Development 121, 2177-2186 (1995)
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Xwnt-8b: a maternally expressed *Xenopus Wnt* gene with a potential role in establishing the dorsoventral axis

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SUMMARY

In amphibian embryos, establishment of dorsal-ventral asymmetry is believed to involve dorsal-ventral differences in vegetally derived mesoderm-inducing signals and/or differences in the competence of animal hemisphere (ectodermal) cells to respond to these signals. Previous studies have shown that certain Wnt proteins can generate an ectopic dorsal axis when misexpressed, and that they do so by modifying the response of ectodermal cells to inducers. None of these Wnt proteins are expressed at an appropriate time to do so in vivo. In this study, we describe the isolation and characterization of a full length cDNA for the *Xenopus Wnt* gene, *Xwnt*-8b, whose biological activity and expression pattern suggest that it may be involved in establishment of the dorsoventral axis. Both maternal and zygotic *Xwnt*-8b transcripts undergo alternative splicing to generate mRNAs which encode two different forms of Xwnt-8b protein. During early cleavage stages *Xwnt*-8b transcripts are confined primarily to animal hemisphere blastomeres, while zygotically derived *Xwnt*-8b transcripts are restricted almost exclusively to a band of cells in the prospective forebrain of neurula and tailbud stage embryos. Ectopically expressed *Xwnt*-8b can completely rescue dorsal development of embryos ventralized by exposure to ultraviolet light, and can induce a complete secondary axis in wild-type embryos. Axis induction is observed only if *Xwnt*-8b is supplied prior to the onset of zygotic gene transcription. This biological activity, together with the presence of maternal *Xwnt*-8b transcripts in cells that will be induced to form the dorsal mesoderm, is consistent with the possibility that *Xwnt*-8b may be the endogenous agent that establishes asymmetry in the response of ectodermal cells to mesoderm-inducing signals, thereby initiating dorsal development.

Key words: *Xenopus*, *Xwnt*-8b, dorsoventral patterning, forebrain

INTRODUCTION

Wnts are a family of structurally related cell-cell signalling molecules. Although the original member of this gene family (*Wnt*-1) was isolated as a proto-oncogene involved in mammary tumorigenesis, it is now better known as a development regulator. Over a dozen *Wnt*-1 related genes have been identified in diverse species, and have been shown to be expressed in unique, highly restricted patterns during development. Based on their expression patterns, it has been suggested that individual family members may be involved in shaping distinct regions of the embryo. Consistent with this hypothesis, loss of expression or misexpression of specific Wnts in mouse, *Drosophila*, or *Xenopus* embryos leads to distinct patterning defects (reviewed by McMahon, 1992; Moon et al., 1993a). One intriguing phenotype that is observed following transient misexpression of a number of different Wnts in *Xenopus* embryos is the induction of a complete secondary dorsal axis in normal embryos, or the induction of a primary axis in embryos that have been ventralized by exposure to ultraviolet (UV) light (reviewed by Christian and Moon, 1993a).

In amphibians, dorsal development is initiated by a cytoplasmic rearrangement (referred to as cortical rotation) which activates an inductive center, termed the Nieuwkoop center, on the dorsal side of the zygote. Cortical rotation can be blocked by exposure of zygotes to UV light, and these embryos lack all dorsal and anterior structures. During normal development, signals from the Nieuwkoop center induce a subpopulation of cells near the equator of the embryo to initiate dorsal mesodermal development. Equatorial cells outside of this zone are induced to initiate ventral mesodermal differentiation. The Nieuwkoop center functions prior to the time that zygotic gene expression begins at the midblastula stage. Thus, components of this signalling center must be derived from the maternal genome and must be present in the egg prior to fertilization.

A second dorsal inductive center, known as the Spemann organizer, begins operating shortly after the onset of zygotic gene expression. The Spemann organizer is composed of newly induced dorsal mesodermal cells. These cells emit signals that dorsalize the fate of adjacent ventral mesodermal cells, and that induce overlying ectodermal cells to adopt a neural fate.

Blastomere and cytoplasm transplantation studies have shown that signals from either the Nieuwkoop center (Gimlich, 1986) or the Spemann organizer (Spemann and Mangold, 1924) are sufficient to organize a secondary dorsal axis when introduced at the appropriate developmental stage. The axes induced by ectopically expressed Wnts appear to be due to the activation of a Nieuwkoop center-like signalling pathway, and not to activation of the later acting Spemann organizer signalling pathway. Specifically, axial induction is observed only if appropriate Wnts are introduced prior to the onset of zygotic gene transcription, when the Nieuwkoop center is normally active, and not if these same Wnts are first supplied after this time (Christian and Moon, 1993b).

Although ectopic expression of Wnts leads to the formation of dorsal mesoderm, Wnt signals cannot directly induce presumptive ectodermal cells to form dorsal mesoderm. Instead, Wnts appear to modify the response of cells to endogenous mesoderm inducing signals such that dorsal, rather than ventral, mesoderm differentiates (Christian et al., 1992; Sokol and Melton, 1992). Endogenous mesoderm-inducing signals may include members of the fibroblast growth factor (FGF) or transforming growth factor beta (TGFβ; e.g. Vg-1, activins, bone morphogenetic proteins) families (reviewed by Sive, 1993).

To date, *Xenopus* Wnt-1, Wnt-3A and Wnt-8 have all been shown to possess full axis inducing activity and yet none of these are expressed in a pattern consistent with their involvement in the maternal steps of dorsal development (reviewed by Moon et al., 1993a). Two maternally expressed *Xenopus Wnt* genes, *Xwnt*-5A and *Xwnt*-11, have been described, neither of which can induce a complete dorsal axis (Ku and Melton, 1993; Moon et al., 1993b).

In this report we describe the sequence, expression pattern, and dorsal axis inducing activity of a maternally expressed *Xenopus Wnt*, *Xwnt*-8b. During early cleavage stages *Xwnt*-8b transcripts are concentrated in prospective ectodermal cells of the animal hemisphere, while zygotically derived *Xwnt*-8b transcripts are restricted primarily to a narrow band of cells in the prospective forebrain. Ectopically expressed *Xwnt*-8b can completely rescue dorsal development of UV-irradiated embryos and can induce a complete secondary axis in normal embryos. This activity is observed only if *Xwnt*-8b is supplied prior to the onset of zygotic gene transcription. The biological activity of *Xwnt*-8b, together with the expression of *Xwnt*-8b in cells that will be induced to form the mesoderm, is consistent with the possibility that this *Wnt* may function to modify the response of ectodermal cells to mesoderm inducing signals, thereby initiating dorsal development.

