkoop and Faber (1967). For dissections, the vitelline membrane was removed manually using watch-makers forceps and dissections were performed in 1×MBS using tungsten needles.

Ultraviolet irradiation of fertilized eggs was as described by Sato and Sargent (1990) except that embryos were placed in quartz cuvettes for the procedure. Dorsoanterior-enhanced embryos were obtained by incubation in 0.3 M lithium chloride, 0.1×MBS for 10–12 min at the 32-cell stage. In all cases, some embryos were allowed to develop to stage 28 at which time they were assigned a score of dorsoanterior index (DAI) as described by Kao and Elinson (1988). On this scale, a score of zero corresponds to a completely ventralized

embryo, and a score of ten to a completely dorsalized embryo. In each of the four experiments reported in the results, the average DAI for u.v.-irradiated embryos was less than 0.5 while that for lithium-treated embryos was greater than 8.5.

Mesoderm induction assays

Blastula animal caps were obtained and cultured as described by Kimelman and Kirschner (1987) with the exception that explants were cultured in half-strength normal amphibian medium (NAM, Slack, 1984). Recombinant *Xenopus* bFGF was a gift of Dr D. Kimelman (University of Wahington), NGF was a gift of Dr J. Glomset (University of Washington). Conditioned medium from *Xenopus* XTC cells (XTC-CM) was a gift of Dr S. Hauschka (University of Washington). XTC-CM was heated to 95°C for 5 min before use.

RNA analysis

RNA was extracted from embryos or embryos parts and analyzed by northern hybridization to ^{32}P-labelled cDNA probes as previously described (Giebelhaus *et al.* 1987). Hybridization of northern blots with cRNA probes was performed as described by Christian *et al.* (1990).

In situ *hybridization*

Albino *X. laevis* embryos were processed for *in situ* hybridization as described by Wilkinson *et al.* (1987*b*). Single-stranded, ^{35}S-labelled probes in sense and antisense orientations were derived by T7 and SP6 transcription of the partial length *Xwnt*-8 clone described by Christian *et al.* (1991).

In vitro *transcription and microinjection of RNA*

Capped synthetic RNA was generated by *in vitro* transcription of pXwnt-8 or pXwnt-8myc as described by Moon and Christian (1989). Fertilized eggs were injected with capped *Xwnt*-8 synthetic RNA prior to first cleavage as described by Moon and Christian (1989).

Results

Cloning of Xwnt-*8: a novel* Wnt-1 *related gene*

A *Xenopus* neurula-stage cDNA library was screened with the previously described partial length *Xenopus Wnt*-1-related clone designated *Xwnt*-8 (Christian *et al.* 1991). Sixteen clones rescreened positive and the longest of these was sequenced on both strands. This clone, pXwnt8–7.2, contains the complete coding region of *Xwnt*-8, the complete 3′ non-coding region as demonstrated by the presence of a polyadenylation consensus signal followed by a poly(A) tract, and all but a few nucleotides of the 5′ untranslated leader sequence as indicated by the results of primer extension analysis (data not shown). The predicted coding sequence for *Xwnt*-8 is shown in alignment with the published sequence of *Xenopus Wnt*-1 (*Xwnt*-1, Noordermeer *et al.* 1989) in Fig. 1. The two proteins share 40 % amino acid identity overall (48 % if conservative substitutions are included) which is somewhat lower than that observed between the various murine *Wnts* (50–87 %, Gavin *et al.* 1990) or between other *Xenopus Wnts* (47–50 %, Christian *et al.* 1991). However, as with all members of the *Wnt* family identified to date, *Xwnt*-8 contains a hydrophobic putative leader sequence, potential asparagine-linked glycosylation sites and, diagnostic for *Wnt* proteins, a large number of invariant cysteine residues. Of the 21 invariant cysteines shared by *Wnt* polypeptides from various species (Gavin *et al.* 1990), the position of 19 of these is conserved in *Xwnt*-8, supporting its designation as a member of this gene family. Interestingly, two cysteines present in all other *Wnt* proteins are not conserved in *Xwnt*-8 (residues 327 and 331, Fig. 1). Exhaustive analysis of this region of pXwnt8–7.2 indicates that this is not artifactual (see Materials and methods). In addition, four independent bacteriophage clones were shown to encode an identical sequence.

The degree of identity between *Xwnt*-8 and other identified members of the murine *Wnt* family ranges between 34 and 40 %. In contrast, the general level of identity between *Xwnt*-1, -3, -4 and -5A and their murine counterparts ranges from 69 % to 90 % (Noordermeer *et al.* 1989; Christian, Moody, McGrew and Moon, unpublished data). Thus, *Xwnt*-8 appears to be a novel member, not yet identified in mouse, as well as the most divergent member of the *Wnt* family identified to date.

Xwnt-*8 is transiently expressed in a restricted cell population during embryonic development*

The temporal profile of expression of *Xwnt*-8 was determined by northern blot analysis of embryonic total RNA, using a full-length *Xwnt*-8 antisense riboprobe. This analysis (Fig. 2) confirms and extends our previous results (Christian *et al.* 1991). *Xwnt*-8 transcripts are first detected during the blastula stage of development,

