The β-catenin/VegT-regulated early zygotic gene *Xnr5* is a direct target of SOX3 regulation

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Summary

In *Xenopus laevis*, β-catenin-mediated dorsal axis formation can be suppressed by overexpression of the HMG-box transcription factor XSOX3. Mutational analysis indicates that this effect is due not to the binding of XSOX3 to β-catenin nor to its competition with β-catenin-regulated TCF-type transcription factors for specific DNA binding sites, but rather to SOX3 binding to sites within the promoter of the early VegT- and β-catenin-regulated dorsal-mesoderm-inducing gene *Xnr5*. Although B1-type SOX proteins, such as XSOX3, are commonly thought to act as transcriptional activators, XSOX3 acts as a transcriptional repressor of *Xnr5* in both the intact embryo and animal caps injected with *VegT* RNA. Expression of a chimeric polypeptide composed of XSOX3 and a VP16 transcriptional activation domain or morpholino-induced decrease in endogenous XSOX3 polypeptide levels lead to an increase in *Xnr5* expression, as does injection of an anti-XSOX3 antibody that inhibits XSOX3 DNA binding. These observations indicate that maternal XSOX3 acts in a novel manner to restrict *Xnr5* expression to the vegetal hemisphere.

Supplemental data available online

Key words: *Xenopus*, SOX3, Nodal-related protein, *Xnr5*, β-Catenin, VegT

Introduction

The *Xenopus* egg has a distinct animal-vegetal asymmetry characterized at the molecular level by the distribution of maternal mRNAs and proteins (Chan and Etkin, 2001; St Amand and Klymkowsky, 2001; Pandur et al., 2002). The radial symmetry of the egg is broken by sperm entry. Fertilization induces microtubule-dependent cortical rotation. Subsequent cytoplasmic rearrangements lead to the asymmetric stabilization of β-catenin, which generates a second symmetry axis, known as the dorsal-ventral or organizer-contraorganizer axis (Kumano and Smith, 2002). The asymmetry in cytoplasmic β-catenin, estimated at ~1.5 times between pro-dorsal and pro-ventral sides of the blastula stage embryo (R. Moon, personal communication) leads to changes in gene expression through interactions with maternally supplied LEF/TCF-type transcription factors. Three of four members of the vertebrate LEF/TCF family are supplied maternally in *Xenopus*, *XTCF1* (Roel et al., 2003), *XTCF3* (Molenaar et al., 1996) and *XTCF4* (Houston et al., 2002). A number of experiments suggest that XTCF3 acts as a repressor of target genes. The asymmetry in β-catenin activity acts to derepress dorsalizing genes (Houston et al., 2002; Klymkowsky, 1997).

How the rotation-induced asymmetry in β-catenin and other cytoplasmic components interacts with the pre-existing animal-vegetal asymmetries generated during oogenesis and meiotic maturation is the subject of intense study. The earliest zygotic regulatory landmark identified to date is the expression of the transforming growth factor β (TGFβ) family, Nodal-related *Xnr5* and *Xnr6* genes. Both are first detected at the 256-cell stage, well before the beginning of 'general' transcription at the mid-blastula transition (MBT), which occurs at stage 8.5 (Yang et al., 2002). Takahashi et al. (Takahashi et al., 2000) reported that *Xnr5* and *Xnr6* RNAs are present throughout the vegetal hemisphere in a shallow dorsal-vegetal gradient, although they did not see expression of either gene before the MBT. The activation of *Xnr5* and *Xnr6* expression in animal caps (Rex et al., 2002) and whole embryos (Yang et al., 2002) is dependent upon the activation of β-catenin and the vegetally localized maternal T-box transcription factor VegT (Zhang et al., 1998; Zhang and King, 1996), also known as Xombi (Lustig et al., 1996), Antipodean (Stennard et al., 1996) and Brat (Horb and Thomsen, 1997).

Studies of *Xnr5* and *Xnr6* expression (Yang et al., 2002) and the isolation of a minimal *Xnr5* promoter (Hilton et al., 2003) suggest that *Xnr5* is directly regulated by β-catenin and maternal TCFs. As such, it joins *Siamois* (Brannon et al., 1997) and *Twin* (Laurent et al., 1997), which encode homeobox-containing proteins expressed in the dorsal endoderm or Nieuwkoop center (Carnac et al., 1996; Lemaire et al., 1995), *Xbra* (Vonica and Gumbiner, 2002), which encodes a T-box containing protein expressed in mesoderm, and *Xnr3* (McKendry et al., 1997), which encodes a Nodal-related protein expressed within the Spemann organizer, as targets of β-catenin/TCF regulation in the early *Xenopus* embryo.

Another family of maternal and early zygotic factors that might influence β-catenin-regulated genes are the SOX proteins. SOXs and LEF/TCF proteins are part of a larger family of

sequence specific DNA binding proteins that contain a single high mobility group (HMG) box DNA binding motif (Bowles et al., 2000) (Klymkowsky, 2004). The HMG boxes of SOX proteins share at least 50% identity with the HMG box of the mammalian male sex determining polypeptide SRY. There are over 20 different SOXs in mammals, and these have been divided into ten subgroups based on similarities within their HMG box regions. Outside of the HMG box, SOX proteins of the same subgroup share little primary sequence similarity (Bowles et al., 2000). As in the case of LEF/TCF, the binding of SOX proteins to their target sites induces DNA bending (Bewley et al., 1998; Giese et al., 1992; Love et al., 1995). Inhibitory interactions between β-catenin-regulated gene expression and SOX3, SOX17α and SOX17β were first described by Zorn et al. (Zorn et al., 1999). In *Xenopus*, ectopic expression of these SOXs ventralizes embryos, blocks β-catenin-mediated axis duplication and inhibits β-catenin-induced activation of LEF/TCF-responsive reporters in cultured cells.

XSOX3 was described initially by two groups. Koyano et al. (Koyano et al., 1997) reported that XSOX3 was expressed in oocytes but that both RNA and polypeptide disappeared in mature eggs and early embryos. Penzel et al. (Penzel et al., 1997) reported that *XSOX3* RNA was present maternally and expressed within the neural plate. XSOX17α and β are expressed zygotically, regulated by VegT (Engleka et al., 2001) and required for endodermal differentiation (Hudson et al., 1997). Based on DNA gel shift and in vitro protein binding studies, Zorn et al. (Zorn et al., 1999) concluded that SOX3, SOX17α and SOX17β inhibited β-catenin-signaling by competing directly with TCFs for binding to β-catenin.

XSOX3 is a member of the B subgroup of SOX proteins, which have been further subdivided into B1 (1, 2 and 3) and B2 (14 and 21) subgroups. The B1 SOXs are thought to act as transcriptional activators and the B2 SOXs as transcriptional repressors (Uchikawa et al., 1999). Studies in the mouse suggest that the B1 SOXs are functionally redundant and that differences in phenotypes associated with mutations in these genes are due largely to regulatory differences (Avilion et al., 2003).

The maternal nature of XSOX3 suggests that it could be directly involved in patterning the early embryo. We have extended our previous studies explore this possibility and to define further the mechanism by which XSOX3 modulates β-catenin-mediated gene regulation. Using an affinity-purified antibody directed against the C-terminus of XSOX3 and point mutations in the HMG box region of the polypeptide, we find that XSOX3 binds to sites within the *Xnr5* promoter, distinct from TCF-binding sites. At these sites, it unexpectedly acts as a repressor. In addition to its apparently direct effects on *Xnr5*, injection of *XSOX3* RNA leads decreased levels of *Siamois*, *Twin*, *Xnr3* and *Xbra* RNAs. Depletion of XSOX3 by morpholino injection, expression of an activating form of XSOX3 or injection of an anti-XSOX3 antibody leads to increased accumulation of *Xnr5* RNA, suggesting that the normal function of maternal XSOX3 is to restrict Xnr5 expression to the vegetal hemisphere of the embryo.

Materials and methods

Plasmids and mutant construction

The pCDNA-XSOX3-V5His plasmid was described in Zorn et al. (Zorn et al., 1999), the pCS2myc-tagged ΔG-Xβ-catenin and green fluorescent protein (GFP) plasmids in Merriam et al. (Merriam et al., 1997), the XLEF1 and XTCF3 plasmids in Molenaar et al. (Molenaar et al., 1996; Molenaar et al., 1998), the XTCF4 plasmid in Konig et al. (Konig et al., 2000), and the XSOXD plasmid by Mizuseki et al. (Mizuseki et al., 1998). Plasmids encoding VegT were supplied by M. L. King and J. Heasman (Zhang and King, 1996). We subcloned the XSOX3-V5H_6 sequence into the pCS2 plasmid to create pCS2-XSOX3-V5H_6; the XSOXD sequence was used to generate pCS2-XSOXD-V5H_6. PCR was used to amplify the XSOX3 coding sequence, omitting the C-terminal 20 amino acids recognized by the anti-XSOX3c antibody; this sequence was cloned into both pCS2mt-VP16 (viral transcription activation domain) and pCS2mt-EnR (Engrailed transcriptional repressor domain) plasmids to create pCS2mtXSOX3ΔC-VP16 and pCS2mtXSOX3ΔC-EnR. Point mutations in the XSOX3 HMG box were generated using a QuickChange site-directed mutagenesis kit (Stratagene) following manufacturer's instructions. The entire coding region of each mutated DNA was sequenced to insure that only the desired changes had been introduced. Capped RNA was synthesized from linearized plasmids using Ambion mMessage mMachine kits. Morpholinos against XSOX3 and XSOX7 sequences were synthesized by Gene-Tools. They were resuspended in 0.1×Ringer's saline to a concentration of 10 mM. Antibodies were dialysed against PBS and injected into fertilized eggs as described previously (Klymkowsky et al., 1992). Antibody concentration was measured using a modified Lowry reaction (BioRad) with bovine serum albumin as a standard.

