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The *Xenopus* homeobox gene *Twin* mediates Wnt induction of *Goosecoid* in establishment of Spemann's organizer

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SUMMARY

We describe the isolation of the *Xenopus* homeobox gene *twin* (*Xtwn*), which was identified in an expression cloning screen for molecules with dorsalizing activities. Injection of synthetic *Xtwn* mRNA restores a complete dorsal axis in embryos lacking dorsal structures and induces a complete secondary dorsal axis when ectopically expressed in normal embryos. The sequence homology, expression pattern and gain-of-function phenotype of *Xtwn* is most similar to the previously isolated *Xenopus* homeobox gene *siamois* (*Xsia*) suggesting that *Xtwn* and *Xsia* comprise a new subclass of homeobox genes important in dorsal axis specification. We find that *Xtwn* is able to activate the Spemann organizer-specific gene *goosecoid (gsc)* via direct binding to a region of the *gsc* promoter previously shown to mediate Wnt induction. Since *Xtwn* expression is strongly induced in ectodermal (animal cap) cells in response to overexpression of a dorsalizing Wnt molecule, we examined the possibility that *Xtwn* might be a direct target of a Wnt signal transduction cascade. First, we demonstrate that purified LEF1 protein can interact, in vitro, with consensus LEF1/TCF3-binding sites found within the *Xtwn* promoter. Second, these binding sites were shown to be required for Wnt-mediated induction of a *Xtwn* reporter gene containing these sites. As LEF1/TCF3 family transcription factors have previously been shown to directly mediate Wnt signaling, these results suggest that *Xtwn* induction by Wnt may be direct. Finally, in UV-hyperventralized embryos, expression of endogenous *Xtwn* is confined to the vegetal pole and a *Xtwn* reporter gene is hyperinduced vegetally in a LEF1/TCF3-binding-site-dependent manner. These results suggest that cortical rotation distributes Wnt-like dorsal determinants to the dorsal side of the embryo, including the dorsal marginal zone, and that these determinants may directly establish Spemann's organizer in this region.

Key words: *twin*, *goosecoid*, *siamois*, *Xenopus*, homeobox, Wnt, cortical rotation, Spemann's organizer, Nieuwkoop Center, dorsal determinants, LEF1, TCF3, gastrulation

INTRODUCTION

Discovery of the amphibian organizer played a central role in shaping the concept that inductive interactions are important in the establishment of the dorsoventral axis. In 1924, Spemann and Mangold discovered that heterotopic transplantation of the dorsal blastopore lip, or organizer, into the lateral/flank region of a host embryo could induce a secondary axis comprised predominantly of host tissue (Spemann and Mangold, 1924). Formation of the organizer has been traced back to the events of fertilization (reviewed by Gerhart et al., 1989). The site of sperm entry determines the direction of rearrangement of egg cytoplasm (Danilchik and Denegre, 1991) including the rotation of cortical cytoplasm relative to the deep endoplasm which thereby defines the future dorsal side of the embryo (Gerhart et al., 1989). Treatments that block cortical rotation (e.g. UV-irradiation, cold-shock, or nocodazole) result in embryos lacking dorsal structures such as notochord, somites and neural tissue. A normal body axis can be rescued by manually tipping the embryo 90° along its animal-vegetal axis, underscoring the importance of cortical rotation. Similarly, equatorial injection of vegetal cytoplasm into UV-hyperventralized embryos results in axis rescue indicating that cortical rotation is important in the transport of dorsal determinants from the vegetal pole to the dorsal side of the embryo (Holowacz and Elinson, 1993, Kageura, 1997).

Much effort has been placed on characterizing and elucidating the nature of the inductive events and molecules involved in establishment of Spemann's organizer. Conjugation experiments of animal pole ectoderm and vegetal pole endoderm suggested that mesoderm forms in the marginal zone as the result of inductive signals released from the underlying vegetal pole endoderm (Nieuwkoop, 1973). In addition, it was found that ventrovegetal fragments induce only ventral mesoderm while dorsovegetal fragments, also referred to as the Nieuwkoop Center (NKC), are capable of inducing dorsal

mesoderm (prospective organizer) (Nieuwkoop, 1973; Dale and Slack, 1987; Gimlich and Gerhart, 1984). The 'three signal model' incorporates these observations to suggest that three inductive signals play a role in dorsoventral specification (Dale and Slack, 1987). During blastula stages, a signal from the ventrovegetal endoderm and a second signal from the dorsovegetal endoderm (possibly released at midblastula transition; Wylie et al., 1996) induce the overlying marginal zone cells to become ventral and dorsal mesoderm, respectively. Once induced by the NKC, the organizer releases a third signal, during gastrulation, which promotes dorsalization of the adjacent lateral mesoderm to further pattern the mesoderm. While the three signal model explains the embryological data, it is worth noting that more than three signals (Sive, 1993) are likely to be involved in dorsal-ventral patterning of the mesoderm and the localization of molecules underlying each of these signals may overlap in the embryo.

Candidate mesoderm-inducing factors (MIFs) include activin, Vg1, Xnr1 and Xnr2, members of the transforming growth factor (TGF-β) superfamily, which can induce dorsal mesoderm in isolated naive ectodermal explants (animal caps) (Asashima et al., 1990; Thomsen et al., 1990; Smith et al., 1990; Dale et al., 1993; Jones et al., 1995). Microinjection of synthetic mRNA encoding activin or BVg1 (a processible form of Vg1) into the ventral blastomeres of early embryos leads to secondary axes, thereby mimicking the NKC/organizer transplantation phenotypes (Thomsen et al., 1990; Thomsen and Melton, 1993; Dale et al., 1993). Another class of secreted factors, termed competence modifiers, has been implicated in mesodermal patterning. *XWnt8*, a member of the Wnt superfamily of growth factors, is a potent inducer of secondary axes when its mRNA is injected ventrally but, in contrast to MIFs, is an ineffective mesoderm inducer in animal cap assays (Christian et al., 1992). When animal caps are treated with low concentrations of MIFs, the animal caps differentiate into ventral mesoderm. However, with the addition of *XWnt8*, ectodermal cells adopt a more dorsal fate (organizer), suggesting that Wnt-like factors synergistically interact with MIFs on the dorsal side of the embryo to establish Spemann's organizer (Christian et al., 1992; Kimelman et al., 1992; Sokol and Melton, 1992; Sokol, 1993).

Support for a synergistic interaction between Wnt-like signals and MIFs in organizer specification was provided by analysis of the regulation of the organizer-specific homeobox gene, *goosecoid* (*gsc*). Molecular studies revealed that *gsc* expression is mediated by the combined effects of Wnt-like and activin/BVg1-like signals via two *cis*-acting regulatory elements within its promoter, the proximal element (PE) and the distal element (DE), respectively (Watabe et al., 1995). While DE-mediated activin induction of *gsc* was determined to be direct (not requiring de novo protein synthesis), the nature of the PE-mediated induction by Wnt could not be determined due to the lack of soluble Wnt protein.

The Wnt signal transduction cascade has been linked to cortical rotation. The dorsal determinants relocalized to the dorsal side by cortical cytoplasmic rotation have been shown to behave like competence modifiers, suggesting the possibility that the dorsal determinants may be Wnts themselves or downstream components of a Wnt signaling cascade (Holowacz and Elinson, 1995). Consistent with this hypothesis, recent results suggest that β-catenin protein, a downstream effector of Wnt signaling, may be transported to the dorsal side by cortical rotation where it becomes dorsally enriched (Larabell et al., 1997; Rowning et al., 1997; Schneider et al., 1996). In the absence of β-catenin protein, embryos produce mesoderm, but fail to develop dorsal axial structures (Heasman et al., 1994). β-catenin interacts with members of the LEF1/TCF3 family of HMG box transcription factors (Molenaar et al., 1996; Behrens et al., 1996; Huber et al., 1996) and this interaction is required for dorsal determination in *Xenopus* embryos (Molenaar et al., 1996). The importance of the β-catenin-LEF1/TCF3 interaction for Wnt signaling has also recently been confirmed in *Drosophila* (Brunner et al., 1997; van der Wetering et al., 1997). These observations imply that Wnt-like dorsal determinants, localized to the dorsal side by cortical rotation, may act directly or indirectly on the PE (in concert with activin-like signaling via the DE) to induce *gsc* transcription and hence establish Spemann's organizer.

In this paper, we report the isolation of the *Xenopus* homeobox gene *twin* (*Xtwn*) by expression cDNA library screening for novel *Xenopus* axis-inducing molecules. Both the spatiotemporal expression profile and the axis-inducing activity of *Xtwn* are consistent with a role for *Xtwn* in the establishment of Spemann's organizer. Interestingly, expression of *Xtwn* in animal caps is strongly induced by *XWnt8*, but only poorly by activin. We suggest that a Wnt signaling cascade indirectly induces expression of *gsc* via a direct induction of *Xtwn* expression which, via its direct binding to the Wnt-responsive element (PE) within the *gsc* regulatory region, activates *gsc* transcription. That the former induction is direct is supported by a requirement for LEF1/TCF3-binding sites within the *Xtwn* promoter for its responsiveness to Wnt signals. Finally, when cortical rotation was inhibited, we found that endogenous *Xtwn* expression was extinguished from the marginal zone and confined to the vegetal pole, and that this vegetal expression was dependent on the same LEF1/TCF3-binding sites. These data, in light of the known localization and behavior of dorsal determinants, suggest that establishment of Spemann's organizer occurs as a consequence of direct inheritance of dorsal determinants by cells within the dorsal marginal zone.