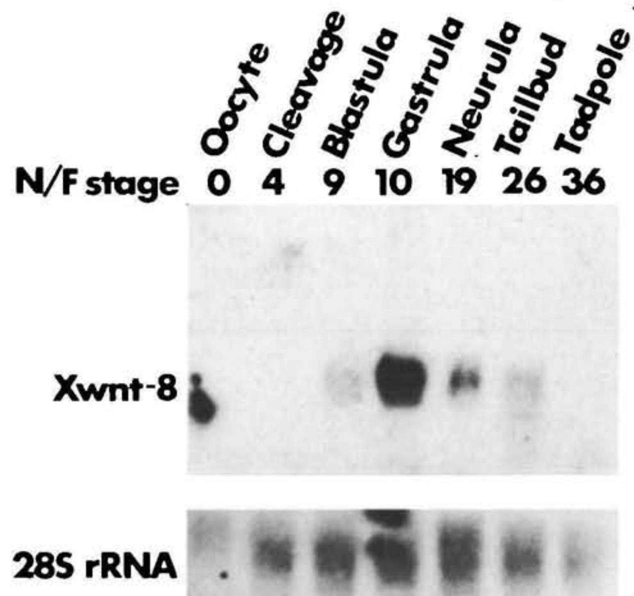

Fig. 2. Expression of *Xwnt*-8 transcripts through early *Xenopus* development. Northern blot containing 20 μg of total RNA isolated from oocytes or embryos at the indicated developmental stages (N/F: Nieuwkoop and Faber, 1967) probed with a ^{32}P-labelled antisense *Xwnt*-8 RNA probe. The filter was rehybridized with a probe for 28S ribosomal RNA to demonstrate the presence of RNA in all lanes.

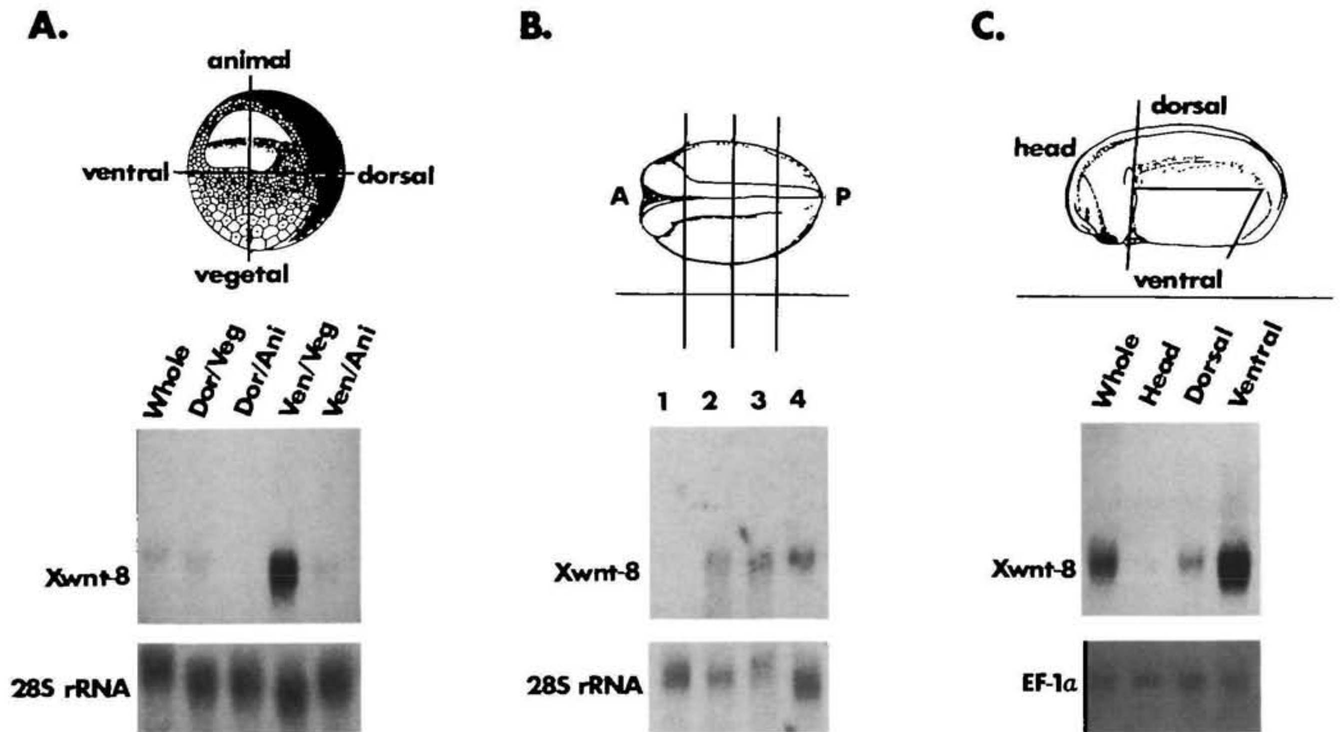

Fig. 3. Spatial distribution of *Xwnt*-8 transcripts in gastrula and neurula stage embryos. Early gastrula (stage 10, panel A), mid-neurula (stage 18, panel B) and late neurula (stage 22, panel C) stage embryos were dissected as illustrated at the top of each panel. Northern blots containing 15 μg of total RNA from the region indicated above each lane were hybridized with the *Xwnt*-8 probe. The bottom of each panel shows the same blots rehybridized with the probe indicated to the left to demonstrate equivalent loading in each lane. (A) Dor, dorsal; Ven, ventral; Ani, animal; Veg, vegetal. (B) Each number corresponds to the region directly above it on the overlying schematic diagram. A, anterior; P, posterior.

following which the level of expression increases rapidly, reaching a maximum by early gastrula (stage 10). Transcript abundance declines during the neurula and tailbud stages, becoming undetectable by the tadpole stage. At all stages examined, a single transcript approximately 2.1 kb in length is detected. The same filter was rehybridized with a cDNA probe for *Xenopus* 28S ribosomal RNA to demonstrate that all lanes contain fairly equivalent amounts of RNA (Fig. 2).