Promoter reporter reagents

The *Siamois*-promoter/firefly-luciferase and *Siamois*-null/luciferase plasmids (Brannon et al., 1997) were supplied by R. Moon and D. Kimelman (University of Washington), wild-type and *TcfA*, *TcfB* and *TcfA*/*TcfB* mutated *Xnr5*-promoter/luciferase plasmids (Hilton et al., 2003) were supplied by E. Hilton and R. Old (University of Warwick, Warwick, UK). A mutated version of the *Xnr5* reporter in which the two SOX3 binding sites upstream of the distal TCF site were removed was generated using the Quickchange site-directed mutagenesis kit. The optimized TOPFLASH and FOPFLASH reporters (Korinek et al., 1997) were supplied by R. Vogelstein (Johns Hopkins University). The pRL-TK plasmid was used to normalize both embryonic and cell cultured experiments using a Promega Dual Luciferase Assay system.

Embryonic and axis duplication studies

Eggs were obtained from hormone-stimulated female *X. laevis*, fertilized, dejellied and injected following established lab procedures (Bachant and Klymkowsky, 1997). Embryonic stages were defined according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Ultraviolet (UV) ventralization of fertilized eggs was performed as described previously (Zorn et al., 1999). Animal caps were generated using a Gastromaster™ apparatus (Xenotek Engineering) and healed in 1×MMR; after healing, they were maintained in 20% MMR. Injected embryos were cultured at 16°C and analysed by immunoblot, immunoprecipitation or whole-mount immunocytochemistry.

Antibodies and immunocytochemistry

Mouse monoclonal anti-V5-epitope antibody was purchased from Invitrogen. An affinity purified rabbit antibody against *Xenopus* β-catenin was raised by Bethyl Laboratories, using purified His_6-β-catenin polypeptide isolated from baculovirus infected cells. Affinity purified rabbit polyclonal antibodies were raised against the N-terminal 20 amino acids of XTCF3 (anti-XTCF3n), the C-terminal 20 amino acids of XTCF3 (anti-XTCF3c) or the C-terminal 20 amino acids of XSOX3 (anti-XSOX3c) by Bethyl Laboratories. The mouse monoclonal anti-Myc antibody 9E10 (Evan et al., 1985) was used to visualize Myc-tagged polypeptides.

Immunochemical analyses

For immunoblot and immunoprecipitation studies, embryos were washed with lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.05% NP-40, 0.5% Triton X-100, 1 mM EGTA, 5 mM NaF and protease inhibitors (Roche)] and homogenized in 20 μl lysis buffer per embryo, typically in groups of 20-25. Homogenates were extracted with Freon and the aqueous layer was recovered and either used immediately or stored frozen at –80°C. Alternatively, embryos were recovered, the excess liquid removed, and the embryos stored at –80°C until used to generate lysates. For SDS-PAGE/immunoblot analysis, 20 μl of lysate (approximately one embryo equivalent) was mixed with 5 μl of 5×sample buffer, heated at 90°C for 10 minutes. For immunoprecipitation analysis, from 100-300 μl of lysate (5-15 embryo equivalents) were incubated with 0.5-1.0 μl of affinity-purified antibody for 1-2 hours. Then, 25 μl of protein-A/agarose (Sigma) was added and incubated overnight at 4°C. Agarose beads were recovered by low speed centrifugation, washed sequentially in lysis buffer, high salt (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.1% NP-40, 0.05% sodium deoxycholate) and low salt (50 mM Tris-HCl pH 7.5, 0.05% NP-40, 0.05% sodium deoxycholate) buffers, recovered by centrifugation, and resuspended in 1×SDS-PAGE sample buffer. After electrophoresis, polypeptides were transferred to membranes. After Ponceau S visualization, blots were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.5% Tween 20 (NFTT) for at least 20 minutes. Blots were incubated for at least 1 hour with primary antibodies diluted into NFTT. The following dilutions were used: anti-Xβ-catenin, 1:2500; antiXTC3c, 1:2000; anti-XTCF3n, 1:5000; anti-XSOX3c, 1:5000; antiV5, 1:5000 and anti-myc supernatant, 1:5. Blots were washed three times in 0.1% Tween-20 TBS and then incubated in goat anti-rabbit/horseradish-peroxidase (HRP) or goat anti-mouse/HRP secondary antibodies (BioRad) diluted 1:20,000 in TBST, and washed 3× in TBS-Tween. Bound antibodies were visualized using the Pierce PicoWestern ECL reagent on Kodak XL1 film. For immunocytochemistry, embryos were stained following established laboratory protocols (Dent et al., 1989) (our current protocol can be found at http://spot.colorado.edu/~klym/Methods/wholemount.htm).

Cell transfection/luciferase assays

HeLa cells grown in Dulbecco's modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum and antibiotics were transfected using FuGene6 (Roche) following the manufacturer's protocol. Typically, 1 μg pCS2-SOX-expressing plasmid was transfected along with 0.2 μg pCS2mt-ΔG-Xβ-catenin plasmid, 0.2 μg of TOPFLASH plasmid and 0.2 μg pRL-TK plasmid, which expresses *Renilla* luciferase under the control of a thymidine kinase promoter (Promega) as a normalization control. 'Empty' pCS2mt plasmid DNA was then added so that, in each experiment, a total of 2 μg plasmid DNA was transfected. Cultures were lysed 18-24 hours after transfection in 100 μl of chilled passive lysis buffer (Promega) supplemented with protease inhibitor cocktail (Roche). 10 μl of lysate was added to 100 μl of luciferase assay reagent II (Promega) and read for 10 seconds using a Turner TD-20/20 luminometer to obtain the firefly luciferase reading. 100 μl of Stop-N-Glo (Promega) substrate was then added and a 10 second reading was made to quantify the level of *Renilla* luciferase. Data was normalized by dividing the firefly by the *Renilla* luciferase readings. All readings were made in duplicate and each assay repeated at least twice.

Electrophoretic mobility shift assays

Proteins were synthesized using a TnT™ in vitro transcription and translation kit (Promega) according to the manufacturer's directions. Protein yield was verified by anti-V5 SDS-PAGE/immunoblot. Labeled DNA probes were prepared by annealing complementary oligonucleotides followed by end labeling with ^{32}P-ATP using T4 polynucleotide kinase. Electrophoretic mobility shift assays were performed using the protocol of Kamachi and Kondoh (Kamachi and Kondoh, 1993). 2 μl of TnT reaction was incubated with probe in binding buffer (20 mM Hepes pH 7.9, 100 μM KCl, 16% glycerol, 1 mM DTT, 1 mM EDTA, 0.5 μg salmon sperm DNA) in a final volume of 12 μl. In antibody supershift experiments, 0.5 μl anti-V5 antibody was added to the reaction. Products were separated on 4% native polyacrylamide gels, dried and visualized by autoradiography.

Biotinylated-DNA 'fishing' analysis

For each biotinylated DNA target, one oligonucleotide was synthesized with a 5′ biotin group, the other was unmodified. For short sequences (such as DC5, 5′Biot-catggtaggtgagcaacAACAATgaatattt-3′; TCF, 5′ Biot-gtgtcatcagaatcATCAAAGgacctccct-3′; and the distal Xnr5 site, 5′Biot-gtcacctgac<u>attgttgtattGTT</u>TGATgttgc-3′) the two oligonucleotides were annealed together. In DC5 and TCF, SOX/TCF sites are capitalized; in the distal Xnr5 oligonucleotide, the two SOX sites are underlined and the TCF site is capitalized. For the longer *Siamois* and *Xnr5* promoter fragments (~200-400 base pairs), the desired regions were amplified by PCR using a biotinylated and unbiotinylated primer pair and Vent polymerase (New England Biolabs). All primers were synthesized by Invitrogen.

Biotinylated DNAs were incubated with streptavidin-agarose beads (Sigma) in coupling buffer (150 mM NaCl, 25 mM sodium phosphate, pH 6.9) for ~1 hour at room temperature with constant mixing. The beads were then washed twice with binding buffer (20 mM HEPES, pH 7.9, 50 mM KCl, 5 mM $MgCl_2$, 12% glycerol, 0.5 mM EDTA and 0.1% Triton X-100). 100-300 μl lysate (5-15 embryo equivalents) was made 1× in binding buffer, 1 mM DTT and 0.5 μg ml^{-1} herring sperm DNA. After a 10 minute incubation at room temperature, 50 μl of DNA-streptavidin-agarose beads were incubated with the lysate for 10 to 20 minutes at room temperature with constant gentle mixing. The beads were then recovered by centrifugation and washed twice with binding buffer, and bound protein was eluted with 2×SDS sample buffer, denatured and analysed by SDS-PAGE and immunoblot.