MATERIALS AND METHODS

Embryo manipulations and RNA injections

Eggs were fertilized in vitro, dejellied and resultant embryos cultivated as described previously (Cho et al., 1991). Staging was according to Nieuwkoop and Faber (1967) and the extent of dorsoanterior development was determined according to the dorsoanterior index (DAI) of Kao and Elinson (1988). UV-hyperventralized embryos were obtained by irradiating vegetal poles of dejellied embryos 40 minutes after fertilization for 1 minute with a handheld UV source (UVP, model UVGL-25). Hyperdorsalized gastrulae were obtained by treating 32-cell-stage embryos in 120 mM LiCl for 40 minutes. Dorsoventral polarity was determined and indicated amounts of synthetic mRNAs were injected into two ventral blastomeres of 4- to 8-cell-stage embryo as previously described (Klein, 1987; Cho et al., 1991). For *Xtwn* and *Xsia* mRNA injection studies, plasmid templates were linearized with *Sal*I and *Kpn*I and transcribed with SP6 and T3 RNA polymerases, respectively.

Construction of a cDNA library and expression screening

LiCl-treated embryos were harvested at stage 10.25 equivalent.

RNA was purified using the acid guanidium-isothiocyanate method (Chomczynski and Sacchi, 1987). Oligo(dT)-primed cDNAs were generated using a cDNA synthesis kit (Amersham). cDNAs less than 500 bp were eliminated from the cDNA pool by size exclusion chromatography and directionally cloned into pBluescript KSII+ (Stratagene) digested with *Eco*RI and *Not*I. The average size of cDNAs from 30 random minipreparations of plasmid DNAs was approximately 1.3 kb. The pool of cDNAs were transformed into *E. coli* XL-1Blue and plated onto 50 150 mm LB-ampicillin plates. Each plate represented a fraction of the library containing approximately 2000 individual cDNAs (colonies). Bacterial colonies were scraped from the plates and grown in separate liquid cultures for large-scale plasmid isolation by standard alkaline lysis/CsCl gradient centrifugation (Sambrook et al., 1989). Pools of plasmid DNA templates were linearized with *Not*I and synthetic mRNA was generated using the T3 Megascript Kit (Ambion). mRNA synthesized from each cDNA pool was microinjected into UV-ventralized embryos and screened for axis-inducing activity. The DAI scores (Kao and Elinson, 1988) of injected embryos were obtained when control embryos reached tadpole stage 35. Sixteen fractions possessed significant axis-inducing activity (data not shown). To determine which fractions possess known axis-inducing molecules, slot-blot hybridization using probes for the genes *XWnt1, XWnt3A, XWnt5A, XWnt8, XWnt8b*, and *XWnt11*, the nodal-related genes *Xnr1, Xnr2* and *Xnr3*, *Xenopus activins A* and *B*, *β-catenin*, *goosecoid*, *chordin*, *siamois* and *noggin* was performed (Wolda and Moon, 1992; Cui et al., 1995; Ku and Melton, 1993; Smith et al., 1995; Lemaire et al., 1995; Jones et al., 1995; Blumberg et al., 1991; Sasai et al., 1994; Smith and Harland, 1992; Christian et al., 1992; Ueno et al., 1990; Thomsen et al., 1990; K. Inoue and K.W.-Y. Cho, unpublished). Nine of the sixteen fractions did not hybridize to these cDNA probes (data not shown) suggesting that these fractions contain as yet unidentified axis inducers. Four subsequent rounds of sib selection were performed to isolate a cDNA encoding the dorsalizing activity.

DNA sequence determination and analysis

The *Xtwn* cDNA was sequenced using a Sequenase 2.0 kit (US Biochemicals) according to the protocol provided by the manufacturer. Sequence analysis was performed using GCG sequence analysis software (Wisconsin Package Version 9.0, Genetics Computer Group, Madison, Wisc.). Sequence comparisons to all available known nucleic acids and proteins were performed using the National Center for Biotechnology Information's Basic Local Alignment Search Tools (BLASTX, BLASTN, tBLASTX and tBLASTN).

Preparation of RNA and northern blot hybridizations

To examine the temporal expression profile of *Xtwn*, total embryonic RNA was isolated from *Xenopus* embryos according to Chomczynski and Sacchi (1987). 20 μg of total RNA was loaded into each lane of a formaldehyde gel and ethidium bromide fluorescence confirmed that all lanes were loaded approximately equally (data not shown). To examine the spatial expression profile of *Xtwn*, embryos that exhibited a dorsal-to-ventral shift in animal pigmentation, and had their first cleavage plane bisect this pigmented area into two equal parts, were asymmetrically marked with Nile blue sulfate to indicate the direction of cortical rotation and hence the future dorsal-ventral axis (Klein, 1987; see also Cho et al., 1991). Dye-marked blastula-stage 8.5-9 embryos were dissected with a hairloop to collect ectoderm, endoderm, dorsal, lateral and ventral marginal zone fragments. 32 fragments per region were collected and total RNA was loaded into each lane of a formaldehyde agarose gel. RNA was transferred to nylon filters according to standard procedures (Sambrook et al., 1989). Filters were hybridized at 65°C according to Church and Gilbert (1984) with a random-primed 344 bp fragment consisting of nucleotides 74-418 of *Xtwn* which was generated by PCR amplification using *Xtwn*-specific primers described for RT-PCR analysis.

Construction of expression plasmids

A GST fusion containing the *Xtwn* homeodomain was constructed in pGEX-KG (Smith and Johnson, 1988) by PCR amplification of the *Xtwn* homeodomain using the following primers 5′-CATGCCATGGACAGGTGTAGGAGAAGA-3′ and 5′-GCGGAAGCTTGGCTCTGAGCAGATG-3′ containing *Nco*I and *Hin*dIII sites (underlined), respectively. This fragment was digested and subcloned between the *Nco*I and *Hin*dIII sites of pGEX-KG. pXβm-*Xtwn*, a plasmid containing the 5′ and 3′ UTRs of β-globin and the *Xtwn* coding region, was constructed by subcloning a 732 bp *Sma*I-*Rsa*I fragment of *Xtwn* into pSP64TBX (kindly provided by Naoto Ueno). A partial *Xlhbox7* cDNA (containing the homeodomain) was fused to *lacZ* in the pTRB vector as described in Oliver et al. (1988).

RT-PCR analysis

RT-PCR analysis was carried out as described previously (Blitz and Cho, 1995). An exception was made in Fig. 6 in which twenty cycles were used. The *Xtwn* primers used were 5′-TCCTGTGTTCTGCCCACCA-3′ and 5′-CTGTTGGGTGCCGATGGTA-3′. *Xsia* primers were 5′-ACCCCACCAGGATAAATCTG-3′ and 5′-GGTACTGGTGGCTGGAGAA-3′. The histone H4 primers were described in Blitz and Cho (1995).

Primer extension analysis

1 ng of *Xtwn* upstream primer (5′-CTTGTAGGGTCAGCGCTGT-3′) was annealed to 15 μg of blastula-stage embryonic total RNA. Extension was performed using 15 units of MMLV reverse transcriptase (Gibco, BRL), 0.3 mM each of dCTP, dGTP and dTTP, and 0.33 μM α-^{32}P-dATP at 37°C for 45 minutes. Samples were phenol/chloroform extracted and ethanol precipitated. Pellets were resuspended in 98% formamide loading dye and analyzed by autoradiography following electrophoresis on an 8% polyacrylamide gel.