MATERIALS AND METHODS

Isolation of cDNA clones and plasmid construction

A partial length *Xwnt*-8b cDNA clone (Wolda and Moon, 1992) was used to screen a *X. laevis* neurula (stage 17) λgt10 library (gift from D. Melton). The largest phage clone that rescreened positive was subcloned, as two *Eco*RI fragments, into pGEM1 (Promega). The two cDNAs were sequenced on both strands using the dideoxy chain termination method and T7 DNA polymerase (Sequenase, United States Biochemical). Neither of these cDNAs contained a sequence encoding the amino terminus of Xwnt-8b as evidenced by the absence of a methionine start site at the beginning of the open reading frame. A restriction fragment from the extreme 5′ end of the existing *Xwnt*-8b cDNA was used to rescreen the original cDNA library, two additional *Xenopus* neurula and tailbud stage cDNA libraries (gifts from T. Sargent and M. Saha), and a *Xenopus* genomic library (gift from A. Ribera). None of the clones isolated in these screens extended the existing sequence at the 5′ end. Sequence encoding the amino-terminal 22 amino acids of Xwnt-8b was obtained using the polymerase chain reaction (PCR)-based Rapid Extension of cDNA Ends (RACE) protocol (Frohman, 1993). PCR products were subcloned into pGEM-T (Promega) and four independent cDNAs from different PCR reactions were sequenced to verify that the sequence was reproducible and thus likely to be error free.

A cDNA containing the full coding region of *Xwnt*-8b was generated by recombining the original two cDNAs using unique restriction sites, and appending the RACE-generated cDNA onto the 5′ end using the Gene Splicing by Overlap Extension strategy (Horton, 1993). Those portions of the recombinant clone that were generated by PCR-based amplification of other cDNAs were resequenced to verify that errors had not been introduced during the amplification step. The full coding sequence was subcloned into the plasmid expression vector CSKApT (Christian and Moon, 1993b) to generate CSKA-*Xwnt*-8b. A *Sal*1-*Sma*1 fragment of CSKA-*Xwnt*-8b, which contains the full *Xwnt*-8b protein coding region, was subcloned into the transcription vector pSP64T (Kreig and Melton, 1984) to generate SP64T-*Xwnt*-8b.

Ribonuclease protection and in situ hybridization analysis

Total nucleic acids were isolated from developmentally staged *Xenopus* embryos as described by Moon and Christian (1989) and RNA selectively precipitated in 4 M LiCl. Ribonuclease protection analysis of 100 μg of total oocyte RNA, 50 μg of embryonic RNA or 10 μg of yeast tRNA was performed according to the method of Ausebel et al. (1994). To quantify levels of endogenous or ectopically expressed *Xwnt*-8b transcripts, RNA extracted from oocytes, from neurulae, or from cleaving embryos, which had been injected with synthetic *Xwnt*-8b RNA, was analyzed by ribonuclease protection along with known amounts of synthetic target *Xwnt*-8b RNA. Protected bands were visualized with a Molecular Dynamics phosphorimager and were quantified using the Macintosh IP lab gel program. The level of endogenous *Xwnt*-8b transcripts was calculated based on the assumption of approximately 5 μg of total RNA for early embryos (Gurdon and Wickens, 1983)

In situ hybridization of whole embryos to digoxigenin probes was performed as described by Harland (1991). Following the visualization step, embryos were dehydrated, embedded in paraffin, sectioned and stained as described by Kelly et al. (1991).

Analysis of RNA by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared by acid guanidinium thiocyanate-phenol-chloroform extraction as described by Chomczynski and Sacchi (1987), but precipitations were carried out in ethanol rather than isopropanol. Total RNA samples were treated with RNase-free DNase, phenol-chloroform extracted, and ethanol precipitated. Oligo dT-primed first strand cDNA was prepared from 5 μg of total RNA with an AMV reverse transcription kit (Life Sciences) in a 25 μl reaction according to the manufacturer's instructions. Negative controls excluding reverse transcriptase were prepared in parallel for each RNA sample. 50 μl PCR reactions were carried out using AmpliTaq DNA polymerase (1 unit/reaction) and PCR buffer, with 200 μM dNTP's, 1 μCi of (α-^{32}P)dCTP, 15 pmol of each primer, and 1.5 μl of first strand cDNA. In order to ensure equivalent loading of all components of the PCR reaction, 'master mixes' were prepared for each cDNA sample with all components of the reaction except primers. Aliquots of this mix were then transferred to the reaction tubes to which primers had been added. The initial denaturation was carried out at 94˚C for 4 minutes and was followed by three-temperature

cycling: denaturation at 94˚C for 30 seconds, annealing for 30 seconds at 54˚C, and extension at 72˚C for 30 seconds. The final extension was carried out at 72˚C for 2 or 4 minutes. Cycle number was determined empirically for each primer pair, so that PCR products were examined during the exponential phase of amplification. Histone H4, EF-1α, Vg1 and *Xwnt*-11 primers were all used at 20-24 cycles. At these cycle numbers, amplification occurred in the exponential range for the samples with low levels of the cDNA of interest (data not shown). *Xwnt*-8b and *Xwnt*-8 PCR amplification was carried out for 40 cycles in all trials. After amplification, 13% of each reaction was run on a 5% polyacrylamide gel which was dried and exposed to film at –75˚C with an intensifying screen.

The sequences of primers used for RT-PCR are listed here, in 5′ to 3′ orientation. *Xwnt*-8b: upstream, (TTTAGATCT – *Bgl*II site) TGACTTGAACATCCATTCT and downstream, (*Hin*dIII site) TGGAGAAAGGAATCCTGTA; *Xwnt*-8 (Christian and Moon, 1991): upstream, AATGGAATTGAGGAGTGT and downstream, GCTCCTCTGTTGTCAGC; Histone H4 (Niehrs et al., 1994): upstream, CGGGATAACATTCAGGGTA and downstream, TCCATGGCGGTAACTGTC; *Xwnt*-11 (Ku and Melton, 1993): upstream, GAAGTCAAGCAAGTCTGCTGGG and downstream, GCAGTAGTCAGGGGAACTAACCAG; Vg1 (Weeks and Melton, 1987): upstream, CCCTCAATCCTTTGCGGTG and downstream, CAGAATTGCATGGTTGGACCC; EF-1α (Hemmati-Brivanlou and Melton, 1994): upstream, CAGATTGGTGCTGGATATGC and downstream, ACTGCCTTGATGACTCCTAG.