Quantitative real time PCR analysis

Total RNA was prepared from groups of five embryos homogenized in 1 ml of Trizol reagent (Invitrogen). Homogenates were extracted with 200 μl of chloroform and the upper layer was precipitated in two-thirds of a volume of isopropanol at –20°C for at least 1 hour. After centrifugation at 4°C, 16,000 ***g*** for 15 minutes, the pellet was washed with 75% ethanol, dried and dissolved in 50 μl RNase-free H_2O. Samples were treated with RNase-free DNase I (Ambion) at 37°C for 1 hour and then purified again using a RNeasy Kit (Qiagen) according to manufacturer's instructions.

cDNA synthesis was performed from 1 μg purified RNA using random primers and ImProm-II Reverse Transcription System (Promega) according to the manufacturer's directions. Real time PCR was carried out using a DNA Engine Opticon System (M J Research). A 20 μl PCR reaction contains 1×SYBR Green I nucleic acid gel stain (Molecular Probes), used to quantify amplified DNA, 1 μl cDNA, 1 μM each upstream and downstream primer, 2 mM $MgCl_2$, 0.2 mM dNTPs and 1 unit Taq DNA polymerase (Promega). A standard curve was generated as described in Kofron et al. (Kofron et al., 2001). A dilution series (cDNA: H_2O=100%, 75%, 50%, 25% and 10%) was made from uninjected (control) embryo cDNA samples. Each sample was normalized to the expression level of elongation factor 1α (EF1α). Melting curve analysis was performed on each specific product. The primer sets used are listed in the Table 1. The cycling conditions used are as follows: step 1, 94°C for 4 minutes; step 2, 94°C for 30 seconds; step 3, 55°C or 60°C for 30 seconds (primer dependent); step 4, 72°C for 30 seconds; step 5, 83°C for 1 second; step 6, plate read; step 7, go to step 2 (34 more times); step 8, 72°C for 10 minutes; step 9, melting curve analysis (60-95°C in 0.5°C increments, 1 second hold for each step); step 10, end.

Results

To define how XSOX3 inhibits β-catenin-mediated dorsal axis

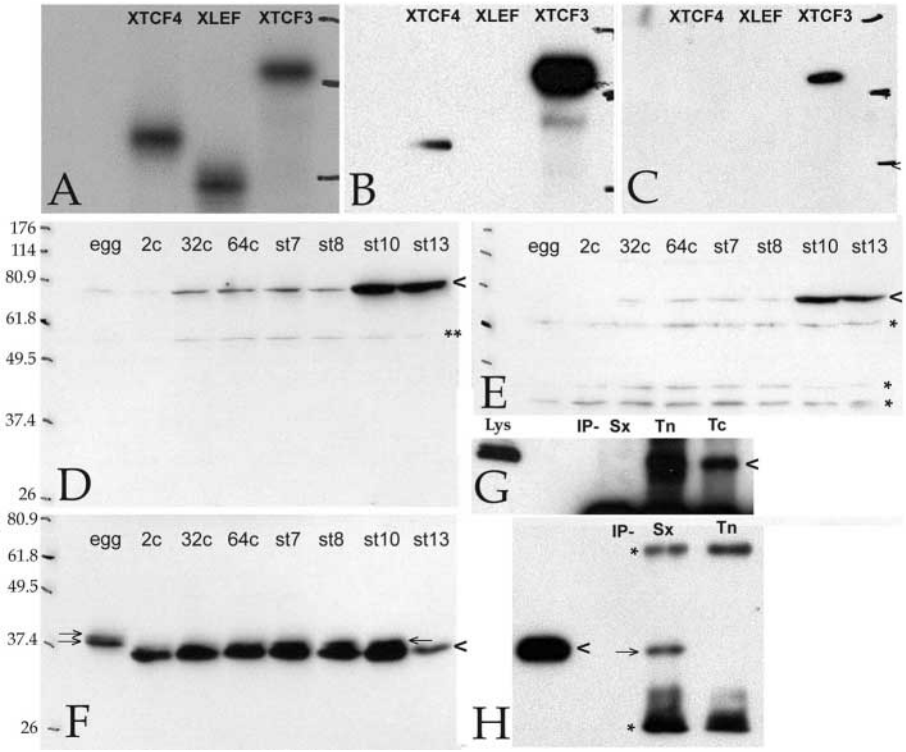

Fig. 1. Antibody specificity. (A) An autoradiogram of SDS-PAGE-separated TnT-synthesized XTCF4, XLEF and XTCF3 polypeptides. (B) A blot of a parallel gel probed with the anti-XTCF3n antibody; the antibody reacts strongly with XTCF3 and more weakly with XTCF4. (C) A blot of a parallel gel probed with the anti-XTCF3c antibody; the antibody reacts with XTCF3 but not XTCF4. Neither anti-XTCF3 antibody reacts with XLEF. (D-F) Embryonic lysates, prepared from unfertilized eggs (egg), two-cell (2C), 32-cell (32c), 64-cell (64c), stage 7 (st7), stage 8 (st8), gastrula/stage 10 (st10) and neurula/stage 13 (s13) embryos were separated by SDS-PAGE, blotted and probed using the anti-TCF3n (D), anti-TCF3c (E) or anti-XSOX3c (F) antibodies. Each lane is loaded with one embryo equivalent. Both anti-XTCF antibodies reacted with a ~67 kDa polypeptide that we presume to be XTCF3 (D,E, arrowhead). The anti-XTCF3n antibody reacted weakly with a 54 kDa polypeptide (D, **) that might be maternal XTCF4 polypeptide. The XTCF3c antibody reacted with distinct sets of lower molecular weight bands (E, *). Anti-XSOX3c (F) reacts strongly with a ~35 kDa polypeptide (arrowhead) throughout early development. The level of this polypeptide decreases in neural stage embryos. In the unfertilized egg (egg) anti-XSOX3c reacts with slower migrating bands (arrows). Occasionally, such slower migrating, anti-XSOX3c-reactive polypeptides are seen in embryonic lysates (arrow). (G) Anti-XTCF3n (Tn) and anti-XTCF3c (Tc) antibodies immunoprecipitated (IP) a common anti-XTCF3n-reactive ~67 kDa polypeptide (arrowhead) present in embryonic lysates (Lys, arrowhead). This polypeptide was not immunoprecipitated by the anti-XSOX3c antibody (Sx). (H) Anti-XSOX3c antibody precipitates a single ~35 kDa, anti-XSOX3c-reactive polypeptide (arrow) that migrates with the 35 kDa polypeptide seen in lysates (Lys, arrowhead). No such band is seen in anti-XTCF3n (TCF3n) or anti-XTCF3c (data not shown) immunoprecipitates. The asterisk (*) marks the heavy and light chains of the precipitating antibody.

Table 1. PCR primer sets for real-time, quantative RT-PCR

PCR primers	Sequence	Reference
Xnr3	F, 5′-CTTCTGCACTAGATTCTG-3′; R, 5′-CAGCTTCTGGCCAAGACT-3′	Kofron et al., 1999
Xnr5	F, 5′-TCACAATCCTTTCACTAGGGC-3′; R, 5′-GGAACCTCTGAAAGGAAGGC-3′	Yang et al., 2002
Xnr6	F, 5′-TCCAGTATGATCCATCTGTTGC-3′; R, 5′-TTCTCGTTCCTCTTGTGCCTT-3′	Takahashi et al., 2000
Siamois	F, 5′-CTCCAGCCACCAGTACCAGATC-3′; R, 5′-GGGGAGAGTGGAAAGTGGTTG-3′	Brannon et al., 1996
Twin	F, 5′-AGTCGTGCCTTTGAAGCCACT-3′; R, 5′-CGCCGCTTGCATAGAAACAGT-3′	This work
EF1a	F, 5′-CAGATTGGTGCTGGATATGC-3; R, 5′-ACTGCCTTGATGACTCCTAG-3′	This work

specification in *Xenopus*, we generated affinity-purified antibodies against XSOX3 (anti-XSOX3c) and XTCF3 (anti-XTCF3n and anti-XTCF3c). Each antibody recognized the appropriate bacterially and baculovirus-infected insect cell synthesized polypeptides (data not shown). Immunoblot analyses against in vitro synthesized polypeptides revealed that anti-XTCF3n reacts with XTCF3 and weakly with XTCF4, whereas anti-XTCF3c reacts with XTCF3 only (Fig. 1A-C), as might be expected from a comparison of the XTCF3 and XTCF4 sequences (data not shown). Immunoblot analyses of early embryonic development (Fig. 1D,E) reveal that both anti-XTCF3 antibodies react with a ~67 kDa polypeptide. The calculated molecular weight of XTCF3 is 60,262 Da. The amount of the 67 kDa anti-TCF3-reactive polypeptide increases following the mid-blastula transition (stage 8.5). Anti-XTCF3n antibody also recognized a polypeptide of ~54 kDa (Fig. 1D). Given that *XTCF4* is a maternal RNA (Houston et al., 2002) and the XTCF4 polypeptide has a predicted molecular weight of 53,062 Da, it is possible that this smaller, anti-XTCF3n-reactive polypeptide is XTCF4. The anti-XTCF3c antibody reacted with a distinct set of smaller polypeptides (Fig. 1E) that might be proteolytically processed forms of XTCF3. The anti-XTCF3c antibody occasionally reacts, apparently non-specifically, with a low molecular weight polypeptide present in embryonic extracts. This polypeptide was not immunoprecipitated by the anti-XTCF3c antibody (data not shown).