Reporter gene constructs and luciferase assays

To clone the genomic region corresponding to sequence upstream of the *Xtwn* cDNA, a *Xenopus* genomic library (Leroy and De Robertis, 1992) was screened using the 344 bp PCR fragment, described above, corresponding to nucleotides 74-418 of the *Xtwn* cDNA. The –357*Xtwn*/Luc reporter was constructed by subcloning a PCR product containing sequences from –357 to +24 of the *Xtwn* gene (position –1 corresponds to the first nucleotide upstream of the transcriptional start site) between the *Bam*HI and *Hin*dIII sites of the promoter-less luciferase vector pOLuc (de Wet et al., 1987). The 'upstream' and 'downstream' primers used for PCR were 5′-GGGGGATCCTAACTGGTTTATAGTTGCATGTT-3′ and 5′-GGGAAGCTTGTGCTGCTAAGGGCAACTC-3′ and contain *Bam*HI and *Hin*dIII sites (underlined), respectively. The –357*Xtwn*(Δ3)/Luc was generated by a multistep PCR regimen as follows. The 'downstream' primer described above was used in a PCR reaction in conjunction with the primer 5′-GTCATGTaccgagagggtgAGAGACcgaccctgttggagagggtggCCCCAAATCATATTCTGGCC-3′ (containing nucleotides changes from the wild-type promoter in lower-case print) to produce a 3′ fragment containing mutations in two potential LEF1/TCF3 consensus binding sites. The 'upstream' primer described above was used in a separate PCR reaction in conjunction with the primer 5′-TGTCTCTacaaagagattgACATGACACCTGTACCCCTT-3′ to produce a 5′ fragment. Gel-purified 5′ and 3′ fragments were mixed together and used as template for a third PCR reaction using the 'upstream' and 'downstream' primers as outside primers to amplify the mutated –357(Δ3) fragment with terminal *Bam*HI and *Hin*dIII sites. The amplified fragment was subsequently subcloned between the *Bam*HI and *Hin*dIII sites of pOLuc and sequenced. Luciferase assays were performed as described previously (Watabe et al., 1995).

DNase I protection assays

To prepare a double-stranded DNA probe, –226 *gsc*/Luc was digested with *Hin*dIII, ^{32}P-end labeled using Klenow DNA polymerase

(Sambrook et al., 1989), and digested with *Bam*HI. A 241 bp asymmetrically end-labeled fragment containing the distal and proximal elements of the *gsc* promoter (Watabe et al., 1995) was released and gel purified. Crude fusion protein extracts were prepared as described by Hoey and Levine (1988) and Oliver et al. (1991) with minor modifications. Probe was incubated on ice with crude protein extracts (final concentration of 20 ng/μl) containing GST or β-galactosidase fusion proteins and poly(dIdC) (final concentration of 20 ng/μl) and DNase I footprinting was performed according to Osborne et al. (1987). The samples were extracted with phenol/chloroform, precipitated with ethanol and analyzed by autoradiography following denaturing polyacrylamide gel electrophoresis. Similarly, a 385 bp *Bam*HI-*Hin*dIII fragment of –357*Xtwn*/Luc was ^{32}P-end-labeled, incubated with purified human LEF1 protein (a generous gift of M. Waterman, University of California, Irvine) and subjected to analysis by DNAse I footprinting.

RESULTS

Isolation of a novel axial determinant, *Xenopus twin*

In order to identify novel genes important in dorsoventral axial patterning during early vertebrate embryogenesis, we adopted the elegant expression cloning strategy developed by Smith and Harland (1991, 1992). Here we describe the characterization of a novel cDNA that exhibits strong axis-inducing activity (see Materials and Methods). Injection of mRNA encoded by this cDNA in amounts as low as 2 pg was sufficient to induce partial secondary axes while 5 pg was sufficient to induce complete secondary axes, which included anterior head structures (data not shown). The potency of this molecule was further demonstrated in the UV rescue assay in which injection of 10 pg of mRNA was sufficient to rescue a normal body axis (avg. DAI of 4.6, *n*=18) from UV-hyperventralized embryos (avg. DAI 0.2, *n*=30), including the most anterior dorsal structures such as eyes and cement glands (data not shown).

DNA sequence analysis (Fig. 1A) revealed that this cDNA encodes a 234 amino acid homeodomain protein with closest similarity to *Xenopus siamois* (*Xsia*) (Lemaire et al., 1995). The overall amino acid sequence identity with *Xsia* is 50% with 88% identity between the two homeodomains (Fig. 1B). Similarity outside of the homeodomains is confined to three short blocks of sequence located in the

A

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1    GCAGCACAGAC ATG ACT TGT GAC TCT GAA CTT GAG CAA ATC ATC TAC ACA
                 M   T   C   D   S   E   L   E   Q   I   I   Y   T

51   GCG CTG ACC CTA CAA GAT GAC TAT CCT GTG TTC TGC CCA CCA CAG AGG
      A   L   T   L   Q   D   D   Y   P   V   F   C   P   P   Q   R

99   GAC CAA ACC AAA TCC TGC TCC AGC TCT TTT GGT ATG TTT CCT GAT TCT
      D   Q   T   K   S   C   S   S   S   F   G   M   F   P   D   S

147  TAT CCA GGG GTG GGA AAC CAG GGA ATC TTG CAG GAG ACT ATA AGA GAA
      Y   P   G   V   G   N   Q   G   I   L   Q   E   T   I   R   E

195  CTT TAT TCT GTC CTT GGG ATC CCA CAA GAT TCT CAT TTT AAC AGA AGC
      L   Y   S   V   L   G   I   P   Q   D   S   H   F   N   R   S

243  ATG AAG CAT CAT CTC CTA GAA CCC AAG AAG GCG ACA CTA TCT ACT GGG
      M   K   H   H   L   L   E   P   K   K   A   T   L   S   T   G

291  ATC TAC GCC AAA CCA ACC TGC AAT CAG ACA CCT AAA GCC TGT AAA CGG
      I   Y   A   K   P   T   C   N   Q   T   P   K   A   C   K   R

339  CCA TTT TGT GAA GAG GAG CAG AGG GAA GGT AAA AAG CCC AGA ATA GAG
      P   F   C   E   E   E   Q   R   E   G   K   K   P   R   I   E

387  ATG GAT CAT TTC CTA CCA TCG GCA CCC AAC AGG TGT AGG AGA AGA ACC
      M   D   H   F   L   P   S   A   P   N   R   C   R   R   R   T

435  ATT TAT TCA AAG GAG CAA ATC CTC TTC CTC CAG AAT CAA TTT GAT CTC
      I   Y   S   K   E   Q   I   L   F   L   Q   N   Q   F   D   L

483  AAT CCC TAT CCA GAC TTT GTG AAG AGA TGC CAC ATT GCA AAG ATA ACT
      N   P   Y   P   D   F   V   K   R   C   H   I   A   K   I   T

531  GGG ATC CCA GAG CCC AGG ATT CAG GTT TGG TTC CAG AAC AGA AGA GCC
      G   I   P   E   P   R   I   Q   V   W   F   Q   N   R   R   A

579  AGG CAT CTG CTC AGA GCC ATC AAT TCT CAG GTT CCC CAA GAA AAG AGA
      R   H   L   L   R   A   I   N   S   Q   V   P   Q   E   K   R

627  TCA GCA GCT GCA GAA GAA CCC AGA TGC TTT ACC TAC AGA GAA CCC CAG
      S   A   A   A   E   E   P   R   C   F   T   Y   R   E   P   Q

675  TAT CCC AGA CAT GTG GGG CTA AGC AAA ATA CTC CCT TAC ACG TAG
      Y   P   R   H   V   G   L   S   K   I   L   P   Y   T   *

720  CATGTACCCAAAGTCATGACTGAATCACTGCAGTGACATTTGTACCTTGAGACACCTATATGC
783  AAAGAACTTTCAAATTACTTTTTTTTTGGCTGAAATATTTGTCACTATGTACATAGAGAAATT
844  AACTACTACTTTAAATTTGACGTCCCTTAATGTATAACGTAAATATTTTAAATATTAGAATTA
906  AAGGTATGGGATAGGTTCATATACTCTATTATTACTCTTTATTTAAACAAGTTTCATTTACTG
970  AAAGTGTATCGCTTTAGTCCATAAAAAAACATGTTTATATGATATAAAGTCAGTATTTAAATT
1033 TATCAATAATTTATTCATTATTGTTCCCATGGGACCTTATATAAATATAATTGCTTTATCAGC
1096 ACTTCTCACTGTATTGTGTTATAAGGAGTACAGTTTAAGATACGCATGGCAACACACAGTACT
1159 TGATTTCAGAAGTCAATTTGTTTTATCTTAAGACAATAGTTGGCATTTAATGGTATAAATATA
1222 TGTATTTTAATTTTTGTGATATTGCTTGGTAATCAATAAAATTGTATCTTTTTTT
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B