Embryo culture and manipulation

Xenopus eggs were obtained and embryos were cultured as described by Moon and Christian (1989). All embryonic stages are according to Nieuwkoop and Faber (1967). UV irradiation of zygotes was performed as described by Christian et al. (1991).

In vitro transcription and microinjection of synthetic RNA or expression plasmids

Capped synthetic RNA was generated by in vitro transcription of SP64T-*Xwnt*-8b and synthetic RNA or plasmid DNA was injected into cleaving *Xenopus* embryos as described by Moon and Christian (1989).

RESULTS

Sequence analysis of Xwnt-8b

The predicted amino acid sequence of Xwnt-8b is shown in Fig. 1, aligned with the sequence of the most closely related Wnt family member, Xwnt-8. Xwnt-8b contains a hydrophobic putative leader sequence and, diagnostic for Wnt proteins, a large number of invariant cysteine residues. The position of 19 out of 22 cysteine residues is shared between Xwnt-8b and members of the Xwnt family outside of Xwnt-8, and overall amino acid identity ranges from 27-31% (data not shown). Among Wnts, subsets of proteins have been identified that display a much higher degree of homology with each other, but which are expressed in distinct temporal and/or spatial patterns, suggesting that they serve non-redundant functions. The amino acid sequence of Xwnt-8b is 56% identical to that of Xwn*t*-8, and the positions of all 22 cysteine residues are conserved. If conservative amino acid substitutions are taken into consideration, the degree of homology between Xwnt-8 and Xwnt-8b is 67%. In addition, both proteins possess a highly basic 20-22 amino acid carboxy-terminal extension relative to other known Xwnts (residues 414-435 of Xwnt-8b, Fig. 1). These data support the designation of Xwnt-8b as a submember of the Xwnt-8 family. Recently, a one nucleotide error in the originally reported sequence of *Xwnt*-8 was discovered. The corrected DNA sequence introduces an earlier stop codon such that the predicted translation product (Fig. 1) is shorter than that reported by Christian et al. (1991).

Developmentally regulated alternative splicing of *Xwnt*-8b transcripts

The predicted amino acid sequence of Xwnt-8b includes a 73 amino acid insert (residues 84-156, Fig. 1) which is not conserved among other known Wnts. Sequence encoding this insert is located at a predicted intron/exon junction (Nusse and Varmus, 1992), the location of which was confirmed by sequencing portions of a *Xwnt*-8b genomic clone across this boundary (Fig. 2A). Ribonuclease protection analysis of embryonic RNA, using a probe which overlaps the boundary between conserved and nonconserved Wnt sequence (Fig. 2B), revealed the developmentally regulated expression of two unique *Xwnt*-8b transcripts (Fig. 2C). Messenger RNAs that protect an 139 nucleotide fragment of the probe used in this assay would be generated by splicing out the unique exon, and these mRNAs would encode a form of Xwnt-8b protein in which the non-conserved 73 amino acid insert is absent.

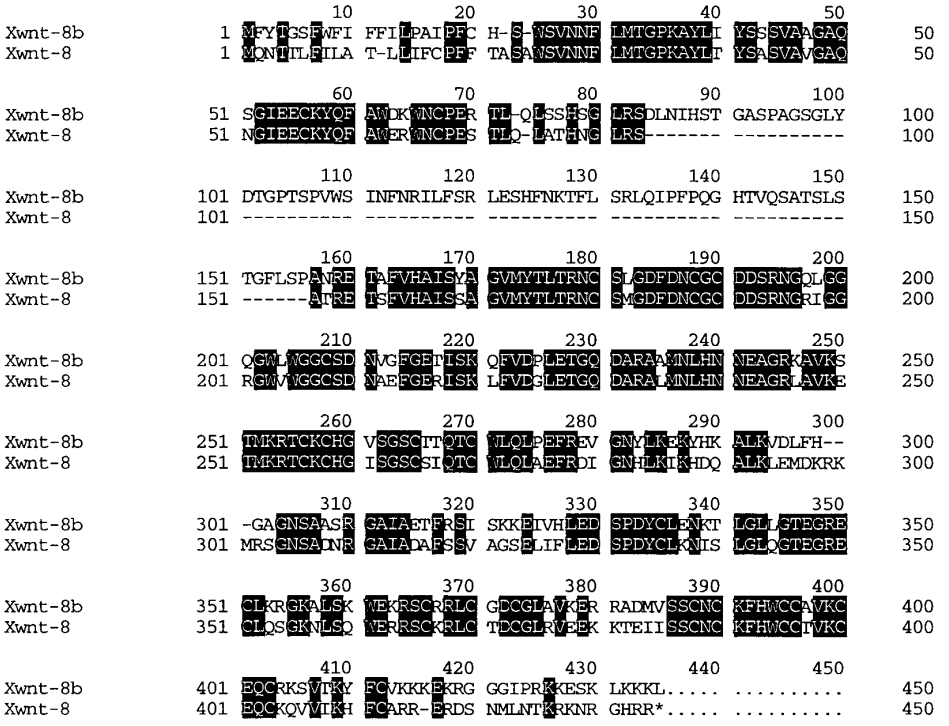

Fig. 1. Comparison of predicted Xwnt-8b and Xwnt-8 protein sequences. Gaps introduced to align the sequences are shown as dashes and identical residues are shaded.

Zygotic RNAs encoding this shorter form of Xwnt-8b were first detected near the onset of gastrulation (st. 10.5), increased in abundance by the end of gastrulation (st. 13), and peak levels were observed in swimming tadpoles (st. 38; Fig. 2C). A second class of transcripts, which would protect a 240 nucleotide fragment of the probe used in this assay, would be generated by splicing in the unique exon, and these would encode the relatively longer form of Xwnt-8b protein which includes the nonconserved core of amino acids as shown in Fig. 1. Zygotic transcripts encoding the longer form of Xwnt-8b were first detected at the end of gastrulation (st. 13), were present at fairly equivalent levels throughout the tailbud stages (st. 32) and then declined to barely detectable levels in tadpoles (st. 38).