The anti-XSOX3c antibody recognized a polypeptide of ~35 kDa (the calculated molecular weight of XSOX3 is 34,012 Da) in immunoblots of embryo lysates (Fig. 1F). Both anti-XTCF3n and anti-XTCF3c immunoprecipitated a ~67 kDa polypeptide from *Xenopus* embryo lysates (Fig. 1G). AntiXSOX3c immunoprecipitated a 35 kDa polypeptide (Fig. 1H). Occasionally a high molecular weight, presumably

unrelated, background band of ~110 kDa was recognized by anti-XSOX3c in immunoblots; this polypeptide was not immunoprecipitated by the antibody (data not shown).

The level of 35 kDa anti-XSOX3c reactive polypeptide remains stable throughout early development and declines upon the onset of gastrulation (Fig. 1F). In unfertilized eggs, anti-XSOX3c recognizes a set of slower migrating bands (Fig. 1F); these bands disappear and are replaced by a 35 kDa band within 20 minutes of fertilization or following the prick activation of the egg (data not shown). The faster of these slower-migrating, anti-XSOX3c-reactive bands can sometimes be resolved in cleavage-stage embryos (Fig. 1F). DNA binding studies indicate that the slower, anti-XSOX3c-reactive polypeptide bind a SOX3-DNA target sequence (data not shown). Whether these slower migrating bands are due to the CDK-mediated phosphorylation of XSOX3 described by Stukenberg et al. (Stukenberg et al., 1997) has not yet been determined.

In situ hybridization analysis reveals that the maternal *XSOX3* mRNA is concentrated in the animal hemisphere of early cleavage stage embryos (Penzel et al., 1997) (Fig. 2A). Whole-mount immunocytochemistry with anti-XSOX3c revealed intense staining of the animal hemisphere that was abolished by pre-incubating the antibody with the peptide-conjugate against which it was raised (Fig. 2B). XSOX3 appears to be primarily cytoplasmic in early embryos (Fig. 2B). By the 64/128-cell stage, staining is clearly nuclear as well as cytoplasmic and its nuclear localization becomes increasingly pronounced as development proceeds (Fig. 2C,F). Cytoplasmic staining can be see in mitotic cells throughout development (Fig. 2F). The initial cleavages that separate animal from vegetal blastomeres occur within the animal hemisphere (Nieuwkoop and Faber, 1967), leading to the partitioning of XSOX3 protein to vegetal blastomeres (Fig. 2C). The anti-XTCF3 antibodies, anti-XTCF3c (Fig. 2D) and anti-TCF3n (Fig. 2E), produced staining patterns that were similar to each other, and to the pattern seen with anti-XSOX3c. Anti-XSOX3c stained nuclei are found in all regions of the embryo through gastrulation, including the most vegetal cells located within the yolk plug of the gastrula stage embryo (Fig. 2G).

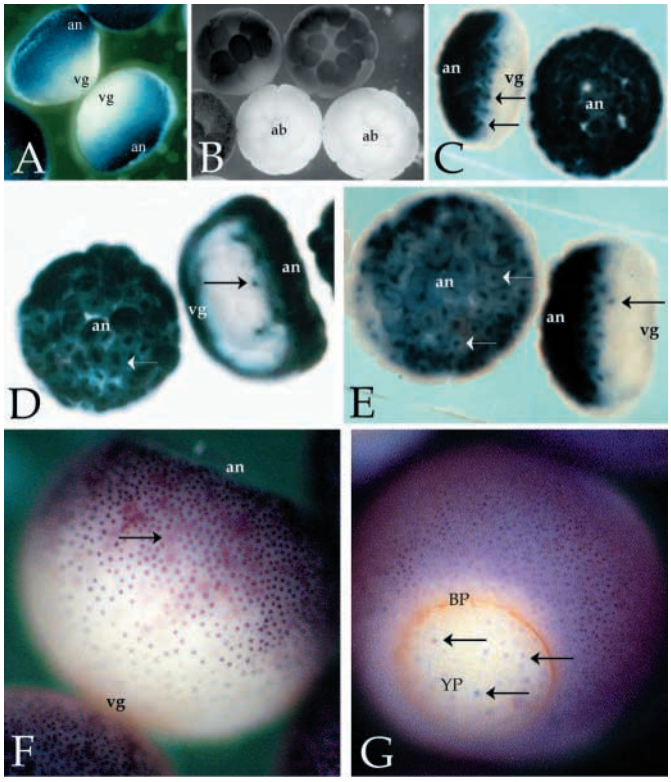

Fig. 2. Immunocytochemical analysis of XSOX3. (A) In situ hybridization of fertilized eggs with an antisense probe for *XSOX3* RNA reveals that *XSOX3* mRNA is localized primarily to the fertilized egg's animal hemisphere ('an' and 'vg' mark the animal and vegetal hemispheres, respectively, in all parts). (B) Whole-mount immunocytochemistry of a 64-cell embryo with the anti-XSOX3c antibody reveals a strong cytoplasmic reaction with the animal hemisphere; preincubation of the antibody with the peptide conjugate against which it was raised completely abolished staining (ab). (C) The nuclear nature of the staining becomes more pronounced as development proceeds but can be clearly seen in early stage embryos (~128-cell stage). The protein is still primarily localized to the animal hemisphere but nuclei in vegetal blastomeres (arrows) clearly contain the immunoreactive polypeptide. Staining of early stage embryos with either anti-XTCF3n (D) or anti-XTCF3c (E) produced a pattern of staining similar to that seen for anti-XSOX3c. Nuclei are marked by arrows. (F) By mid-blastula stages, the XSOX3 polypeptide appears to be nuclear except in mitotic cells (arrows). Nuclear XSOX3 staining is seen throughout the embryo. (G) During gastrulation, anti-XSOX3 staining can be seen in the nuclei (arrows) of yolk plug (YP) cells. The blastopore (BP) is clearly visible and XSOX3 staining is seen throughout the surface ectoderm.

Making mutations in XSOX3

The structure of the DNA-binding HMG box consists of three short α-helical domains (Fig. 3A) and is characterized by nine invariant positions (Fig. 3B), as noted in the NCBI Conserved Domain Database record for pfam00505.6 (HMG box). Based on the conserved residues common to all HMG boxes and an analysis of mutations known to disrupt DNA binding in TCF/LEF and SOX polypeptides, we generated six point mutations in the XSOX3 HMG-box region (Fig. 3B,C). Two of these mutations, m7 and m8, are analogous to mutations made in the HMG box of mouse SOX2 (Scaffidi and Bianchi, 2001).

Although the HMG box is clearly involved in DNA binding, there is also evidence that it can mediate interactions with other polypeptides (Harley et al., 1996; Wilson and Koopman, 2002). We tested whether the m7 or m8 mutations altered the interaction with β-catenin by co-transfecting plasmids encoding a Myc-tagged *Xenopus* β-catenin and the V5-tagged SOX proteins into HeLa cells or by injecting RNAs encoding these polypeptides into fertilized *Xenopus* eggs. Immunoprecipitation analysis indicates that neither mutation altered the ability of XSOX3 to interact with β-catenin (data not show) (see Fig. S1 at http://dev.biologists.org/supplemental/).