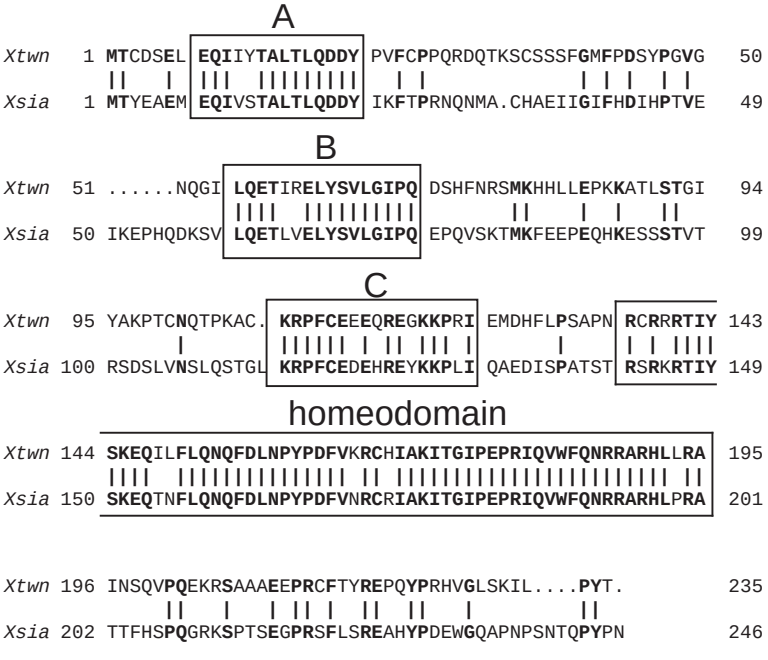


Fig. 1. *Xtwn* encodes a homeobox gene most closely related to *Xsia*. (A) Nucleotide sequence and deduced amino acid sequence of the *Xtwn* cDNA. The homeodomain is underlined. (B) Comparison of the amino acid sequences of *Xtwn* and *Xsia*. Vertical lines indicate amino acid identity shared between the two proteins. Boxes A, B, and C are regions of high identity between *Xtwn* and *Xsia* with 86%, 88%, and 76% identity, respectively. GenBank accession numbers for the *Xenopus Xtwn* cDNA and promoter sequences are AF020333 and AF020520, respectively.

amino terminal portion of the proteins (labelled A, B and C in Fig. 1B) which possess 86%, 88% and 76% amino acid identity, respectively. Since ectopic expression of this mRNA can induce twinned embryos possessing complete secondary axes, and the encoded protein is related most closely to *Xenopus siamois*, we have named this gene *Xenopus twin* (*Xtwn*). The similarity in homeodomain sequence between *Xtwn* and *Xsia* suggests the possibility that these proteins share similar DNA-binding specificity, but the functional significance of the short amino terminal blocks of sequence (which have no significant similarity to any other known sequence) is unknown. Due to the pseudotetraploidy of *Xenopus* (Graf and Kobel, 1991), an obvious question is whether the *Xtwn* cDNA represents a gene distinct from *Xsia* or whether *Xtwn* and *Xsia* are alternative copies of the same gene. The dissimilarities between *Xtwn* and *Xsia* suggest that *Xtwn* is a distinct gene. Therefore, we suggest that *Xtwn* and *Xsia* represent a novel subclass of homeobox genes that are important in the early dorsal patterning events in *Xenopus*.

Developmental expression profile of *Xtwn*

To determine the temporal expression profile of *Xtwn*, northern blot analysis was performed with total embryonic RNA isolated from a series of staged *Xenopus* embryos. A single transcript was detected that had a migration consistent in size with the 1.3-kb *Xtwn* cDNA isolated (data not shown). *Xtwn* is initially expressed at midblastula stage 8.5 (the onset of zygotic transcription) and is maximally expressed during late blastula stage 9.0. *Xtwn* expression decreases during early to midgastrula stages (stage 10-11) and, by northern analysis, *Xtwn* transcripts are no longer detected by mid- to late gastrula (stage 12; data not shown). Maternal *Xtwn* mRNA was also detected by RT-PCR analysis of total embryonic RNA prepared from pre-MBT-stage embryos (data not shown). RNA analysis of the expression pattern also demonstrates that, like the homeobox gene *Siamois*, the maximal expression of *Xtwn* precedes that of *gsc*, a marker for Spemann's organizer which is maximally expressed at early to midgastrula (stage 11; data not shown).

The spatial expression profile of *Xtwn* was analyzed by both whole-mount in situ hybridization of blastula- and gastrula-stage embryos and northern blot analysis of dissected tissue fragments. These results demonstrate that low levels of *Xtwn* expression are specifically detected in the dorsal marginal zone (data not shown). From these results, we conclude that the spatial expression pattern of *Xtwn* is very similar to that of *Xsia*, which has also been shown to be expressed within the dorsovegetal region of early gastrula embryos (Lemaire et al., 1995). RT-PCR analysis shows that *Xtwn* is maximally expressed in the dorsal marginal zone, but can also be detected in the vegetal pole fragment and very weakly in the animal cap (data not shown).

Induction of *Xtwn* by *XWnt8*

Since the expression of both *Xtwn* and *gsc* appears to over-lap within the dorsal marginal zone, we hypothesized that the regulation of *Xtwn* may be similar to that of *gsc*. As both Wnt- and activin/BVg1-like signals regulate *gsc* expression, we investigated the effects of *XWnt8* and activin on *Xtwn* expression. Synthetic mRNA encoding either *XWnt8* or activin was microinjected into the animal pole of 2-cell-stage embryos and *Xtwn* expression was analyzed by RT-PCR of RNA derived from blastula-stage (stage 9) animal cap explants. As shown in Fig. 2, *Xtwn* expression is strongly induced by as little as 5 pg of *XWnt8* mRNA (lane 5), whereas 50 pg of activin mRNA only very poorly induces *Xtwn* (lane 4 and data not shown). These data indicate that the mode of *Xtwn* regulation differs from that of *gsc*. *Xsia* is similarly strongly induced in response to *XWnt8*, but only poorly by activin (Carnac et al., 1996; Brannon and Kimelman, 1996; Fagotto et al., 1997, and our unpublished data). These findings also suggest that *Xtwn* and *Xsia* expression may be regulated in a similar manner by members of the Wnt superfamily and/or downstream components of the Wnt signalling cascade.

Regulation of *gsc* by *Xtwn* is mediated via the PE

Several lines of evidence suggest that *gsc* expression may be regulated by *Xtwn*: (1) *Xtwn* expression can be detected within the dorsal marginal region of the embryo, (2) ectopic expression of *Xtwn* leads to the formation of a secondary axis presumably through the formation of an ectopic organizer and (3) *Xtwn* expression precedes that of *gsc*. To explore this possibility in a more direct manner, we examined the effects of *Xtwn* and *Xsia* expression on various *gsc* reporter genes. These experiments were performed using the animal cap explant assay in which 4-cell-stage embryos were injected with *gsc* reporter gene constructs either alone, or coinjected with varying amounts of *Xtwn* or *Xsia* synthetic mRNA. Animal cap explants were isolated at blastula stages (stage 8.5-9), cultured for 2-3 hours and assayed for relative luciferase activity at early gastrula stage. Relative levels of induction (fold-induction) were obtained by comparing the luciferase activity of embryos injected with reporter gene constructs alone, or reporter gene constructs coinjected with *Xtwn* on *Xsia* mRNAs. As shown in Fig. 3B, *Xtwn* and *Xsia* each induced the expression of –226*gsc*/Luc (13-fold and 7.2-fold respectively), a construct containing both the distal element (DE) and the proximal element (PE) (Fig. 3A), which responds to activin/BVg1 and Wnt-like signals, respectively (Watabe et al., 1995). *Gsc*

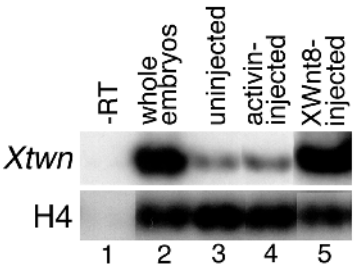


Fig. 2. *Xtwn* expression is induced in ectodermal (animal cap) explants in response to *XWnt8*. The blastomeres of 4-cell embryos were microinjected animally with varying amounts of either *XWnt8* or activin mRNA. RNA from animal cap explants was analyzed for *Xtwn* and histone H4 expression by RT-PCR. Animal cap explants from embryos injected with as little as *XWnt8* mRNA (5pg) express *Xtwn* (lane 5). In this experiment, injection of activin mRNA (50 pg) did not induce *Xtwn* (lane 4), however, in other experiments activin did induce *Xtwn* expression weakly (data not shown). All animal caps were isolated at stage 8.5-9 and RNA was harvested immediately for RT-PCR. Note that low levels of *Xtwn* mRNA are detected in uninjected and activin-injected animal caps (lanes 3 and 4) and may be due to maternal *Xtwn* expression (our unpublished data).