The spatial distribution of *Xwnt*-8 transcripts was examined by northern blot analysis of equivalent amounts of RNA extracted from various regions of gastrula to tailbud stage embryos. Embryos that had just initiated gastrulation (stage 10) were dissected into four fragments by cutting along the animal–vegetal and prospective dorsal–ventral midlines as illustrated in Fig. 3A. *Xwnt*-8 expression is localized almost exclusively to cells within the ventral vegetal quadrant of gastrulae (Fig. 3A). This correlates approximately with a future posteroventral cell fate, although it must be noted that the correlation between animal–vegetal polarity and anterior–posterior cell fate is not exact since fate mapping shows that some animal and vegetal pole cells may contribute to posterior and anterior structures, respectively (Dale and Slack, 1987).

To determine whether *Xwnt*-8 transcripts exhibit a graded distribution from head to tail, stage 18 neurulae were dissected into four regions along the anterior–posterior axis as shown in Fig. 3B. *Xwnt*-8 RNA is not detected in the anteriormost fragment but expression appears to be fairly equivalent along the posterior 75 % of the axis (Fig. 3B). Late neurula-stage embryos (stage 22) were also dissected into dorsal, ventral and head fragments which were analyzed for *Xwnt*-8 expression. Consistent with the spatial distribution of transcripts observed during gastrulation, the *Xwnt*-8 gene is expressed predominantly in the ventral region of neurula-stage embryos, and expression is not detected in the head (Fig. 3C). We obtained an identical result when earlier stage neurulae (stage 18) were dissected to yield dorsal and ventral regions (data not shown). In each case, filters were rehybridized with either a 28S ribosomal RNA probe or with an EF-1α cDNA probe to establish the presence of equivalent amounts of RNA in each lane.

To localize further *Xwnt*-8 expression during gastrulation, single-stranded ^{35}S-labelled *Xwnt*-8 probes were hybridized to sections from stage 10–11 albino *Xenopus* embryos. In sections hybridized with antisense *Xwnt*-8 transcripts, silver grains were concentrated primarily within deep mesodermal tissue (Keller, 1986) on the ventral side of the embryo (Fig. 4A, B, D and E). It is possible that some hybridization also occurred within the endoderm, although to a lesser degree. The ventrally localized signal was not detected by the sense probe (Fig. 4C). Thus, the results of these studies confirm and refine the conclusions reached by analysis of dissected gastrulae, in that *Xwnt*-8 transcripts are

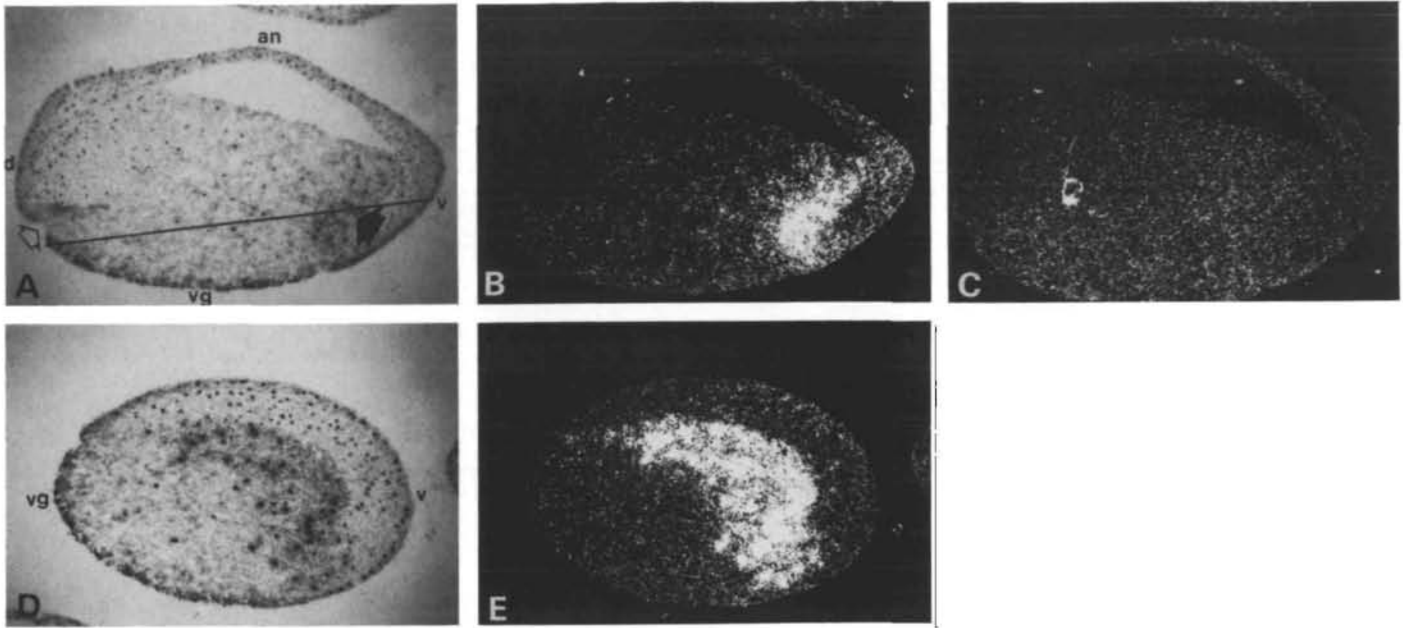

Fig. 4. Localization of *Xwnt*-8 transcripts in gastrula stage embryos by *in situ* hybridization. Sections of gastrulae (stage 10–11) hybridized with ^{35}S-labelled single-stranded *Xwnt*-8 RNA probes. (A,B,C) Sagittal sections; (D,E) para-equatorial section; plane of section indicated by black line in panel A. Arrows in A denote dorsal blastopore lip (open arrow) and deep ventral mesoderm, which expresses high levels of *Xwnt*-8 RNA (filled arrow). (B,C,E) Dark-field micrographs; (A,D) Light-field micrographs corresponding to B and E. The section in C was hybridized with a sense-strand probe, the remaining sections were hybridized with an antisense strand probe. an, animal; vg, vegetal; d, dorsal; v, ventral.

observed to be enriched in the ventral vegetal quadrant of embryos, and specifically within ventral mesodermal cells and perhaps some endodermal cells.