LEF/TCF and SOX proteins bind to the core consensus binding site 5′-(A/T)(A/T)CAA(A/T)G-3′, although optimal binding sites are likely to be 10-12 base pairs long (Klymkowsky, 2004) (Mertin et al., 1999; van Beest et al., 2000) (see below). We examined the effects of the mutations on the binding of XSOX3 to the consensus sequence 5′-ATTGTT-3′ found within DC5, an enhancer element found in

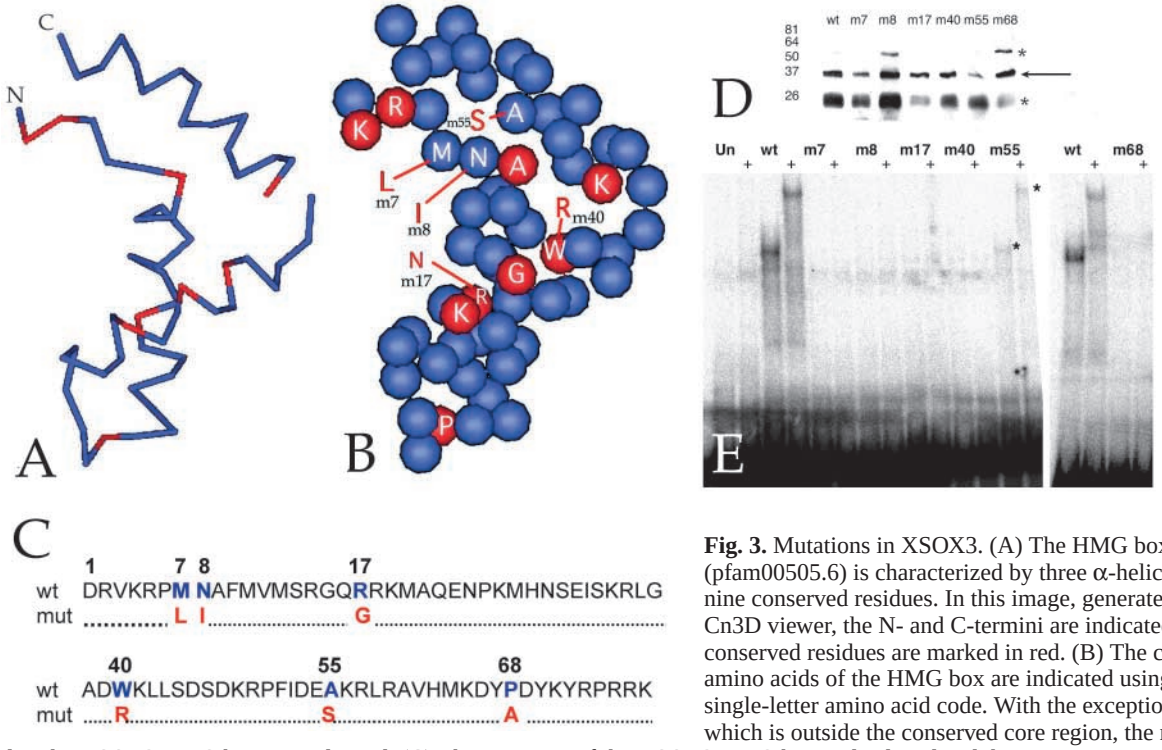

Fig. 3. Mutations in XSOX3. (A) The HMG box (pfam00505.6) is characterized by three α-helices and nine conserved residues. In this image, generated using the Cn3D viewer, the N- and C-termini are indicated and conserved residues are marked in red. (B) The conserved amino acids of the HMG box are indicated using the single-letter amino acid code. With the exception of m68, which is outside the conserved core region, the mutations we generated in the XSOX3 HMG box are indicated. (C) The sequence of the XSOX3 HMG box is displayed and the mutations generated for this analysis are indicated. The first residue of this sequence, D, corresponds to amino acid 38 of the full-length XSOX3 sequence. (D) XSOX3-$V5H_6$ polypeptides (wild type, m7, m8, m17, m40, m55 and m68) generated by in vitro transcription/translation (TnT) were analysed by SDS-PAGE/immunoblot using the anti-V5 antibody. All accumulated to similar levels (arrow). The nature of the extraneous bands (*) are not known. (E) TnT-synthesized proteins were used in oligonucleotide gel-shift studies with the DC5 SOX-binding oligonucleotide. Unprogrammed lysate (Un) showed no shift and no effect upon the addition of antiV5 antibody (+). Oligonucleotide gel shift and antibody-induced supershift were observed upon addition of XSOX3 (wt) and m55 (m55) polypeptides, but not with m7, m8, m17, m40 or m68 polypeptides.

the chick δ-crystalline promoter (Kamachi et al., 1998; Kamachi et al., 1999). All mutated versions of XSOX3, except m55, abolished the polypeptide's affinity for the DC5 oligonucleotide in gel shift assays (Fig. 3D,E). There was no apparently binding of wild-type or mutated XSOX3 to the TCF binding sequence 5′-ATCAAAG-3′ or to the TCF sites present in the *Siamois* promoter (Brannon et al., 1997) under these conditions (data not shown).

In vivo analyses of mutated XSOX3 polypeptides

The TOPFLASH reporter (Korinek et al., 1997) is widely used to assay Wnt and β-catenin-regulated TCF transcriptional activation (Williams et al., 2000). In HeLa cells, a mutationally stabilized form of *Xenopus* β-catenin strongly activated TOPFLASH; this activation was suppressed by the co-expression of wild-type XSOX3-$V5H_6$ (Fig. 4A). β-Catenin did not activate the FOPFLASH reporter, which lacks TCF-binding sites (data not shown). All mutated forms of XSOX3 suppressed the β-catenin-induced activation of TOPFLASH (Fig. 4A). Western blot analysis revealed that these plasmids lead to similar levels of exogenous protein accumulation (data not shown). This result supported the hypothesis that XSOX3 acts to suppress β-catenin-mediated activation of TOPFLASH by binding to β-catenin.

The ability of certain SOX proteins to suppress β-catenin-mediated dorsalization of *Xenopus* embryos was first demonstrated by Zorn et al. (Zorn et al., 1999). To test the ability of mutated forms of XSOX3 to suppress β-catenin signaling in vivo, dorsal blastomeres of four-cell-stage embryos were injected equatorially with a total of 650 pg *XSOX3* RNA together with RNA encoding GFP. At stage 12, embryos were sorted based on green autofluorescence and a subset were homogenized, immunoprecipitated and analysed by immunoblot; all polypeptides accumulated to similar levels (Fig. 4B). Embryos that were allowed to develop to approximately stage 25 were scored using the dorsoanterior index (Kao and Elinson, 1988) (Fig. 4C). The injection of RNAs encoding XSOX3-$V5H_6$, XSOX3m7-$V5H_6$, XSOX3m40-$V5H_6$, XSOX3m55-$V5H_6$ or XSOX3m68-$V5H_6$ ventralized embryos, whereas those encoding XSOX3m8-$V5H_6$ and XSOXm17-$V5H_6$ did not (Fig. 4D; Table 2). This difference between mutant XSOX3 polypeptides was unexpected given their common ability to inhibit β-catenin activation of TOPFLASH (see above).

XSOX3 regulation of *Siamois*

The disparity between in vitro TOPFLASH and in vivo ventralization assays lead us to examine the ability of exogenous XSOX3 to modulate the activity of *Siamois*, a homeobox gene whose expression in late blastula stage embryos is regulated by β-catenin. Brannon et al. (Brannon et al., 1997) characterized an ~800 bp fragment of the *Siamois* promoter that is negatively regulated by XTCF3 and positively regulated by β-catenin. Fertilized eggs were injected with 50

Table 2. XSOX3 ventralization assay

Injection	% Ventralized	Average DAI	*n*
Uninjected	0	5.0	118
Sox3	76	2.6	127
Sox3m7	64	2.7	55
Sox3m8	1	5.0	147
Sox3m17	8	4.8	51
Sox3m40	67	2.9	36
Sox3m55	60	3.6	40
Sox3m68	85	2.1	58

Table shows activity of XSOX3 point mutants in the embryo ventralization assay.

Embryos were scored according to the DorsoAnterior-Index (DAI) of Kao and Ellinson (Kao and Ellinson, 1988).

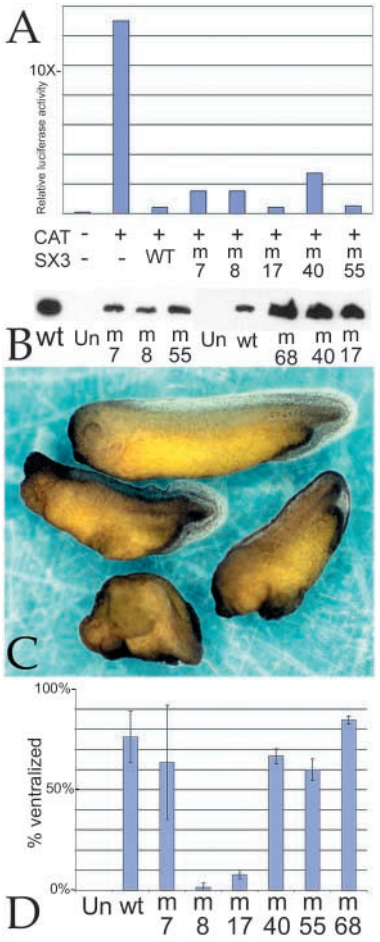

Fig. 4. SOX3 mutant activities. (A) HeLa cells were transfected with plasmids encoding the various XSOX3 mutant polypeptides, along with a plasmids encoding a mutationally stabilized form of *Xenopus* β-catenin, the pRL-TK plasmid for the normalization of transfection efficiency and the TOPFLASH reporter. Co-expression of β-catenin (CAT +) activated TOPFLASH and this activation was suppressed by the co-expression of XSOX3-V5H$_6$ (WT). The co-expression of the mutant XSOX3 polypeptides (m68 was not tested) led to the inhibition of the β-catenin-induced activation of TOPFLASH. (B) Embryos were injected equatorially into the two dorsal blastomeres of four-cell embryos with RNA encoding V5H$_6$-tagged forms of XSOX3. At stage 12, embryos were homogenized and the lysates immunoprecipitated using the mouse antiV5 antibody and then analyzed by SDS-PAGE/immunoblot using the mouse antiV5 antibody. Two distinct experiments are displayed. In each case, similar amounts of the exogenous polypeptides were found to accumulate. No signal was detected in uninjected (Un) embryos. (C) Control and RNA-injected embryos were allowed to grow out to stage ~25. A wild-type (DAI 5) embryo is show at the top of the panel; three embryos displaying various levels of ventralization are shown below. (D) The proportion of embryos ventralized by the injection of XSOX3-V5H$_6$ RNA or its mutated variants is displayed. The exact numbers and extent of ventralization observed are given in Table 2. Wild-type embryos are 0% ventralized.