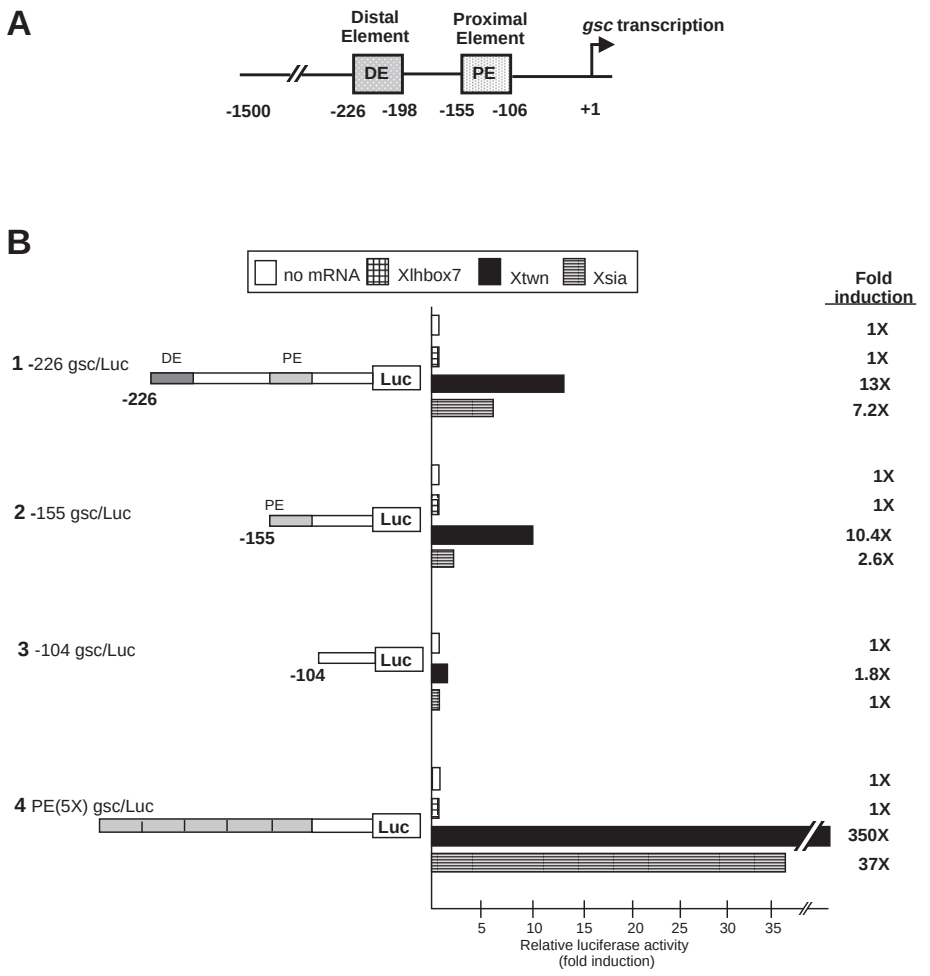


Fig. 3. *Xtwn* and *Xsia* activate *gsc* expression via the PE. (A) The *gsc* promoter contains two *cis*-acting elements, the distal element (DE) and the proximal element (PE) (Watabe et al., 1995). (B) Animal cap assays using various *gsc* promoter constructs driving luciferase. The animal poles of 4-cell-stage embryos were coinjected with reporter gene constructs and 240 pg of either *Xtwn* or *Xsia* mRNA. Animal cap explants were collected at blastula stages, cultured for three hours, and assayed for luciferase activity. Both –226*gsc*/Luc (line 1) and –155*gsc*/Luc (line 2) respond specifically to *Xtwn* (black) and *Xsia* (striped), but not *Xlhbox7* (checkered). Deleting sequences between –155 and –104 blocked the ability of the gsc promoter to respond to *Xtwn* and *Xsia* in animal caps (compare lines 2 and 3). A multimer construct containing five copies of the PE (PE(5×)*gsc*/Luc, line 4) was highly induced in response to *Xtwn* and *Xsia* (350-fold and 37-fold, respectively) but not *Xlhbox7*. Fold induction of reporter genes was calculated as the ratio between *Xtwn*- or *Xsia*-injected and uninjected animal caps. Experiments were repeated several times and one representative experiment is shown. The levels of induction seen by *Xsia* on –155*gsc*/Luc in this particular experiment were unusually low since several repetitions of this experiment indicate that both *Xtwn* and *Xsia* induce this construct within a range of 8- to 15-fold. Note that while the results of injection of 240 pg of these mRNAs of are shown here, similar results were obtained at 5 pg of *Xtwn* RNA.

induction was specific for *Xtwn* and *Xsia* since injection of mRNA encoding the homeobox genes *Xlhbox7* (Bittner et al., 1993; Fig. 3B), *Xlhbox2* (Wright et al., 1987) or *Xlhbox6* (Sharpe et al., 1987) did not significantly induce luciferase activity (data not shown).

Since *Xtwn* and *Xsia* activate a *gsc* promoter containing both the DE and the PE, we wished to determine the region of the *gsc*/Luc construct that was responsive to activation by these genes. When –155*gsc*/Luc, a construct lacking the DE but containing the PE was tested, both *Xtwn* and *Xsia* activated this construct 10.4-fold and 2.6-fold, respectively (Fig. 3B, line 2; Fan and Sokol, 1997). A construct lacking both the DE and the PE (–104*gsc*/Luc) was non-responsive (or minimally responsive) to *Xtwn* and *Xsia* (Fig. 3B, line 3). These results suggest that *gsc* activation by *Xtwn* or *Xsia* is mediated predominantly via the PE since activation levels between constructs containing or lacking the DE do not vary significantly (Fig. 3B, line 1 versus line 2). To further demonstrate that the PE is sufficient for *Xtwn* induction of *gsc*, we constructed a luciferase reporter gene PE(5×)*gsc*/Luc containing five copies of the PE. While both *Xtwn* and *Xsia* are able to activate this reporter gene (350-fold and 37-fold respectively; Fig. 3B, line 4), *Xtwn* mRNA consistently appears to be a more efficient activator of this construct. However, the biological relevance of this difference in activity is unclear. While comparison of the activation potential between *Xtwn* and *Xsia* on the PE(5×)*gsc*/Luc reporter reveals an approximately 9.5-fold higher activity (350-fold/37-fold) for *Xtwn*, this difference is only approximately 2-fold when using the –226*gsc*/Luc reporter. Therefore, the 9.5-fold difference seen using the PE(5×)*gsc*/Luc may be due to an amplification of a much smaller difference in potency between these mRNAs. From these results, we conclude that *gsc* activation by *Xtwn* and *Xsia* is mediated through the PE.

Since *gsc* expression is regulated by *Xtwn*, we wished to determine whether this regulation was direct or indirect. A 241 bp fragment of the *gsc* promoter (containing both the DE and the PE) was asymmetrically ^{32}P-end-labelled and subjected to DNase I protection assays using crude extracts of the *Xtwn* homeodomain fused to the glutathione-S-transferase (GST) protein. These results revealed that a portion of the PE (from –114 to –127) was protected from DNase I digestion after incubation with crude GST-*Xtwn* fusion protein extracts (Fig. 4, lanes 2-4). This binding was specific for the *Xtwn* homeodomain since incubation with either crude GST protein extracts or β-gal-*Xlhbox7* fusion protein did not protect the *gsc* promoter from DNase I digestion (Fig. 4, lanes 6-11). The sequence of the PE protected by *Xtwn* is 5′-ATTACATTAAATCT-3′ and contains two 5′-ATTA-3′ consensus binding sites (underlined) for homeodomain proteins (reviewed by Gehring et al., 1994). Based on these results and the 88%

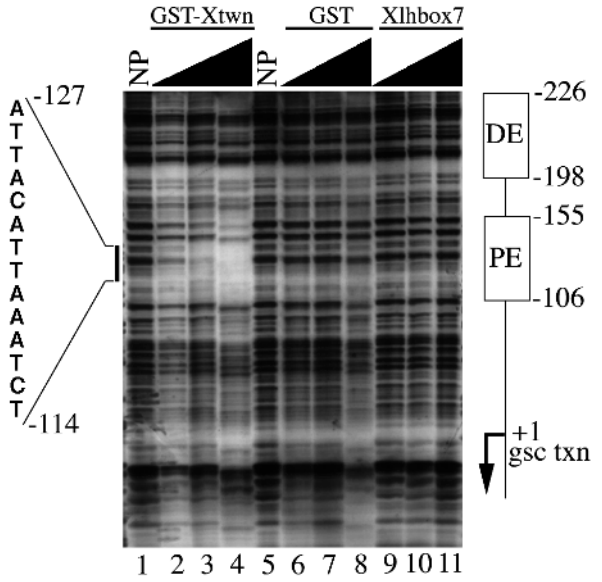


Fig. 4. The *Xtwn* homeodomain binds directly to the Wnt-responsive element (the PE) of the *gsc* promoter. A *Bam*HI-*Hin*d II fragment of −226*gsc*/Luc was ^{32}P end-labelled and incubated with increasing amounts of crude extracts expressing GST-*Xtwn* homeodomain fusion protein (lanes 2-4), GST protein (lanes 6-8), and β-gal-*Xlhbox7* fusion protein (lanes 9-11). NP (lanes 1 and 5) indicates control DNAse I digestion in the absence of protein extract. A footprint is evident in lanes 3 and 4 and this protected region corresponds to −114 to −127 of the *gsc* PE.

identity between the *Xtwn* and *Xsia* homeodomains, we suggest that the homeodomains of both *Xtwn* and *Xsia* can bind directly to the PE to activate *gsc* transcription. Consistent with this notion, we find that mutation of the *Xtwn*-binding site (5′-ATTACATTAAATCT-3′ was changed to 5′-CGGCA-GATCTTGAA-3′) in the context of the PE (−155*gsc*/Luc; Watabe et al., 1995) completely abolishes Wnt-responsiveness of the *gsc* reporter gene (data not shown).