Mesoderm induction is required for Xwnt-*8 expression*

In *Xenopus* embryos, mesoderm is formed in the equatorial region of blastulae in response to an inductive signal emanating from cells of the vegetal hemisphere (reviewed by Smith, 1989*a*, 1989*b*; Gurdon *et al.* 1985). To test whether expression of *Xwnt*-8 is activated by mesodermal induction, we incubated animal caps from early blastula stage embryos in the presence of two known mesoderm-inducing growth factors, bFGF (Slack *et al.* 1987; Kimelman *et al.* 1988) and medium conditioned by the *Xenopus* XTC cell line which contains XTC-MIF, a potent mesoderm inducer recently identified as an amphibian homologue of mammalian activin A (Smith, 1987; Smith *et al.* 1990; van den Eijnden-Van Raaij *et al.* 1990). As a negative control, caps were incubated with a non-inducing growth factor (nerve growth factor; NGF). In each case, mesoderm induction was verified by demonstrating that a cardiac actin gene had been activated (Gurdon *et al.* 1985) or by documenting elongation of explants (at the gastrula stage, prior to the onset of expression of cardiac actin). When control whole embryos reached the gastrula or late neurula (stage 20) stage of development, the isolated animal caps were assayed for expression of *Xwnt*-8. Fig. 5A shows that animal or vegetal pole explants cultured in medium in the absence of any growth factor are not induced to form mesoderm and do not express *Xwnt*-8 transcripts. The same result is obtained when explants are incubated in medium to which NGF has been added (Fig. 5B). In contrast, incubating caps in medium conditioned by XTC cells (XTC-CM), or with $50\,\mathrm{ng\,ml^{-1}}$ of *Xenopus* bFGF (Kimelman *et al.* 1988) results in activation of the *Xwnt*-8 gene (Fig. 5A). Expression of *Xwnt*-8 is also induced in animal caps incubated with lower doses of bFGF ($20–40\,\mathrm{ng\,ml^{-1}}$, Fig. 5B) and this expression is evident at least by the early gastrula (stage 10) stage of development (data not shown). Although mesoderm induction leads to *Xwnt*-8 expression, the converse does not appear to be true. Ectopic expression of *Xwnt*-8 polypeptides resulting from injection of synthetic *Xwnt*-8 RNA into fertilized eggs (see later) does not activate cardiac actin expression in isolated animal caps, nor does it induce elongation of explants, both of which are characteristic signs of mesoderm induction (Smith, 1989*b*) (data not shown). Whether specific types of ventral mesoderm are induced in this experiment is presently unclear.

Xwnt-*8 expression in ultraviolet-irradiated and lithium-treated embryos*

Xwnt-8 transcripts are highly localized to ventral cells and are not detected in the extreme anterior region of embryos (Fig. 3). To examine the relationship between *Xwnt*-8 expression and mesodermal cell fate along the anterior–posterior and dorsal–ventral axes, the relative abundance of *Xwnt*-8 mRNA in lithium-treated or ultraviolet (u.v.)-irradiated embryos was compared to

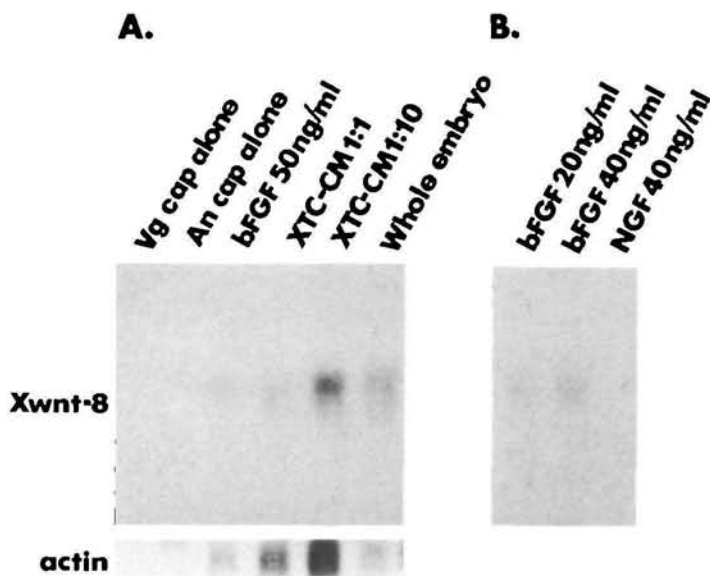

Fig. 5. Induction of *Xwnt*-8 mRNA by mesoderm-inducing peptide growth factors. Northern blots containing RNA from animal (An) or vegetal (Vg) caps isolated from stage 6 blastula and incubated in the absence of growth factors (alone) or from animal caps incubated in the presence of bFGF, NGF or XTC-conditioned medium (XTC-CM) diluted as indicated above each lane. Caps were cultured until control embryos reached neurula (stage 20). (A) The upper blot, which contains RNA from 25 caps or from 1/2 of one whole neurula stage embryo per lane, was hybridized with a *Xwnt*-8 riboprobe and exposed to film for two weeks. Each lane of the lower blot, which was probed with a cardiac actin cDNA, contains RNA from 40 caps incubated alone or with bFGF, from 8 caps incubated with each dilution of XTC-CM or from 1/2 of one neurula stage embryo. (B) Each lane contains RNA from 10 animal caps incubated with bFGF at 20 ng ml^{-1} or from 16 caps incubated with bFGF or NGF at 40 ng ml^{-1}. The blot was hybridized with a *Xwnt*-8 riboprobe and exposed to film for two weeks. Low doses of bFGF do not induce adequate levels of cardiac actin for detection using our cDNA probe.