pg pRL-TK plasmid DNA with S01234-luc (50 pg) together 1 ng of RNAs encoding wild-type or mutated XSOX3s. Approximately at stage 9, the embryos were homogenized and assayed for luciferase activity. In the absence of *XSOX3* RNA, the reporter was preferentially active on the pro-dorsal side of the embryo (Fig. 5A). Although a factor that inhibits dorsal axis formation might be expected to inhibit *Siamois* reporter activity, both wild-type and m7 forms of XSOX3 activated the reporter on the dorsal but not the ventral side of the embryo (Fig. 5B). Injection of RNA encoding the m8 form XSOX3 had no effect of *Siamois* reporter activity in either dorsal or ventral blastomeres (Fig. 5B). The unexpected ability of exogenous XSOX3 to activate the *Siamois* reporter led us to examine the effects of XSOX3 on the endogenous *Siamois* gene. *Siamois* expression is suppressed in embryos ventralized by UV illumination of the vegetal hemisphere during the first cell cycle, and its expression can be rescued by the injection of RNA encoding a stabilized β-catenin (Fig. 5C) (Zorn et al., 1999). Co-injection of wild-type or m7, but not m8, *XSOX3* RNA suppressed β-catenin's ability to rescue *Siamois* expression (Fig. 5C), as would be expected for ventralizing factors.

DNA binding of SOX3s in embryonic lysates

We found no evidence for the binding of XSOX3 to sites within the *Siamois* promoter region using conventional gel shift assays with in vitro synthesized proteins (data not shown). It is known, however, that the binding of SOX proteins to DNA is often dependent on, or enhanced by, accessory factors (Kamachi et al., 2000; Weiss, 2001; Wilson and Koopman, 2002). We therefore examined the binding of endogenous XSOX3 and XTCF3 to biotinylated double-stranded DNAs. In this assay, which we term DNA fishing, biotinylated oligonucleotides are bound to streptavidin-agarose beads and then incubated with lysates derived from stage 7/8 embryos. Beads were recovered by centrifugation and bound proteins were analysed by SDS-PAGE and immunoblot. This method was originally used by Gabrielsen et al. (Gabrielsen et al., 1989) to isolate the yeast transcription factor τ. Under these conditions, endogenous XTCF3 bound to both TCF and *Siamois* promoter target sequences, but not to a *Siamois*-derived sequence in which the three TCF site had been mutated (Fig. 5D). The addition of a 10- or 20-fold excess of unbiotinylated *TCF* oligonucleotide abolished XTCF3 binding to both biotinylated *TCF* and *Siamois*-derived oligonucleotides (Fig. 5D). Endogenous XSOX3 bound to the DC5 oligonucleotide but not to either wild-type or mutated versions of the *Siamois* promoter sequence; XSOX3 binding to DC5 was abolished by the presence of a tenfold excess of unbiotinylated DC5 (Fig. 5D). These data indicate that DNA fishing is a rapid and reliable method for assaying protein-DNA interactions, and confirm that XSOX3 does not bind to the TCF consensus site or the *Siamois* promoter sequence even in the context of embryo lysates.

Xnr5 as a target of XSOX3 regulation

Yang et al. (Yang et al., 2002) reported that zygotic expression of the Nodal-related genes *Xnr5* and *Xnr6* was detectable at the 256-cell stage, well before the mid-blastula transition and significantly before other known β-catenin-regulated

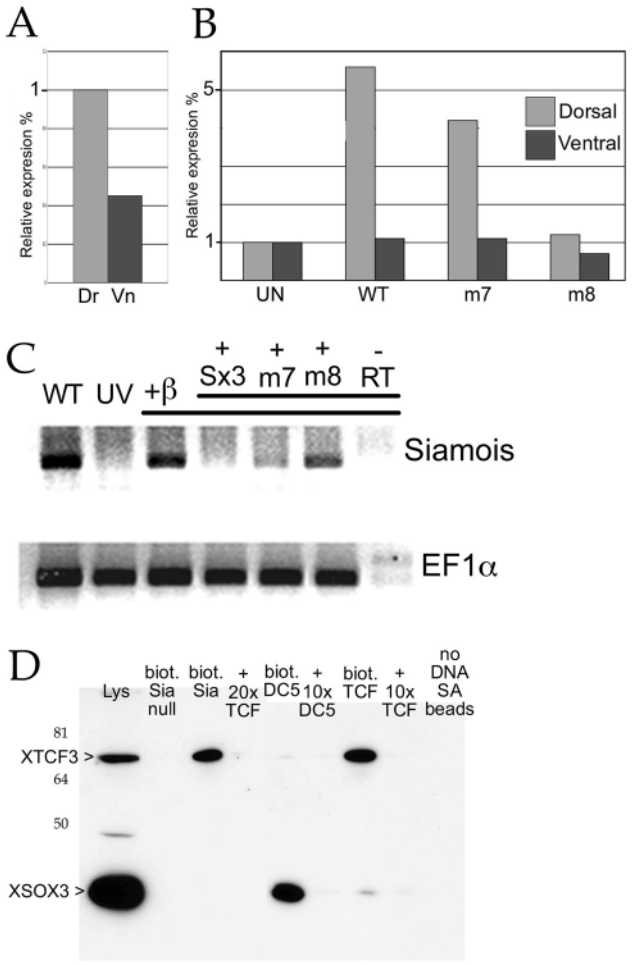

Fig. 5. XSOX3 effects on *Siamois*. (A) The ~800bp *Siamois*/firefly-luciferase reporter together plasmid encoding *Renilla* luciferase were injected into either the dorsal or ventral side of fertilized eggs. Embryos were homogenized at stage ~9 and luciferase activities were measured. Activity was normalized using *Renilla* luciferase levels and the dorsal activity was set to 1. (B) Fertilized eggs were injected with the *Siamois*/luciferase reporter plasmid together with 1 ng of *XSOX3-V5H6* RNA. Reporter activity on the dorsal or ventral sides of the embryo in the absence of exogenous *XSOX3* RNA (UN) was set independently to 1. Co-injection of *XSOX3* wild-type or m7 RNAs activated the *Siamois* reporter on the dorsal but not on the ventral sides of embryos; the m8 polypeptide had no effect on either side of the embryo. (C) To determine the effect of *XSOX3* RNA injection on the endogenous *Siamois* gene, embryos were ventralized by UV illumination during the first cell cycle. The dorsal axis was rescued by the injection of mutationally stabilized β-catenin RNA (1 ng). RNA was isolated from wild-type, UV-treated, UV-treated, β-catenin-rescued and UV-treated, *β-catenin*- and *XSOX3*-RNA-injected embryos at stage 9, and RT-PCR was performed to visualize expression of the dorsalizing gene *Siamois*; *EF-1α* was used as a control. *Siamois* is expressed in intact embryos, greatly reduced in UV-ventralized embryos and returned to nearly wild-type levels in β-catenin RNA-injected embryos. The co-injection of β-catenin and either wild type or m7 XSOX3 RNA (1 ng) suppressed the reappearance of *Siamois* expression; co-injection of m8 RNA (1 ng) did not suppress β-catenin-induced *Siamois* expression. (D) Streptavidin-agarose bound biotinylated mutant *Siamois* promoter fragment (biot. Sia null), biotinylated wild-type *Siamois* promoter fragment (biot. Sia), biotinylated DC5 (biot. DC5′) or biotinylated TCF (biot. TCF) DNAs were incubated with stage-8 embryo lysates (Lys). Bound proteins were eluted and analysed by immunoblot using the anti-XTCF3n and anti-XSOX3c antibodies. Neither XTCF3 nor XSOX3 bound to the mutated *Siamois* sequence. XTCF3, but not XSOX3, bound to the wild-type *Siamois* and TCF sequences, and this binding was blocked by incubation with a 10- to 20-fold excess of unbiotinylated TCF oligonucleotide. XSOX3, but not XTCF3, bound to the DC5 sequence and this binding was blocked by incubation with a tenfold excess of unbiotinylated DC5 oligonucleotide. No binding of XTCF3 or XSOX3 was observed when biotinylated DNAs were omitted from the assay (No DNA, SA beads).

dorsalizing genes (e.g. *Siamois*, *Twin* and *Xnr3*). *Xnr5* is expressed throughout the vegetal region of early to mid-blastula stage embryo (Takahashi et al., 2000) in a VegT- and β-catenin-dependent fashion (Rex et al., 2002; Yang et al., 2002). A ~200bp minimal *Xnr5* promoter sequence has been characterized (Hilton et al., 2003). A reporter plasmid in which this Xnr5 promoter sequence is used to drive firefly luciferase expression was generously supplied to us by E. Hilton and R. Old. In our hands, the reporter was expressed somewhat more actively in dorsal than in ventral blastomeres (Fig. 6A) and was more active in vegetal than in animal hemispheres (Fig. 6B). Both wild-type and m7 versions of *XSOX3* RNAs activated the *Xnr5*-luciferase reporter in dorsal, ventral (Fig. 6C), animal and vegetal (Fig. 6D) blastomeres. Expression of the m8 version of XSOX3 had no effect on *Xnr5* reporter activity (Fig. 6C,D). This pattern of activity correlates with the activity of these polypeptides in ventralization and other in vivo assays (see above).