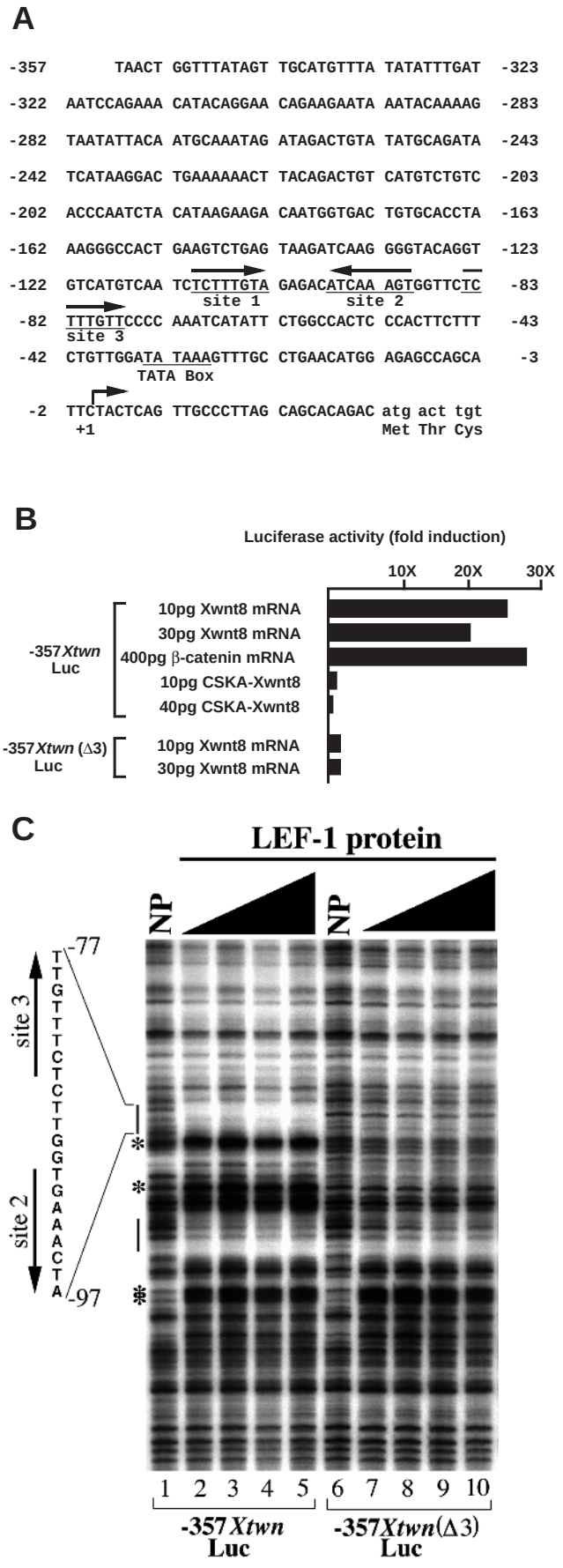
Induction of *Xtwn* expression by *XWnt8* requires LEF1/TCF3-binding sites in the *Xtwn* promoter

Induction of *Xtwn* by Wnt suggests the possibility that *Xtwn* is a direct target of a Wnt signal transduction cascade. Therefore, we sought to provide support for this hypothesis by examining the structure and function of the *Xtwn* promoter. The nucleotide sequence analysis of 357 bp upstream of the *Xtwn* transcription initiation site reveals three sequence motifs (Fig. 5A) conforming to the consensus LEF1/TCF3-binding site (Love et al., 1995; M. Waterman, personal communication). As members of the LEF1/TCF3 family of HMG box transcription factors have been demonstrated to mediate Wnt signaling by direct interaction with β-catenin (Molenaar et al., 1996; Huber et al., 1996; Behrens et al., 1996; Brunner et al., 1997; van de Wetering et al., 1997) and LEF1/TCF3-binding sites have been implicated in mediating Wnt (*wingless*)-induction of *Drosophila Ultrabithorax* (Riese et al., 1997), we examined whether these LEF1/TCF3 sites were important for Wnt induction of the *Xtwn* promoter in the context of a *Xtwn*/Luc reporter gene. The luciferase reporter gene construct −357*Xtwn*/Luc, containing 357 bp upstream of the *Xtwn* transcriptional start site, was microinjected into the animal hemisphere of 4-cell-stage embryos together with varying amounts of *XWnt8* mRNA. Animal caps were dissected at the blastula stage and subjected to luciferase assays. As shown in Fig. 5B, injected *XWnt8* mRNA induces the −357*Xtwn*/Luc reporter gene approximately 20-fold suggesting that maternal Wnt signaling (Christian and Moon, 1993) activates *Xtwn* expression. However, cytoskeletal actin promoter-driven expression of *XWnt8* after midblastula transition (MBT) does not activate the −357*Xtwn*/Luc reporter, consistent with the ventralizing effects of post-MBT expression of *XWnt8* in *Xenopus* (Christian and Moon, 1993). The induction of the reporter gene by *XWnt8* is LEF1/TCF3-dependent since mutation of all three LEF1/TCF3-binding sites (−357*Xtwn*(Δ3)/Luc) abrogates Wnt responsiveness (Fig. 5B). Furthermore, overexpression of β-catenin activated −357*Xtwn*/Luc at levels similar to those of *XWnt8* (Fig. 5B) whereas human LEF1 alone (400 pg; Waterman et al., 1991) weakly activated this promoter (3- to 4-fold).

To verify that the consensus LEF1/TCF3-binding sites in the *Xtwn* promoter indeed interact with proteins of the LEF1/TCF3 transcription factor family, and to examine whether the transversions introduced into the mutant −357*Xtwn*(Δ3)/Luc reporter gene do indeed abrogate protein binding, we performed DNAse I footprinting experiments using purified full-length LEF1 protein. LEF1 produces a strong footprint covering two (sites 2 and 3) of the three consensus LEF1/TCF3-binding sites, along with a strong hypersensitive site between binding sites 1 and 2 (asterisk) (Fig. 5C, see lanes 1-5). We also find another weakly footprinted region and several other hypersensitive sites (asterisks) distal to binding site 1; however, the functional significance of these regions is unclear. Mutation of all three LEF1/TCF3-binding sites abolishes both LEF1 protection of binding sites 2 and 3 and the DNAse I hypersensitive sites between binding sites 1 and 2 (Fig. 5C, lanes 6-10). Taken together with the functional data showing that mutation of the LEF1/TCF3 consensus binding sites abrogates Wnt responsiveness of the *Xtwn* promoter, the footprint data suggests that at least sites 2 and 3 mediate Wnt inducibility of the *Xtwn* promoter.

Inhibition of cortical rotation alters the localization of *Xtwn* expression

Establishment of the *Xenopus* dorsoventral axis requires cortical rotation, which transports dorsal determinants to the prospective dorsal side of the embryo (reviewed in Gerhart et al., 1989). These determinants display characteristics similar to members of the dorsalizing Wnt-signalling pathway (Holowacz and Elinson, 1993) and recent evidence suggests that β-catenin protein, a downstream component of the Wnt-signaling cascade, is dorsally enriched (Schneider et al., 1996; Larabell et al., 1997; Rowning et al., 1997). Blocking of cortical rotation by vegetal UV irradiation of 1-cell-stage embryos results in trapping of the dorsal determinants (and β-catenin protein) in the vegetal pole (Fujisue et al., 1993; Sakai, 1996; Schneider et al., 1996). We sought to examine the behavior of both the endogenous *Xtwn* gene and the reporter gene to determine whether *Xtwn* expression is directed by the dorsal determinants. First, explants of animal cap, marginal zone and vegetal pole tissue were collected from blastula-stage embryos (st. 8.5-9; when *Xtwn* is maximally expressed) and RNA was analyzed by



RT-PCR for *Xtwn* expression (Fig. 6A). *Xtwn* expression was detected within the marginal zone and the vegetal pole explants (Fig. 6A, lanes 7 and 8). To examine the behavior of the wild-type *Xtwn* reporter gene, single blastomeres of 128-cell-stage embryos were injected with –357*Xtwn*/Luc and luciferase activity was measured at early gastrula stages. We found that this reporter gene was strongly active in both dorsal marginal and vegetal pole regions (Fig. 6B, right).

We then examined the effects of blocking cortical rotation (by UV treatment) on the expression of both the endogenous *Xtwn* gene and the luciferase reporter. RT-PCR analysis of explants derived from UV-irradiated embryos (average DAI of 0.03, *n*=150) reveals that *Xtwn* and *Xsia* expression is confined to the vegetal pole cells, and extinguished in the marginal zone (Fig. 6A, lanes 4 and 5; see also Brannon and Kimelman, 1996). Likewise, injection of the wild-type –357*Xtwn*/Luc reporter gene into the vegetal pole of UV-treated 128-cell-stage embryos corroborates that *Xtwn* is hyperinduced in vegetal pole cells (Fig. 6B, left). The activity of the wild-type reporter gene in both marginal and vegetal regions, and in irradiated and unirradiated embryos, was dependent on the LEF1/TCF3-binding sites in the *Xtwn* promoter as –357*Xtwn*(Δ3)/Luc was not activated above the level of the promoter-less luciferase vector (data not shown). These results suggest that movement of dorsal determinants during cortical rotation results in activation of a Wnt-like signaling cascade, or a Wnt signaling cascade component, on the dorsal side, which activates *Xtwn* expression through LEF1/TCF3-binding sites.