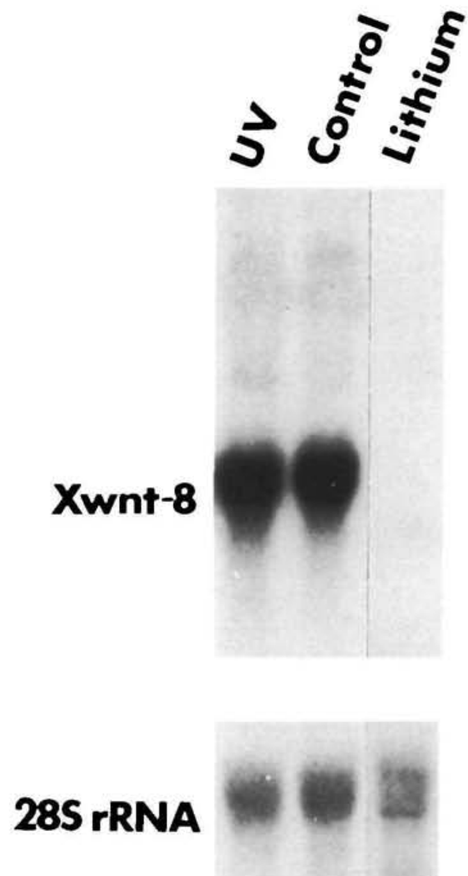

Fig. 6. Expression of *Xwnt*-8 in lithium-treated or u.v.-irradiated embryos. Northern blot containing 20 μg of total RNA from gastrula (stage 11) stage embryos which received no treatment (control) or were treated with u.v. light or lithium as described. The blot was hybridized with the *Xwnt*-8 probe and then with a ribosomal RNA probe to demonstrate equal loading.

that in untreated controls. Incubation of blastula-stage embryos in lithium, which leads to an overcommitment of mesodermal cells to dorsal and anterior structures with no change in the total amount of mesoderm (Kao *et al.* 1986; Kao and Elinson, 1988), causes a dramatic decrease in levels of *Xwnt*-8 transcripts (Fig. 6). In contrast, u.v.-irradiation of fertilized eggs, which results in a respecification of mesoderm along a posterior and ventral pathway (Malacinski *et al.* 1977; Scharf and Gerhart, 1980; Cooke and Smith, 1987; reviewed by Gerhart *et al.* 1989) had essentially no effect on *Xwnt*-8 expression during the gastrula (Fig. 6) and neurula (data not shown) stages of development in four separate experiments, although in one other case expression was increased (data not shown). Reprobing of filters with a 28S ribosomal RNA probe demonstrates approximately equivalent loading of RNA in each lane.

Ectopic expression of Xwnt-8 *transcripts leads to enhanced anterodorsal development*

To determine whether the observed restriction of *Xwnt*-8 expression to ventral mesodermal cells is essential for fulfilling its normal function during embryogenesis, the endogenous distribution of *Xwnt*-8 transcripts was perturbed by injection of synthetic mRNAs into fertilized eggs. That synthetic *Xwnt*-8 mRNA was translationally competent was demonstrated by its translation in a rabbit reticulocyte lysate. This yielded a protein with a relative molecular mass of 41×10^3, which is comparable to that predicted on the basis of sequence analysis (data not shown).

In four separate experiments, using RNA from three different transcription reactions, between 75 and 95 % of embryos expressing synthetic *Xwnt*-8 transcripts exhibited a dose-dependent enhancement of dorso-anterior structures. This phenotype ranged from an anterior bifurcation of the embryonic axis, at doses estimated to be less than 15 pg of RNA per embryo (Fig. 7B), to completely dorsalized embryos with radial retinal pigment and cement gland (DAI of 10; Kao and Elinson, 1988). An example of a dorsalized embryo (DAI of 9) is shown in Fig. 7C. An identical phenotype was seen following injection of synthetic mRNA encoding murine *Wnt*-1 (McMahon and Moon, 1989*b*;