Maternally derived *Xwnt*-8b transcripts are enriched in animal blastomeres of cleaving embryos

To determine whether less abundant *Xwnt*-8b transcripts might be present prior to gastrulation, RNA isolated from unfertilized eggs was assayed by reverse transcription-PCR (RT-PCR). Maternal *Xwnt*-8b RNAs are clearly detectable, although transcript levels increase after the onset of zygotic gene transcription (Fig. 3A). As a positive control for maternal and zygotically expressed genes, the same RNA samples were analyzed for *Xwnt*-11 transcripts, which are known to be present in eggs and early embryos (Ku and Melton, 1993), for *Xwnt*-8 transcripts, which are not detectable until the late blastula stage (Christian et al., 1991), for Histone H4, which serves as a control for approximately equal amounts of RNA in each reaction, and for EF-1α, which is expressed at low levels maternally, and is strongly upregulated at the onset of zygotic gene transcription (Krieg et al., 1989) (Fig. 3A).

To localize *Xwnt*-8b transcripts along the animal-vegetal axis, 8-cell embryos were dissected into animal and vegetal halves and RNA from each was analyzed by RT-PCR. As shown in Fig. 3B, *Xwnt*-8b transcripts are clearly enriched in animal half blastomeres relative to vegetal cells. To control for accuracy in dissections and for equal amounts of RNA in each reaction, the same RNA samples were assayed for expression of vegetally localized (Vg1; Weeks and Melton, 1987) and uniformly distributed (Histone H4; Weeks and Melton, 1987) transcripts (Fig. 3B). Identical results were obtained with RNA isolated from animal and vegetal halves of 32-cell embryos (data not shown).

To quantify levels of endogenous *Xwnt*-8b transcripts during development, total RNA from mature oocytes and neurula (st. 18) embryos was analyzed in an RNase protection assay, using synthetic *Xwnt*-8b RNA to derive a standard curve. The probe used in this assay did not distinguish between the two *Xwnt*-8b splice variants discussed above. The absolute level of *Xwnt*-8b transcripts was estimated as approximately 0.15 pg of RNA per oocyte, increasing to a maximum of approximately 1 pg per embryo by the late gastrula stage (Fig. 3C).

Spatial distribution of zygotic *Xwnt*-8b transcripts

The localization of *Xwnt*-8b transcripts was analyzed by whole-mount in situ hybridization of digoxigenin-labeled ribo-

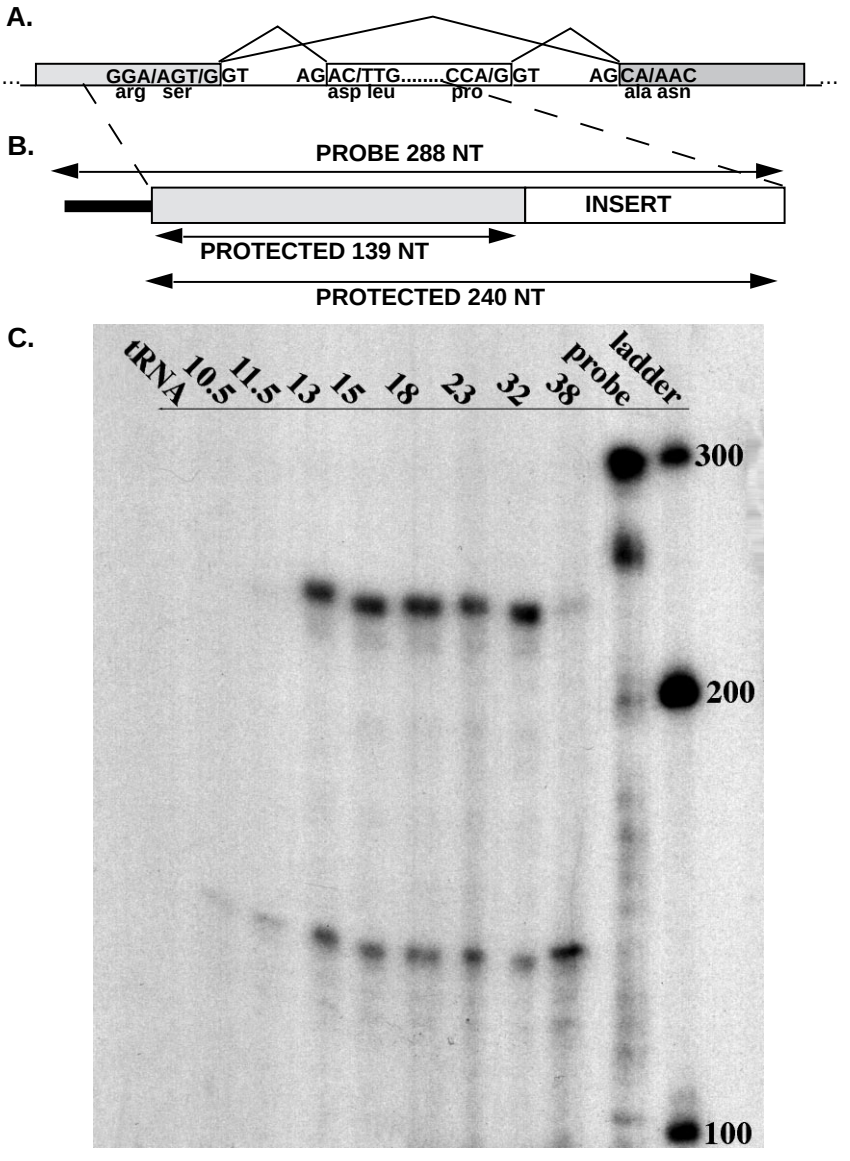

Fig. 2. Developmental expression of two *Xwnt*-8b transcripts generated by differential splicing. (A) Schematic diagram of differential splicing event. Exons are shown as bars and introns as lines. Nucleotides at the intron/exon borders match conserved consensus sequences for eukaryotic splice sites (Mount, 1982) as shown. (B) Schematic diagram of cDNA that was transcribed to generate a probe for ribonuclease protection analysis of *Xwnt*-8b expression. Dotted lines indicate location of 5′ and 3′ limits of the probe template relative to alternatively spliced exon. Solid bar represents vector sequence included in unprotected probe. (C) Ribonuclease protection analysis of 10 μg of tRNA or 50 μg of total RNA isolated from embryos at the developmental stage indicated at the top of each lane (numbers refer to stages according to Nieuwkoop and Faber, 1967).