DISCUSSION

In an ongoing search for molecules involved in axial patterning of the early *Xenopus* embryo, we have identified the homeobox gene *twin* (*Xtwn*). This gene is a potent inducer of complete dorsal axes in both UV rescue and secondary axis induction assays. *Xtwn* is expressed during late blastula stages predominantly in the dorsal marginal zone (with apparent lower level expression in the vegetal pole) prior to the

Fig. 5. *XWnt8* induction of *Xtwn* expression requires LEF1/TCF3-binding sites in *Xtwn* 5′ regulatory region. (A) Sequence of the *Xenopus twin* promoter. The nucleotide sequence of a 385 bp fragment of the *Xtwn* gene is shown. The transcription initiation site (+1) and a TATA box 35 bp upstream are indicated. Three consensus LEF1/TCF3-binding site sequences are underlined. Mutation of LEF1/TCF3-binding sites within the *Xtwn*/Luc reporter gene (–385(Δ3)*Xtwn*/Luc) are described in the Materials and Methods. (B) Schematic representation of the reporter gene assay in animal caps. Embryos are injected into the animal pole of all four blastomeres at the 4-cell stage with reporter gene constructs alone or with the indicated amounts of *XWnt8* or β-catenin mRNA or DNA. At the blastula stage, animal caps are dissected manually, cultured for 2 hours, and subjected to luciferase assays. The level of *Xtwn* induction (fold induction) is obtained by calculating the ratio of luciferase activity between reporter gene-injected caps with or without coinjected mRNA or DNA. Coinjection of the wild-type *Xtwn* reporter gene with *XWnt8* mRNA causes approximately 20-fold induction in animal caps, whereas mutation of the consensus LEF1/TCF3-binding sites (–357(Δ3)*Xtwn*/Luc) abolishes the ability of the *Xtwn* promoter to respond to *XWnt8* in animal cap assays. (C) Identification of LEF1-binding sites. DNAse I protection analysis of a 5′ end-labeled 357 bp fragment of the *Xtwn* promoter reveals a footprint covering sites 2 and 3.

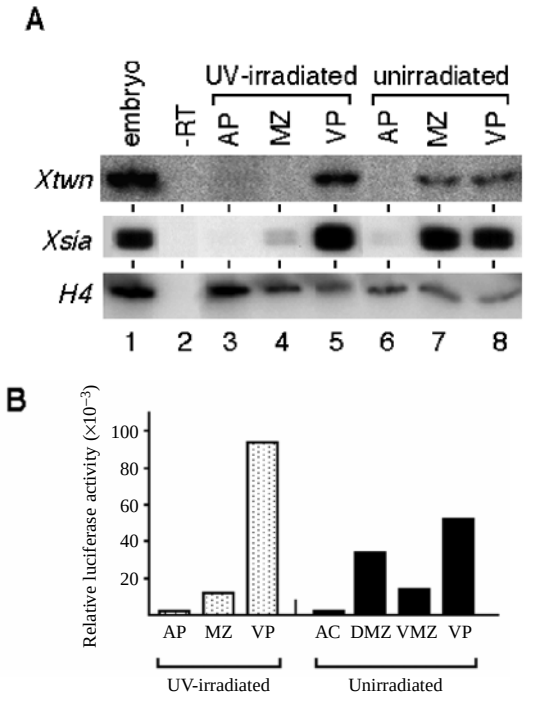


Fig. 6. Inhibition of cortical rotation abolishes *Xtwn* expression in the marginal zone. (A) RT-PCR analysis of *Xtwn*, *Xsia* and histone H4 expression. RT-PCR was performed on tissue fragments (AP, animal pole; MZ, marginal zone; and VP, vegetal pole) isolated at the blastula stage from UV-irradiated (lanes 3-5) and unirradiated embryos (lanes 6-8). *Xtwn* and *Xsia* expression is detected within the marginal zone and vegetal pole fragments from unirradiated embryos (lanes 7 and 8). However, when cortical rotation is blocked by UV-irradiation, *Xtwn* and *Xsia* expression is hyperinduced in the vegetal pole fragments (lane 5). Histone H4 is used as a loading control in the lower panel. Similar results were obtained in each of three independent experiments. (B) Unirradiated and UV-irradiated embryos were injected in a single blastomere at the 128-cell stage with −357*Xtwn*/Luc, and the luciferase activity was measured at early gastrula stage. Injection of the wild-type −357*Xtwn*/Luc reporter gene into embryos also confirms that expression of *Xsia* and *Xtwn* shift from the dorsal marginal zone (DMZ) to the vegetal pole (VP) when cortical rotation is blocked by UV irradiation during the first cell cycle.

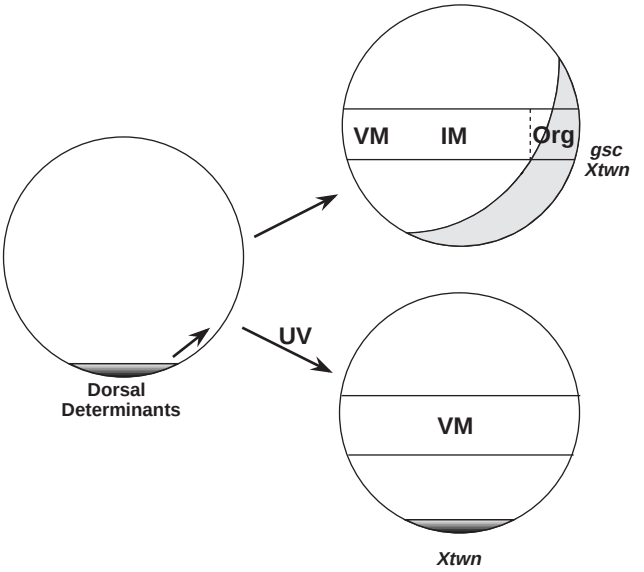


Fig. 7. A dorsal determinant model for establishment of Spemann's organizer in the dorsal marginal zone. Dorsal determinants, perhaps components of the *Wnt* signaling pathway, are localized in the vegetal hemisphere of the unfertilized egg (left). During normal development, fertilization triggers the displacement of these determinants toward the future dorsal side (top right). We propose that cells comprising the prospective dorsal mesoendoderm directly inherit these determinants which activate a *Wnt*-signaling cascade to establish Spemann's organizer. Activation of a *Wnt*-signaling cascade leads to establishment of *Xtwn* expression in the dorsal marginal zone. *Xtwn* directly binds to the *Wnt*-responsive element of the gsc promoter (the PE), and in collaboration with *activin/BVg1*-like signals, activates expression of *gsc.* Treatments that inhibit cortical rotation (e.g. UV irradiation) result in 'trapping' of the dorsal determinants (lower right) in the extreme vegetal endoderm. As a consequence of the lack of distribution of dorsal determinants to the dorsal side, the entire marginal zone becomes specified as ventral mesoderm, no organizer is formed and dorsal-specific marker genes are not expressed 'dorsally'. *Xtwn* expression is instead found in the vegetal pole region. Abbr: VM, ventral mesoderm; IM, intermediate mesoderm; Org, organizer.

expression of *gsc*. Expression of *Xtwn* is strongly inducible by *XWnt8*, but poorly by activin, and can in turn mediate induction of *gsc* gene transcription through its direct interaction via a Wnt-responsive element (the PE) within the *gsc* regulatory region. Wnt induction of *Xtwn* appears to be a direct response to the Wnt signaling cascade since LEF1/TCF3-binding sites within the *Xtwn* promoter are required for *Xtwn* induction by Wnt. Finally, inhibition of cortical rotation, which has previously been demonstrated to result in improper localization of Wnt-like dorsal determinants (Fujisue et al., 1993; Holowacz and Elinson, 1993, 1995; Sakai, 1996; Kageura, 1997), results in loss of *Xtwn* expression from the dorsal marginal zone with concomitant relocalization of expression in the vegetal pole. These latter results lend support for a direct role of dorsal determinants in specification of Spemann's organizer (discussed in more detail below).