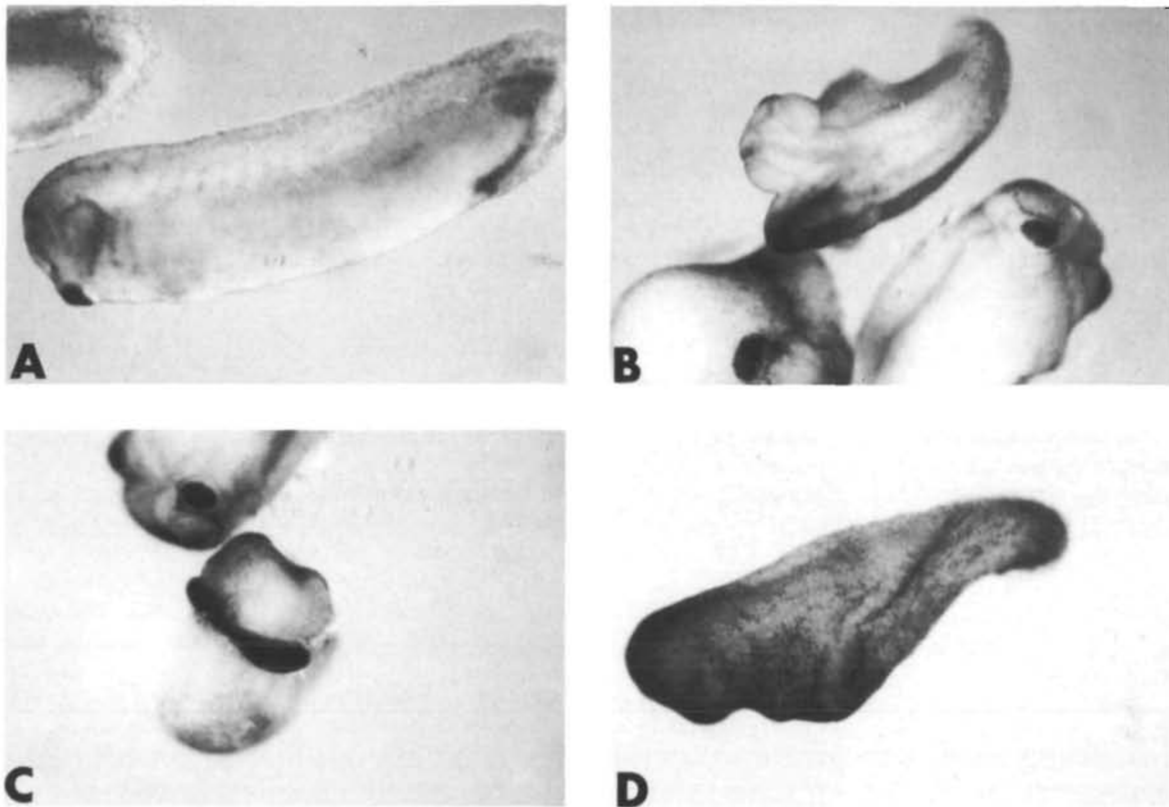

Fig. 7. Duplication of dorsal axial structures following injection of *Xwnt*-8 mRNA into embryos. Approximately 30 pg (B) or 100 pg (C) of *Xwnt*-8 mRNA was injected into the animal hemisphere of a fertilized egg, or 30 pg of RNA was injected into one ventral blastomere of a 16 cell embryo (D). A control (uninjected) embryo is shown in A. All embryos were grown to the tailbud stage.

Table 1. *Survival and phenotype at the tailbud stage of* Xenopus *embryos injected with RNAs encoding Xwnt-8 or Xwnt-8-myc hybrid proteins*

				Phenotype at stage 28				
						Dorsalized (DAI)		
RNA	Estimated dose (pg)	Number injected	Survival to tailbud (st. 28)	WT	ANT BI	6	7–8	9–10
Xwnt-8	10–20	54	49 (91 %)	12 (24 %)	37 (76 %)	0	0	
Xwnt-8	50–75	310	272 (88 %)	0	27 (10 %)	35 (50 %)	108 (40 %)	27 (10 %)
Xwnt-8	100–200	131	97 (74 %)	1 (1 %)	0	16 (16 %)	31 (32 %)	49 (50 %)
Xwnt-8 myc	50–75	250	222 (88 %)	222 (100 %)	0	0	0	0
Xwnt-8 myc	1000	180	172 (95 %)	172 (100 %)	0	0	0	0

All embryos were injected at the one-cell stage and scored at the tailbud stage for normal development (WT), for the presence of an anterior bifurcation (ANT BI) or for a dorsalized phenotype, reported as a score of dorsoanterior index (DAI) as described by Kao and Elinson (1988). Error in scoring is estimated to be ±5 %.

Olson and Moon, unpublished observation) or *Xenopus Wnt*-1 (Edelstein and Moon, unpublished observation) but not following injection of mRNA encoding at least one other member of the *Wnt* family (Moon, McGrew and Christian, manuscript in preparation) or of a variety of RNAs encoding unrelated proteins, such as *Xenopus* vimentin (Christian *et al.* 1990). In addition, injection of mRNA encoding an *Xwnt*-8·myc hybrid protein, which contains a 10 amino acid epitope of human *c-myc* inserted between residues 49 and 50 of *Xwnt*-8, yielded embryos that appear identical to the uninjected embryo shown in Fig. 7A (data not shown). This insertion does not affect translation of *Xwnt*-8·myc RNA in a rabbit reticulocyte lysate (data not shown), but most likely disrupts the secondary structure of the polypeptide thus interfering with its secretion or activity. A summary of the frequency with which various phenotypes were observed following injection of *Xwnt*-8 or *Xwnt*-8·myc mRNA into fertilized eggs in one representative experiment is provided in Table 1. An identical trend of dose-dependent dorsalization was noted in three additional experiments.

To determine whether ectopic expression of *Xwnt*-8 perturbs normal development when this misexpression is restricted to either dorsal or ventral cells of embryos, *Xwnt*-8 transcripts were injected into single dorsal or ventral blastomeres at the 16- to 32-cell stage of development. Interestingly, injection of *Xwnt*-8 mRNA into a single dorsal blastomere yields embryos with an anterior bifurcation of the axis and duplication of anterior structures, identical to that seen following injection at the 1-cell stage as shown in Fig. 7B. In contrast, when *Xwnt*-8 transcripts are injected into a ventral blastomere of a 16- or 32-cell embryo, a more posterior bifurcation of the embryonic axis is observed and a second set of dorsal axial structures, which lack extreme anterior components, develop (Fig. 7D). These results clearly demonstrate the importance of maintaining the normal spatial and temporal pattern of *Xwnt*-8 expression for morphogenesis.