probes to staged embryos. *Xwnt*-8b transcripts are first detected in late gastrula to early neurula stage embryos as diffuse staining of a broad region of the prospective anterior neural ectoderm (data not shown). By mid- to late-neurula stage, the staining is concentrated in a single band of cells located at the predicted junction between the future forebrain and midbrain (Fig. 4A,B, arrowheads). In tailbud stage embryos, *Xwnt*-8b transcripts are detected in a thin, bilaterally symmetric band of cells in the dorsal diencephalon (Fig. 4C,E-H, black arrowheads), and at the forebrain-midbrain boundary (Fig. 4C, white arrowhead). When embryos are stained for prolonged periods specific signal is observed in a few cells in the dorsal mesencephalon, just posterior to the forebrain (Fig. 4C, right side, white arrow). In swimming tadpoles an identical pattern of staining is observed, but the signal is much less intense (data not shown). Staining is not observed in embryos hybridized with a sense *Xwnt*-8b probe (Fig. 4D).

Xwnt-8b has dorsal axis inducing activity when misexpressed prior to the midblastula stage

Synthetic mRNA encoding Xwnt-8b was injected into various blastomeres of 4-8 cell embryos in order to assay for potential functions of endogenous maternally derived *Xwnt*-8b. Injection of approximately 3-10 pg of *Xwnt*-8b RNA near the prospective ventral midline in the marginal zone or vegetal region led to the induction of a secondary dorsal axis, which in many cases was anteriorly complete (Table 1 and Fig. 5A). Injection of an equivalent amount of *Xwnt*-8b RNA into dorsal marginal zone cells did not produce observable phenotypic defects in most embryos (Table 1 and Fig. 5B).

Given the observation that misexpressed *Xwnt*-8b can organize a second dorsal axis in normal *Xenopus* embryos, we further tested its dorsal inducing capacity by asking whether ectopically expressed *Xwnt*-8b could rescue dorsoanterior development in UV-ventralized embryos. Embryos that had been irradiated with UV light prior to first cleavage lacked dorsal axial structures, developing with an average dorsoanterior index (DAI; Kao and Elinson, 1988) of 0.27 (n=33). In contrast, 95% of sibling embryos, which were identically irradiated and then injected with approximately 5 pg of *Xwnt*-8b RNA at the 4 cell stage, showed either complete rescue of dorsoanterior development (DAI 5) or were normal with the exception of slightly reduced eyes and forehead (DAI 4). The average DAI of the *Xwnt*-8b RNA-injected embryos was 4.2 (n=63).

To assay for potential functions of zygotically derived *Xwnt*-8b, a plasmid expression construct (CSKA-*Xwnt*-8b) consisting of the *Xwnt*-8b cDNA cloned downstream of the cytoskeletal actin promoter, was injected into various blastomeres of cleaving embryos. This promoter is first transcriptionally active after the midblastula transition (Harland and Misher, 1988). As a control for non-specific defects, sibling embryos were injected with the plasmid CSKA-CAT, which is designed to express bacterial