The ~200bp *Xnr5* promoter sequence isolated by Hilton et al. (Hilton et al., 2003) contains two TCF ‘off-consensus’ binding sites, a distal ‘TCF/LEFA’ site 5′-GTTTGAT-3′ (Fig. 6E) and a proximal ‘TCF/LEFB’ 5′-ATGAAAG-3′ site (not shown) (off-consensus bases are underlined). Immediately upstream of the TCF/LEFA site are two AATGTT SOX binding sites, SOXa and SOXb, one of which overlaps the TCF/LEFA sequence (Fig. 6E). DNA fishing analyses indicate that both XTCF3 and XSOX3 bind to the wild-type SOXab-TCF/LEFA region (Fig. 6F).

To determine whether XSOX3 binds to the off-consensus TCF/LEFA site, we generated four mutated forms of the region (Fig. 6E). MUT1 removes the SOXa site but leaves the SOXb and TCF sites intact. MUT2 removes the SOXb and TCF sites while leaving the SOXa site intact. MUT3 and MUT4 remove both SOX sites. The orientation of the TCF site is ambiguous; it could be either 5′-TTGTTTG-3′, which is similar to the TCF/LEFB site, or 5′-GTTTGAT-3′. MUT4 was designed to resolve its orientation. DNA fishing with these mutated SOXab-TCF/LEFA sequences (Fig. 6F) indicates that XSOX3 can bind to either SOXa or SOXb sites, although it is not clear whether both sites can be occupied simultaneously. XSOX3 does not appear to bind significantly to the TCF/LEFA site. Similarly, XTCF3 appears to bind to the TCF/LEFA site but not the SOX sites. The binding of XTCF3 to the MUT4 sequence suggests that the site it oriented 5′-GTTTGAT-3′, although it is also possible that XTCF3 can bind to the TTGTTTGAT sequence in either orientation. Studies are ongoing to determine whether XSOX3 and XTCF3 can bind simultaneous to this DNA.

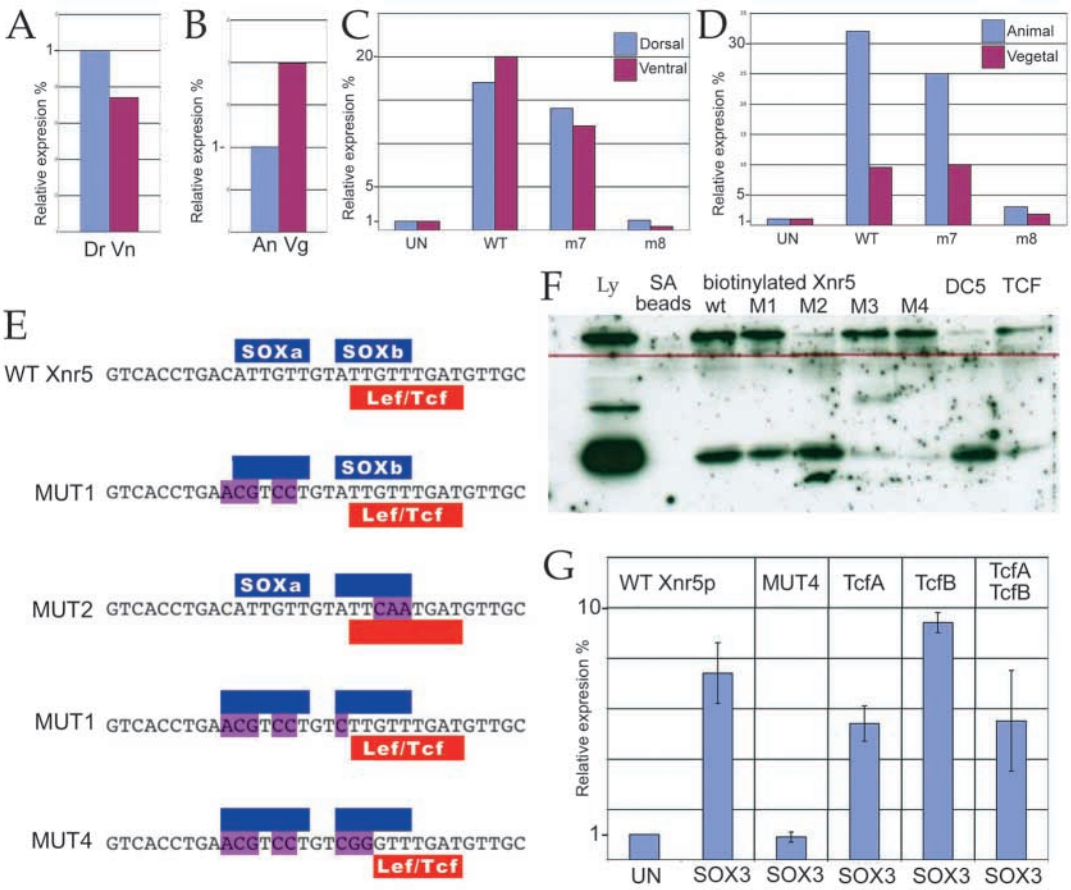

Fig. 6. XSOX3 effects on *Xnr5*. The ~200bp *Xnr5*/firefly-luciferase reporter together plasmid encoding *Renilla* luciferase were injected into either the dorsal or ventral (A) or animal or vegetal (B) hemispheres of fertilized eggs. Embryos were homogenized at stage ~9 and luciferase activities were measured. Activity was normalized using *Renilla* luciferase levels and the dorsal activity was set to 1 in (A), whereas vegetal activity was set to 1 in (B). (C) Co-injection of *XSOX3* wild-type or m7 RNA (1 ng) activated the *Xnr5* reporter to a similar extent on both dorsal and ventral sides of the embryo; m8 RNA (1 ng) had no effect on either side of the embryo. (D) Injection of *XSOX3* wild-type or m7 RNA led to activation of the *Xnr5* reporter in both animal and vegetal hemispheres; the m8 polypeptide produced no significant activation of the *Xnr5*-luciferase promoter. (E) WT Xnr5′ is the sequence of the distal Lef/TcfA site of the *Xnr5* promoter identified by Hilton and Old (E. Hilton and R. Old, unpublished). It contains two SOX binding sites (blue boxes marked SOXa and SOXb) and a LEF/TCF site (red box marked LEF/TCF). MUT1 removes the SOXa site, leaving the SOXb and LEF/TCF sites intact. MUT2 removes the SOXb and LEF/TCF sites, leaving the SOXa site intact. MUT3 removes both SOX sites with no effect on the LEF/TCF site. MUT4 removes both SOX sites, and would remove the LEF/TCF site were it oriented TTGTTTG rather than GTTTGAT. (F) DNA fishing of stage-8 embryonic lysates was used to analyse these mutations. After SDS-PAGE and blotting, the blots were cut. The upper XTCF3 containing region was probed with anti-XTCF3n, the lower *XSOX3*-containing region was probed with anti-XSOX3c. Neither polypeptide bound to streptavidin beads in the absence of biotinylated DNA (SA beads). Both XTCF3 and XSOX3 were bound to wild-type *Xnr5* DNA (wt). Both XTCF3 and XSOX3 bound to the MUT1 DNA (M1), which eliminates the SOXa site. Binding of XTCF3 was eliminated by the MUT2 mutation (M2), but XSOX3 binding remained. Little or no XSOX3 bound to MUT3 (M3) or MUT4 (M4), which eliminate both SOX sites, although both bound XTCF3. XSOX3 but little XTCF3 bound to DC5, whereas the TCF sequence bound XTCF3 but little XSOX3. (G) In whole embryos, the wild-type *Xnr5p* reporter is activated by co-expression of XSOX3. Removal of SOXa and SOXb binding sites (MUT4) abolishes this activation, whereas removal of the TcfA and TcfB sites, either alone or together leaves the reporter responsive to XSOX3, although the TcfA mutation alone or together with the TcfB mutation reduces responsiveness to XSOX3, presumably because this mutation also removes the SOXb binding site.

To confirm that the SOXab sites are responsible for XSOX3's effects on the *Xnr5* reporter, we generated a mutant form of the reporter that carries the MUT4 sequence. The MUT4-Xnr5 reporter was no longer responsive to XSOX3, whereas removal of the TCF/LEFB site (ATGAAAG mutated to ATGCACG) had no effect on its activation by XSOX3 (Fig. 6G). Removal of the TCF/LEFA site (GTTTGAT mutated to GCTCGAT) also removed the SOXb site and produced a partial reduction in the reporter's response to XSOX3 (Fig. 6G). These studies indicate that the responsiveness of the Xnr5 reporter to XSOX3 is due solely to the presence of the SOXab sites.