Comparison of *Xenopus twin* and *siamois*

Expression of *Xtwn* mRNA is primarily localized to the dorsal marginal region of blastula-stage embryos and begins at mid-blastula transition (stage 8.5) preceding expression of the organizer-specific homeobox gene *gsc* (data not shown; Blumberg et al., 1991; Cho et al., 1991). The amino acid sequence of the homeodomain of *Xtwn* is 88% identical (53/60 amino acids) to that of *Xenopus siamois* (*Xsia*), though *Xtwn* and *Xsia* are more divergent outside the homeodomain, suggesting that these proteins likely share DNA-binding specificity. Both *Xtwn* and *Xsia* have spatially similar expression profiles (this study and Lemaire et al., 1995) although the relative positions of *Xtwn* and *Xsia* expression domains have not yet been determined with precision (by double in situ hybridization, for example). The potency of *Xtwn* also compares favorably with *Xsia* (Lemaire et al., 1995) since as little as 5 pg of *Xtwn* RNA is sufficient to induce complete dorsal axes in UV rescue and secondary axis induction assays. Expression of both genes is strongly induced in isolated animal

caps by *XWnt8*, but not by activin, and both *Xtwn* and *Xsia* can induce expression of *gsc* when ectopically expressed (this study, Carnac et al., 1996). These data taken together suggest that *Xtwn* and *Xsia* may have redundant functions during *Xenopus* embryogenesis or act synergistically, perhaps as heterodimers (Mead et al., 1996), to regulate gene transcription.

Xtwn may mediate Wnt induction of *goosecoid* transcription

Establishment of Spemann's organizer has been suggested to require synergistic inputs from both the TGFβ- and Wnt-superfamily signaling cascades (Kimelman et al., 1992; Christian et al., 1992; Sokol and Melton, 1992; Watabe et al., 1995; Cui et al., 1995). In the case of the *goosecoid* promoter, two *cis*-acting DNA sequence elements, the distal and proximal elements (DE and PE), synergistically mediate induction by activin/BVg1 and *XWnt8*, respectively (Watabe et al., 1995). Activin induction, mediated through the DE, occurs in the absence of protein synthesis demonstrating that the DE is a direct target of an activin/BVg1-like signaling cascade (Cho et al., 1991; Watabe et al., 1995). However, a protein synthesis requirement for Wnt-induced transcriptional activation through the PE has not been assessed. Furthermore, no consensus LEF1/TCF3-binding sites exist within the PE suggesting that the *gsc* Wnt-response element does not respond directly to a Wnt signal through LEF1/TCF3 protein family members. Thus, the possibility remains that Wnt induction of *gsc* transcription through the PE may occur indirectly via the Wnt-induced expression of an intermediary transcription factor. In this study, we provide evidence suggesting (1) *Xtwn* expression is induced by *XWnt8* (Fig. 2), (2) this induction requires LEF1/TCF3-binding sites located in the *Xtwn* promoter (Fig. 5), and (3) *Xtwn* protein is capable of specifically interacting with sequences within the Wnt responsive element (the PE) of the *gsc* promoter (Fig. 4). These results suggest that Wnt induction of *gsc* may require the prior Wnt-induced synthesis of the *Xtwn* protein, which in turn mediates the Wnt inductive effects on the *gsc* promoter.

The expression of *Xtwn* in the dorsal marginal zone may be directly conferred by dorsal cytoplasmic determinants

The finding that *Xtwn* mRNA is expressed in the vegetal pole of blastulae of UV-treated embryos is unexpected. It is widely believed that Spemann's organizer is established in the dorsal marginal zone by inductive influences emanating from a distinct signaling center, the Nieuwkoop center (NKC) (Nieuwkoop, 1973; Gerhart et al., 1989; Gimlich and Gerhart, 1984). Fertilization triggers the movement of dorsal cytoplasmic determinants from the vegetal pole to the dorsal vegetal region and cells that inherit these determinants presumably become the NKC (Gerhart et al., 1989). UV treatment during the first cell cycle blocks subcortical cytoplasmic rotation and, according to this view, UV-treated embryos fail to establish a NKC dorsovegetally (Gerhart et al., 1989). Without a NKC, embryos would fail to induce expression of dorsal-specific genes like *gsc* and *Xtwn*, and therefore would fail to establish a functional Spemann's organizer in the dorsal marginal zone. Expression of *Xtwn* and *Xsia* in the vegetal pole of UV-treated embryos suggests that this view requires modification (this study and Brannon and Kimelman, 1996).

At least two alternative models can explain the localization of *Xtwn* in the vegetal pole of UV-treated embryos. According to one view, an extension of the model described above, UV treatment results in the 'trapping' of dorsal cytoplasmic determinants in the vegetal pole (Fujisue et al., 1993; Sakai, 1996) and, consequently, an ectopic NKC may be established in this extreme vegetal location. If *Xtwn* expression marks the NKC (as has been suggested for *Xsia*; Lemaire et al., 1995), establishment of an ectopic NKC in the extreme vegetal pole would therefore be expected to result in ectopic expression of *Xtwn* in this region. However, it is unclear whether *Xsia* does indeed mark the NKC. Fate mapping experiments (Bauer et al., 1994; Vodicka and Gerhart, 1995) demonstrate that the region corresponding to the expression domain of *Xsia* is derived from the B1 and C1 blastomeres of the 32-cell-stage embryo and not from progeny of the D1 blastomere, which are believed to have NKC signaling activity (Nieuwkoop, 1973; Gerhart et al., 1989; Gimlich and Gerhart, 1984). Evidence that *Xsia* may be involved in NKC signaling is suggested by *Xsia* mRNA coinjection experiments with β-gal mRNA into the ventrovegetal region (Lemaire et al., 1995). β-gal staining was observed in the endoderm of the induced secondary axes suggesting that *Xsia*-expressing endoderm can act as a NKC to produce an NKC-like signaling activity which 'dorsalizes' the adjacent marginal zone producing organizer mesoderm. When β-gal mRNA was expressed in the marginal zone (the normal site of *Xtwn* and *Xsia* expression) following coinjection of *Xtwn* or *Xsia* mRNAs into C4 blastomeres (ventral marginal zone), strong staining was seen in axial mesoderm (our unpublished data), suggesting that ventral marginal zone cells expressing *Xtwn* or *Xsia* can be converted to dorsal mesoderm. Interestingly, we find that *Xtwn* and *Xsia* are more potent inducers of complete secondary axes when their RNAs are injected into the ventral marginal zone (C4 blastomere) than when injected into the ventral vegetal region (D4 blastomere) (our unpublished data). While the relative positions of the domains of expression of *Xtwn* and *Xsia* remain to be precisely determined, present data on the localization of NKC activity (Nieuwkoop, 1973; Gerhart et al., 1989; Gimlich and Gerhart, 1984) suggests that the expression of *Xtwn* and *Xsia* may not mark the NKC.

Alternatively, we propose that during normal development, Spemann's organizer is established when prospective mesoendoderm directly inherits the dorsal determinants, without a requirement for reception of NKC signals (see Fig. 7). Therefore, in UV-hyperventralized embryos, trapping of the dorsal determinants in the extreme vegetal endoderm directs *Xtwn* expression in the vegetal pole but this vegetal tissue is unable to function as an ectopic Spemann's organizer. Several lines of evidence support this model. First, dorsal determinants have been shown to be localized in the extreme vegetal pole of the fertilized egg and move to the dorsal marginal zone midway through the first cell cycle (Holowacz and Elinson, 1995; Fujisue et al., 1993; Sakai, 1996; Yuge et al., 1990; Kikkawa et al., 1997). The movement of these determinants is blocked by UV treatment and results in localization of the dorsal determinants vegetally (Fujisue et al., 1993; Sakai, 1996). Second, the B1 and C1 blastomeres of the 32-cell-stage embryo, which are fated to become the prospective organizer region (Bauer et al., 1994; Vodicka and Gerhart, 1995), when transplanted into host embryos, can organize secondary axes (Kageura, 1990; Gimlich and Gerhart, 1984; Gimlich, 1986; Takasaki and Konishi, 1989; Gallagher et al., 1991; Pierce and Brothers, 1992). These data indicate that the B1 and C1 blas-

tomeres of the 32-cell-stage embryo already autonomously possess the necessary 'information' required to pattern the dorsal axis. Although this information may not be expressed until later stages of development, additional studies by Kageura and Yamana (1986) suggest that this activity may have been inherited by the dorsal animal blastomeres as early as the 8-cell stage. Furthermore, progeny of the C1 blastomere express the organizer-specific homeobox gene *gsc* in a cell autonomous fashion arguing that progeny of the C1 blastomere do not require continuous input from NKC factors for *gsc* expression (Lemaire and Gurdon, 1994). Finally, axis-inducing activity is not simply localized to the C1 and D1 blastomeres or their progeny, but is more widely spread over the dorsal side of the embryo (Hainski and Moody, 1992; Holowacz and Elinson, 1993; Kageura and Yamana, 1986; Kageura, 1990; Gimlich and Gerhart, 1984; Gimlich, 1986; Takasaki and Konishi, 1989; Gallagher et al., 1991; Pierce and Brothers, 1992; Kikkawa et al., 1997, Kageura, 1997). These observations support the model that Spemann's organizer is established directly by dorsal cytoplasmic determinants. The distinction between 'Nieuwkoop center signals' and the activities of the widely spread dorsal determinants remains a critical unresolved issue toward understanding the mechanisms underlying dorsoventral axis specification.

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