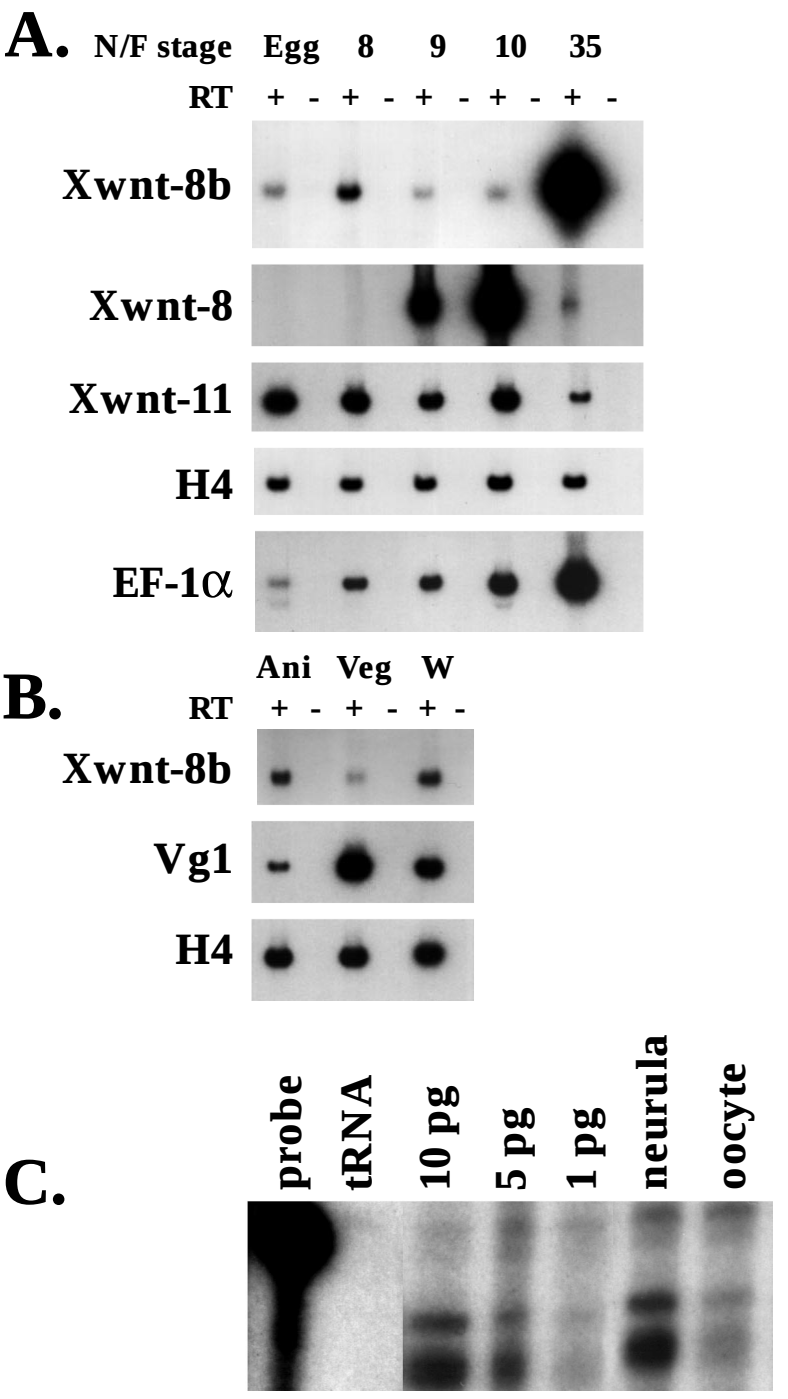

Fig. 3. Expression of maternal *Xwnt*-8b transcripts in eggs and cleaving embryos. (A) Analysis by RT-PCR of the expression of *Xwnt*-8b, *Xwnt*-8, *Xwnt*-11, Histone H4 (H4) and EF-1α in unfertilized eggs (Egg) and embryos at the indicated stages (N/F: Nieuwkoop and Faber, 1967). (B) Analysis by RT-PCR of the expression of *Xwnt*-8b, Vg1 and Histone H4 in blastomeres isolated from the animal (Ani) or vegetal (Veg) half of 8-cell embryos. For each RNA sample, PCRs were performed on duplicate aliquots incubated with (+) or without (–) reverse transcriptase (RT) as indicated above each lane. (C) RNase protection assay for *Xwnt-8b* in total RNA samples from oocytes (100 μg) and st. 18 neurulae (50 μg). Also included in the assay is in vitro synthesized *Xwnt-8b* RNA at the amounts indicated.

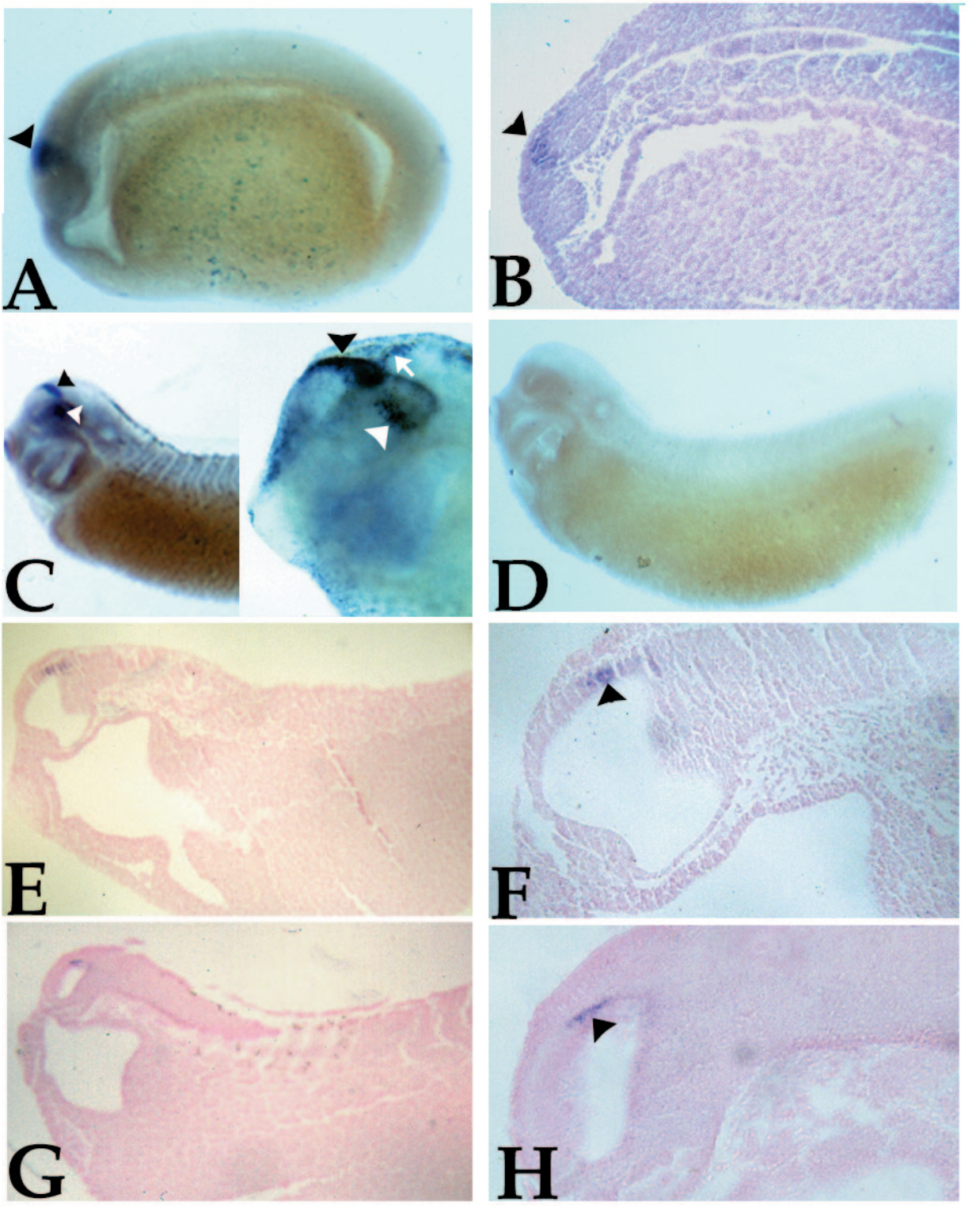

Fig. 4. Localization of *Xwnt*-8b transcripts in neurula and tailbud stage embryos by whole-mount in situ hybridization. (A) Neurula (st. 21), and (B) sagittal section of neurula stage embryo hybridized with a digoxigenin-labeled antisense *Xwnt*-8b probe showing signal at junction between prospective prosencephalon and mesencephalon (arrowheads). (C) Low (left side) and high (right side) magnification views of whole tailbud (st. 33) embryos hybridized with antisense *Xwnt*-8b probe. Staining of dorsal diencephalon (black arrowhead), forebrain-midbrain border (white arrowhead) and dorsal mesencephalon (white arrow) is indicated. Embryo on right was stained overnight to visualize midbrain staining. All of the blue signal with the exception of that indicated by arrows was also observed in embryos hybridized overnight with a sense probe (data not shown) and is thus nonspecific staining. (D) Tailbud (st. 33) embryo hybridized with sense *Xwnt*-8b probe. Low (E,G) and high (F,H) magnification views of sagittal sections of tailbud (st. 33) embryos showing *Xwnt*-8b expression in the dorsal diencephalon (arrowheads).