Discussion

We have identified a novel *Xenopus Wnt*-1 related gene, designated *Xwnt*-8, and have established that it is a growth-factor-responsive gene that is expressed in a spatially and temporally restricted pattern during early development. Members of this family have been implicated in cell–cell signalling during embryogenesis (Nusse, 1988; McMahon and Moon, 1989*a*, 1989*b*; Gavin *et al.* 1990; Christian *et al.* 1991). A number of findings in the present study support a major role for the *Xwnt*-8 gene in amphibian development. Specifically, evidence discussed below suggests that *Xwnt*-8 may be involved in decoding and/or transmitting positional information within the early embryo.

In *Xenopus*, the basic body plan is first imprinted in the mesoderm, which is induced by an interaction between prospective endodermal and ectodermal cells during the blastula stage of development (Spemann, 1938; Nieuwkoop, 1969). Various peptide growth factors have been implicated as the agents responsible for effecting the formation of mesoderm from prospective ectoderm, and for establishing the initial conditions for pattern formation in this tissue (reviewed by Smith, 1989*a*, 1989*b*; Ruiz i Altaba and Melton, 1990). Yet evidence suggests that the delineation of cell fates within the mesoderm is not a direct consequence of exposure to mesoderm-inducing growth factors alone, but requires a secondary signal, or more likely a cascade of signals, produced by the responding cells (Ruiz i Altaba and Melton, 1989, 1990; Smith, 1989*b*). The identities of these putative signals are not yet known, but *Xwnt*-8 fits several criteria for such an agent.

First, a regulatory gene involved in the early patterning events of embryogenesis should be activated soon after the initial induction of mesoderm, as has been demonstrated for the homeobox gene *Mix*.1 (Rosa, 1989). Although animal pole cells are technically competent to respond to mesoderm-inducing signals at least as early as stage 6.5 (64-cell, Jones and Woodland, 1987), activation of new gene transcription in responding cells is delayed until the mid-blastula transition (stage 8, Newport and Kirschner, 1982). *Xwnt*-8 transcripts are first detected between seven and eight hours after fertilization in embryos cultured at 22°C (unpublished observation). This is within one to two hours of the reported time at which the zygotic genome first becomes active (Newport and Kirschner, 1982). Thus, expression of *Xwnt*-8 coincides temporally with the developmental window during which the newly induced mesoderm is believed to be supplied with positional information for establishing the major body axes (Gerhart and Keller, 1986; reviewed by Smith, 1989*a*, 1989*b*). In addition, the transient nature of expression of *Xwnt*-8 is consistent with a role for this gene in programming specific, timed developmental events.

Another requirement for a gene proposed to convey positional information in response to a primary signal, e.g. mesoderm induction, is that expression should occur in responding cells and should be contingent upon these cells receiving the signal. We have shown that activation of the *Xwnt*-8 gene requires mesoderm induction, and that transcripts are localized primarily within the ventral marginal zone mesoderm of gastrulae. During later stages, *Xwnt*-8 RNA remains confined to ventral tissues and is not highly localized along the anterior–posterior axis. For this reason, we stress a role for *Xwnt*-8 in the ventral mesoderm. At the same time, it must be acknowledged that differentiation along the two major body axes cannot be completely disassociated in the pre-neurula-stage embryo, since differences in the timing and extent of gastrulation movements on the dorsal and ventral sides of the embryo lead to coupling of ventral and posterior cell fates, and the same is true of anterior and dorsal differentiation.

Both XTC-MIF and bFGF are activators of *Xwnt*-8 transcription. This result was unexpected given that *Xwnt*-8 transcripts are localized to ventral mesoderm and that XTC-MIF is postulated to contribute primarily the dorsoanterior component, and bFGF the ventroposterior component, of the mesoderm-inducing signal (Green *et al.* 1990). This issue is confusing, however, given that XTC-MIF is capable of inducing ventral mesodermal cell types at low doses (Green *et al.* 1990) and that high doses of bFGF have been postulated to induce dorsal posterior mesoderm (Ruiz i Altaba and Melton, 1989). Our results suggest that low doses of bFGF or XTC-MIF are equal or more potent inducers of *Xwnt*-8 than are high doses of either growth factor. While this is consistent with the ventral localization of *Xwnt*-8 and the proposed role of these factors, it should be noted that the more dilute XTC-MIF-containing medium induces a higher level of cardiac actin, a dorsal mesodermal marker, as well as a higher level of *Xwnt*-8, a ventrally expressed gene. We have not completed a quantitative analysis of expression of *Xwnt*-8 in response to various doses of purified XTC-MIF or bFGF and thus cannot draw firm conclusions as to whether one of these agents is a stronger inducer of *Xwnt*-8 expression than is the other. Preliminary studies show that purified XTC-MIF (Activin A, Smith *et al.*

1990) is capable of inducing expression of *Xwnt*-8, demonstrating that the observed response to XTC-CM is not due solely to the presence of other factors in the media (J. B. Green, J. L. Christian, J. Smith and R. T. Moon, unpublished data). One indication that the *Xwnt*-8 gene may be responsive to the same inductive signals that specify a ventroposterior cell fate, is that the abundance of *Xwnt*-8 transcripts is greatly diminished in embryos exposed to lithium, an agent that respecifies mesodermal cell fate in a dorsoanterior direction (Kao *et al.* 1986; Kao and Elinson, 1988). Unexpectedly, expression of *Xwnt*-8 is not reproducibly augmented by u.v.-irradiation of eggs, which leads to hypertrophy of ventral and posterior mesoderm, possibly indicating that the *Xwnt*-8 gene is regulated independently of the signals that specify ventral mesodermal cell fates. If, however, *Xwnt*-8 is itself a component of a signal involved in the initial specification of a ventral fate, then u.v. irradiation might be expected to have no effect on the level of expression of this gene since the mechanism by which irradiation leads to ventralization is believed to involve the loss of a dorsalizing signal rather than the amplification of a ventralizing signal (Gerhart *et al.* 1989; Vincent and Gerhart, 1987).