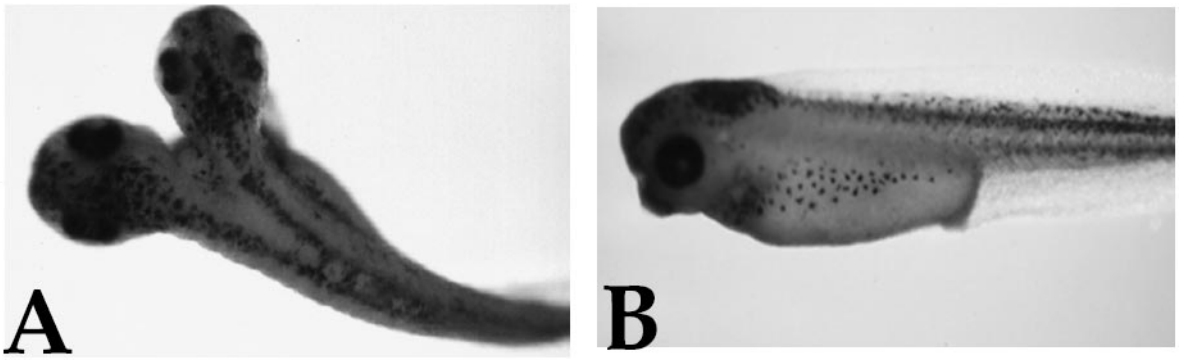

Fig. 5. Injection of synthetic RNA encoding *Xwnt*-8b into ventral blastomeres of 4-cell embryos induces formation of an anteriorly complete secondary dorsal axis. Approximately 10 pg of *Xwnt*-8b RNA was injected near the marginal zone into ventral (A) or dorsal (B) blastomeres of 4-cell *Xenopus* embryos.

Table 1. Phenotype of *Xenopus* embryos injected with RNAs or cDNAs encoding Xwnt-8b

		Phenotype			
RNA/DNA	Injection site	WT	2° Axis	Aceph/ micro	*n*
Xwnt-8b RNA	VMZ	6	94	0	137
Xwnt-8b RNA	DMZ	93	7	0	54
CSKA-X8b	VMZ	100	0	0	67
CSKA-X8b	DMZ	88	0	12	230
CSKA-CAT	DMZ	98	0	2	84

DNA was injected into the marginal zone region of 2 blastomeres of 4-cell embryos, near the prospective dorsal (DMZ) or ventral (VMZ) midline. Embryos surviving until stage 36 were scored as follows: wild type (WT), showing no specific defects; secondary (2°) axis, complete or partial duplication of dorsoanterior structures; acephalic (Aceph) or microcephalic (micro), reduced or absent head. Numbers are expressed as percentages except for *n* which denotes sample size.

chloramphenical acetyl transferase (Christian and Moon, 1993b). Injection of approximately 200 pg of CSKA-*Xwnt*-8b into the equatorial region of 4-cell embryos at either the dorsal or ventral midline produced tadpoles which, in most cases, were indistinguishable from uninjected sibling embryos, or from embryos injected with the CAT expression plasmid (Table 1). A small percentage of embryos injected dorsally with the *Xwnt*-8b expression plasmid showed a reduction in anterior structures similar to, but less severe than, that observed following misexpression of the related protein, Xwnt-8, in dorsal cells after the midblastula transition (Christian and Moon, 1993b). Ribonuclease protection analysis of RNA extracted from CSKA-*Xwnt*-8b-injected embryos revealed that *Xwnt*-8b transcripts were present at levels much higher than endogenous levels at the late blastula through tailbud stages in plasmid-injected embryos (data not shown).

DISCUSSION

Zygotically expressed *Xwnt*-8b may function in neural patterning

We have shown that alternatively spliced transcripts encoding two distinct forms of Xwnt-8b protein are present in *Xenopus* embryos both prior to, and after, the onset of zygotic gene transcription. Zygotically derived *Xwnt*-8b transcripts are restricted to a stripe of cells that presage the formation of a morphologically recognizable forebrain-midbrain boundary. It has been suggested that the vertebrate neural plate may behave as a developmental field analogous to that of the insect parasegment, and that the same molecular strategy may be used to pattern both types of fields (Ruiz i Altaba, 1994). According to this hypothesis patterning molecules produced by cells at boundary regions, including members of the Wnt family, would establish polarity within a neural field by controlling the fate of adjacent cells. The pattern of expression of *Xwnt*-8b during neurulation is consistent with the possibility that *Xwnt*-8b may participate in specifying polarity within the forebrain field.

Xwnt-8b is one of only a few vertebrate genes that is expressed in the most anterior region of the developing central nervous system. As such, it should provide a valuable tool for studying the molecular mechanisms underlying one of the least understood aspects of neural patterning, namely forebrain induction.

Maternally expressed *Xwnt*-8b may function as a dorsal determinant

The presence of maternal *Xwnt*-8b transcripts, coupled with our demonstration that *Xwnt*-8b can induce the formation of a complete dorsal axis if introduced prior to, but not after the midblastula stage, is consistent with the possibility that endogenous *Xwnt*-8b may be involved in establishing the dorsal axis.

The mechanism by which the dorsal side of vertebrate embryos is first specified remains a matter of debate. One model suggests that the dorsal axis is established by a signal derived from dorsal vegetal cells, which both induces the formation of mesoderm, and confers dorsal pattern. This view had its beginnings with studies showing that, in *Xenopus*, the mesoderm arises from the ectodermal (animal) half of the embryo under an inductive influence of the endodermal (vegetal) half (Sudarwati and Nieuwkoop, 1971). When the animal half of early *Ambystoma* embryos is rotated by 180°, dorsoventral polarity is reversed suggesting that mesodermal pattern is determined by the endodermal, or inducing, cells and not by the ectodermal, or responding, cells (Nieuwkoop, 1969).

Several members of the TGFβ family have been shown to

be capable of inducing isolated ectoderm to differentiate as dorsal mesoderm, and one of these, Vg1, is present in endodermal cells at the right time to perform this function in vivo (reviewed by Vize and Thomsen, 1994). Recent studies have shown that if Vg1 is processed to its mature form, it can both induce and dorsally pattern mesoderm (Dale et al., 1993; Thomsen and Melton, 1993). These results are consistent with the possibility that Vg1 activity alone may be sufficient to establish the dorsal axis. Despite this, processed endogenous Vg-1 is not detected (reviewed by Vize and Thomsen, 1994), and embryological evidence suggests that additional dorsalizing signals, as well as the absence of ventralizing signals (reviewed by Harland, 1994), may be required for dorsal patterning.

A second model for axial patterning suggests that mesodermal induction and dorsal patterning are distinct events which require the cooperative action of separate signalling agents (reviewed by Christian and Moon, 1993a). According to this model, dorsal pattern is imprinted upon prospective dorsal ectoderm by a signal that modifies the response of these cells to a mesoderm inducing signal which is uniformly distributed across the axis. In the absence of the modifying signal prospective ventral ectodermal cells are induced to form ventral mesoderm, and in its presence, ectodermal cells on the prospective dorsal side are induced to form dorsal mesoderm. Support for this model comes from studies showing that animal pole ectoderm from prospective dorsal and ventral embryonic regions is biased in its response to mesoderm induction. Specifically, activin and FGF can induce dorsal, but not ventral, ectoderm to form dorsal mesodermal structures (Ruiz i Altaba and Jessel, 1991; Sokol and Melton, 1991, Kimelman and Maas, 1992; Bolce et al., 1992). This bias in ectodermal response is evident prior to the time that mesoderm-inducing signals are first produced (Kinoshita et al., 1993) suggesting that the signals that establish this bias are distinct from inducing signals.

Certain members of the Wnt family can mimic the endogenous signal that patterns the responsiveness of prospective dorsal ectoderm, yet Wnt signals alone are not sufficient to induce ectoderm to form mesoderm (reviewed by Christian and Moon, 1993a). In contrast, Vg1 can induce mesoderm, but does not appear to be involved in modifying the response of ectoderm to inducing signals. Specifically, ectodermal cells in which endogenous Vg1 signalling is blocked (Schulte-Merker et al., 1994) can still form muscle in response to FGF (Hemmati-Brivanlou and Melton, 1992), while ventral or ventralized ectoderm cannot do so (Kimelman and Maas, 1992).

Fate mapping studies have shown that dorsal mesodermal progenitor cells reside within the dorsal equatorial region of gastrula stage embryos (Keller, 1976), and these cells are derived almost entirely from blastomeres which were located in the animal half of cleaving embryos (Bauer et al., 1994). Thus, the expression of *Xwnt*-8b transcripts primarily within animal blastomeres of cleaving embryos is consistent with the possibility that endogenous *Xwnt*-8b may function within these cells to modify their response to vegetally derived, secreted inducing signals. Although blastomere transplantation and cytoplasm transfer experiments clearly demonstrate the existence of a dorsal inductive activity in vegetal cells of early embryos (Gimlich, 1986; Kaguera, 1990; Yuge et al., 1990; Fujisue et al., 1993) these, and similar studies (Cardellini, 1988; Gallagher et al., 1991; Hainski and Moody, 1992) provide evidence that cells in the animal half of the embryo also contain dorsal patterning activity.

Are maternal *Wnt*s required for dorsal development in *Xenopus*?

Although members of the Wnt family are capable of initiating dorsal axial development in *Xenopus* when ectopically expressed, it is has yet to be proved that they actually do so. Another candidate for this role is noggin, a non-Wnt-related protein which, like Wnts, can modify the response of ectodermal cells to mesoderm inducers when ectopically expressed, thereby generating a dorsal axis (Smith and Harland, 1992). Maternal transcripts encoding noggin are present during early cleavage stages (Smith and Harland, 1992) but do not appear to be translated (Schroeder and Yost, 1994) suggesting that noggin does not function in the early steps of dorsal determination.

While the expression pattern and biological activity of Xwnt-8b are consistent with its involvement in dorsal patterning, the quantity of endogenous maternal *Xwnt*-8b RNA is significantly lower than the amount of synthetic RNA which must be injected in order to induce formation of a secondary axis. However, the relative quantity of synthetic and endogenous transcripts does not necessarily reflect relative protein levels since endogenous *Xwnt*-8b transcripts may be translated over a period of weeks or months during oogenesis, whereas injected transcripts must be translated within hours (i.e. prior to the midblastula stage) in order to have a dorsalizing effect upon the embryo. In addition, translational or post-translational regulation may normally be involved in activating Xwnt-8b protein on the dorsal side, as has been proposed for Vg1 (Thomsen and Melton, 1993), such that relatively larger amounts of synthetic transcripts must be injected into ventral cells to compensate for the reduced activation.

In support of the hypothesis that an endogenous Wnt-like activity may be required for dorsal development in *Xenopus*, recent studies have shown that β-catenin, a protein which is believed to be a component of the vertebrate Wnt signal transduction pathway (reviewed by Pfeifer, 1993), is required for dorsal induction. Injection of Fab fragments directed against β-catenin (McCrea et al., 1992), or of mRNA encoding either the intact (Guger and Gumbiner, 1994) or a deletion mutant (Funyama et al., 1994) form of β-catenin into ventral cells of *Xenopus* embryos induces the formation of a secondary axis. Furthermore, when maternal β-catenin transcripts are depleted by introducing antisense oligonucleotides into oocytes, dorsal mesoderm formation is blocked (Heasman et al., 1994). Injection of *Xwnt*-8 RNA into β-catenin-depleted embryos does not rescue any aspect of axial development (Heasman et al., 1994) suggesting that β-catenin is an essential component of Wnt-signalling, and is required for formation of the embryonic axis.

The strongest evidence against the involvement of Wnts in the maternal steps of dorsal axis formation has been the apparent absence of a maternally expressed Wnt which possesses full axis inducing activity. The biological activity of *Xwnt*-8b, together with the presence of *Xwnt*-8b transcripts in cells that will be induced to form the mesoderm, is consistent with the possibility that this Wnt may function to modify the response of ectodermal cells to mesoderm inducing signals,

thereby participating in the establishment of the dorsal axis. Further studies involving ablation of endogenous Xwnt-8b expression or activity will be required to substantiate this hypothesis.

We thank Sharon Wolda for isolating and sequencing *Xwnt*-8b cDNA clones. This work was supported by a grant from the Medical Research Foundation of Oregon and NIH grant R29 HD31087 to J. L. C. and NIH grant HD 27525 to R. T. M.

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(Accepted 30 March 1995)