What is the role of Xwnt-*8 in the ventral mesoderm?*

The results of our localization studies support a function for *Xwnt*-8 in the ventral mesoderm. We were surprised, therefore, to find that ectopic expression of *Xwnt*-8 in the early embryo leads to a dose-dependent enhancement of dorsoanterior structures and, at low doses, a bifurcation of the embryonic axis as observed with ectopic expression of *Wnt*-1 (McMahon and Moon, 1989*b*). This phenotype is outwardly similar to that produced by lithium, which causes the entire marginal zone of the early gastrula to differentiate into the Spemann organizer, a role normally confined to cells of the dorsal marginal zone (Kao and Elinson, 1988). Thus it is difficult to reconcile this phenotype, which resembles that produced by an excess of the putative dorsal organizer morphogen, with the proposed *in vivo* role of *Xwnt*-8 in the ventral mesoderm. Evidence supports the tenet that cells of the ventral marginal zone lack any type of organizing activity (Smith and Slack, 1983). Our results might be explained by postulating that *Xwnt*-8 normally functions in specifying a ventral fate to newly induced mesoderm. Overexpression of *Xwnt*-8 would thus enhance the differentiation of all mesoderm along a ventral pathway, and this mesoderm would then be reassigned a dorsal fate during gastrulation under the control of the organizer. The results of organizer graft experiments are consistent with this hypothesis in that embryos containing two adjacent dorsally located organizers usually develop normally (Cooke, 1972) whereas embryos receiving a second organizer transplanted into the ventral marginal zone develop with a complete second set of dorsal axial structures (Spemann, 1938; Smith and Slack, 1983). Thus, an excess of organizer alone is not sufficient to produce duplicate dorsal structures, unless it is juxtaposed with ventral mesoderm. Our results are also consistent with a number of alternative hypothesized roles for *Xwnt*-8. For example, secretion of *Xwnt*-8 might increase the sensitivity of neighboring cells to a dorsalizing signal, or might function in some capacity as an inhibitory signal. At present, elucidation of a function for *Xwnt*-8 in the ventral mesoderm requires further experimentation.

Interestingly, injection of *Xenopus* activin β_B mRNA into a ventral blastomere of a 32-cell embryo (Thomsen *et al.* 1990) leads to a phenotype which is similar to that observed following a comparable ventral injection of *Xwnt*-8. Activin β_B transcripts are first detected during the late blastula stage of development (Thomsen *et al.* 1990), coinciding with the initiation of transcription of the *Xwnt*-8 gene. It is not yet known whether the activin protein is localized. In light of the fact that Activin A and B have essentially the same inducing properties in a variety of assays (Thomsen *et al.* 1990), and that purified Activin A induces expression of *Xwnt*-8 (J. B. Green, J. L. Christian, J. Smith and R. T. Moon, unpublished data), it is probable that Activin B is also capable of activating the *Xwnt*-8 gene. Taken together, this raises the possibility that the two genes may function consecutively in a single pathway during the embryonic patterning process.

Members of the *Wnt* family are believed to be secreted, raising the possibility that *Xwnt*-8 might function at a distance from its site of synthesis rather than being confined to ventral cells. This is not probable, however, given the evidence that *Wnt*-1 remains closely associated with the extracellular matrix following secretion and is most likely to function in a paracrine or autocrine fashion (Bradley and Brown, 1990; Papkoff and Schryver, 1990).

It is not known whether specific cell-surface receptors exist for various *Wnt*-1 related proteins, or whether the spatial and temporal expression pattern of these putative receptors is coincident with that of their ligands. *Xwnt*-8 receptors may be homogeneously distributed although only receptors in close proximity to the ligand, i.e. near ventral mesodermal cells, would be expected to be activated under normal conditions. Our data demonstrate that ectopic expression of *Xwnt*-8 leads to pattern abnormalities even if transcripts are confined to dorsal or ventral blastomeres rather than being globally distributed. It is always possible that the dorsalized phenotype is not related to the normal function of *Xwnt*-8, but results from the aberrant activation of a heterologous receptor. Our working hypothesis, however, is that ectopic expression of *Xwnt*-8 activates its own nonlocalized receptors. Since ectopic expression of *Wnt*-1 leads to a phenotype which is identical to that observed with low doses of *Xwnt*-8, and since *Xwnt*-1 is first expressed well after the time at which the dorsal–ventral axis is established, we further hypothesize that ectopically expressed *Wnt*-1 is activating *Xwnt*-8 receptors. This is consistent with our original speculation that *Wnt*-1 might be acting through a receptor specific for another family member to produce this phenotype (McMahon and Moon, 1989*b*).

In any case, ectopic expression of *Xwnt*-8 appears to set a signalling pathway in motion at an inappropriate time and/or location, as a result of which the normal embryonic pattern is perturbed.

We are continuing to explore the role and potential interactions of *Xwnt*-8 in the cascade of signalling events involved in pattern formation during embryogenesis.

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