We used DNA fishing to reexamine the relative binding affinities of wild-type, m7 and m8 forms of XSOX3 to the SOXab-TCF/LEFA *Xnr5* promoter sequence (Fig. 7A). Fertilized eggs were injected with RNAs and lysates were prepared from stage 8 embryos. Wild-type and m7 polypeptides bound to DC5 and Xnr5-derived SOX-TCF sequences; under these condition, binding of the m8 polypeptide was clearly reduced compared with the binding of the wild-type and m7 polypeptides.

As in the case of the *Siamois* reporter, we would have expected a ventralizing factor to suppress rather than activate the dorsalizing gene *Xnr5*. To examine the effects of exogenous XSOX3 on endogenous *Xnr5* expression, we used an animal cap assay (Fig. 7B). Although animal caps do not normally express *Xnr5*, they can be induced to so by the injection of *VegT* RNA (Rex et al., 2002). Co-injection of either wild-type or m7 *XSOX3* RNA suppressed the ability of *VegT* RNA to activate *Xnr5* expression in animal caps, whereas m8 was

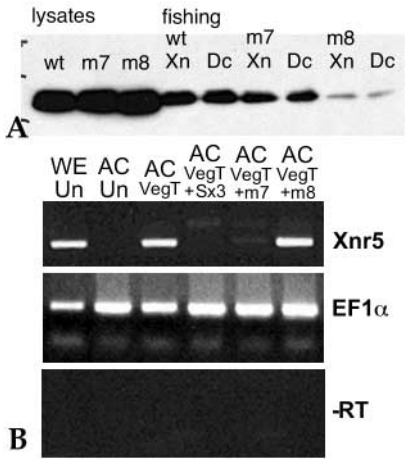

Fig. 7. Wild-type and mutant XSOX3 effects on endogenous *Xnr5*. (A) Fertilized eggs were injected with RNAs encoding wild-type (wt), m7 (m7) or m8 (m8) $V5H_6$-tagged forms of XSOX3. All three polypeptides accumulated to similar levels in stage-8 embryonic lysates (lysates). The binding wild-type and m7 polypeptides to both wild-type *Xnr5* (Xn) and DC5 (DC) sequences (fishing) were much stronger than the binding of m8 under these conditions. (B) Xnr5 is normally expressed in the vegetal region of the embryo in response to the maternal factor VegT. Embryos were injected in the animal hemisphere with *VegT* RNA (1 ng) at the one-cell stage, either alone or together with RNA encoding XSOX3 (1 ng). At stage 8, animal caps were prepared. After a 2 hour incubation, they were homogenized and analysed by RT-PCR. Animal caps from uninjected embryos (AC/Un) did not express *Xnr5* RNA; *Xnr5* RNA was expressed in animal caps derived from embryos injected with *VegT* RNA (AC/VegT). Co-injection of *VegT* and wild-type (AC/VegT/+Sx3) or m7 (AC/VegT/+m7) *XSOX3* RNAs suppressed the accumulation of *Xnr5* RNA, whereas m8 RNA (AC/VegT/+m8) had no effect on *Xnr5* RNA accumulation in response to VegT.

inactive in this assay (Fig. 7B). A similar pattern was seen when intact embryos were examined (Fig. 8A-F). Injection of RNA encoding wild-type, but not m8, XSOX3 into dorsal blastomeres inhibited the accumulation of endogenous *Xnr5*, *Xnr6*, *Siamois*, *Twin*, *Xnr3* and *Xbra* mRNAs as determined by quantitative RT-PCR.

To determine whether the effects of *XSOX3* RNA on endogenous *Xnr5* RNA levels was specific to XSOX3 or was a generic property of SOX proteins, fertilized eggs were injected with RNAs encoding either V5His-tagged XSOX3 or XSOXD. The XSOXD HMG box differs significantly from that box of XSOX3 (Fig. 8G). Whereas expression of XSOXD inhibits β-catenin-activation of the TOPFLASH reporter in cultured cells (Klymkowsky, 2004), it does not ventralize *Xenopus* embryos when injected into dorsal blastomeres (Fig. 8H). It also does not alter *Xnr5* mRNA levels compared with uninjected control embryos (Fig. 8I). These results again emphasize the dichotomy between the heterologous in vitro TOPFLASH reporter assay system and various in vivo assays.

Endogenous XSOX3 acts as a repressor of *Xnr5*

B1 type SOXs such as XSOX3 are commonly assumed to act as transcriptional activators (Bowles et al., 2000; but see Graves, 1998). Injection of *XSOX3* RNA decreases *Xnr5* RNA levels (Fig. 7B, Fig. 8A,B). If endogenous XSOX3 represses *Xnr5* expression, we would predict that depletion of XSOX3 protein would lead to an increase in *Xnr5* RNA levels. Embryos injected with a morpholino directed against the 5′ untranslated region and the translation initiation region of the *XSOX3*

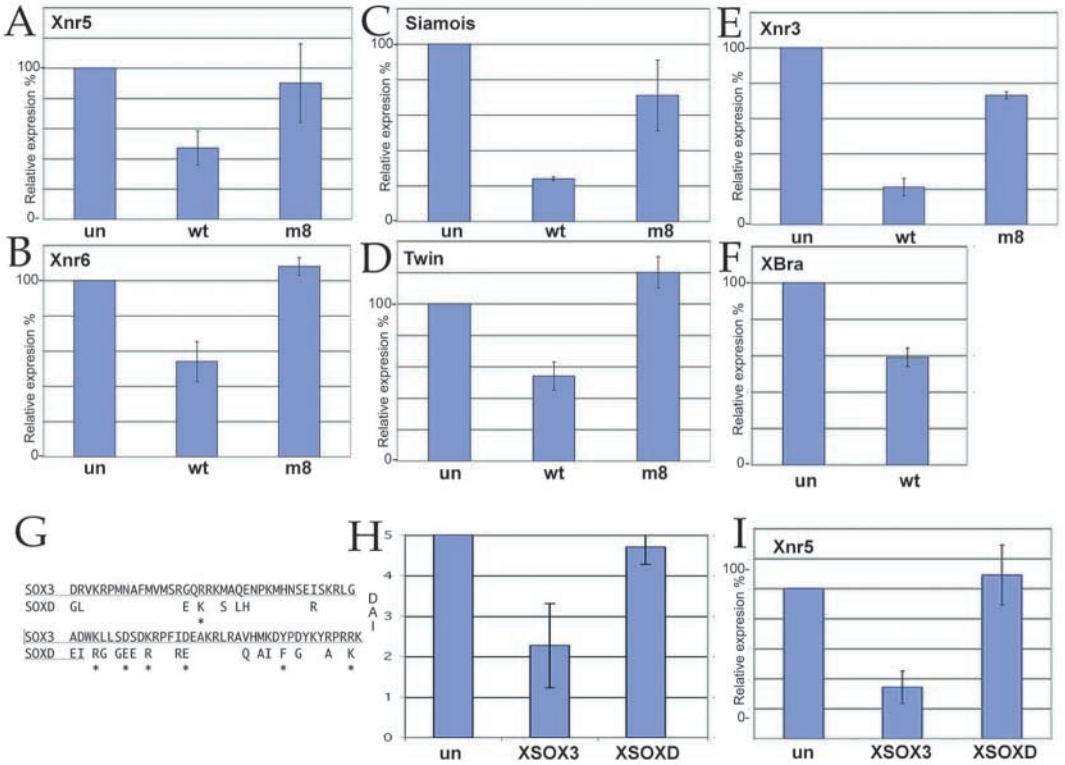

Fig. 8. Quantitative RT-PCR analyses. To determine the effects of XSOX3 expression on endogenous genes, fertilized eggs were injected with wild-type or m8 *XSOX3* RNAs (1 ng), and allowed to develop to stage 9, when they were homogenized and RNA was isolated and subjected to quantitative RT-PCR analysis. Compared with uninjected (un) and m8 (m8) injected controls, injection of wild-type *XSOX3* RNA (wt) led to a decrease in the level of *Xnr5* (A), *Xnr6* (B), *Siamois* (C), *Twin* (D), *Xnr3* (E) and *Xbra* (F) mRNAs. (G) The HMG boxes of XSOX3 and XSOXD differ at several positions [conservative changes are marked with an asterisk (*)]. (H) Compared with the ventralization of embryos following the injection of wild-type *XSOX3* RNA (SOX3), injection of 650 pg mRNA encoding XSOXD-$V5H_6$ (SOXD) failed to ventralize embryos [uninjected embryos (un)]; immunocytochemistry revealed that the XSOXD-$V5H_6$ polypeptide had accumulated (data not shown). (I) Fertilized eggs were injected with 1 ng of either *XSOX3-V5H6* wild-type (wt) or *XSOXD-V5H6* RNA. At stage 9, embryos were homogenized and subjected to quantitative RT-PCR analysis. Injection of *SOXD-V5H6* RNA had no apparent effect on *Xnr5* RNA levels.

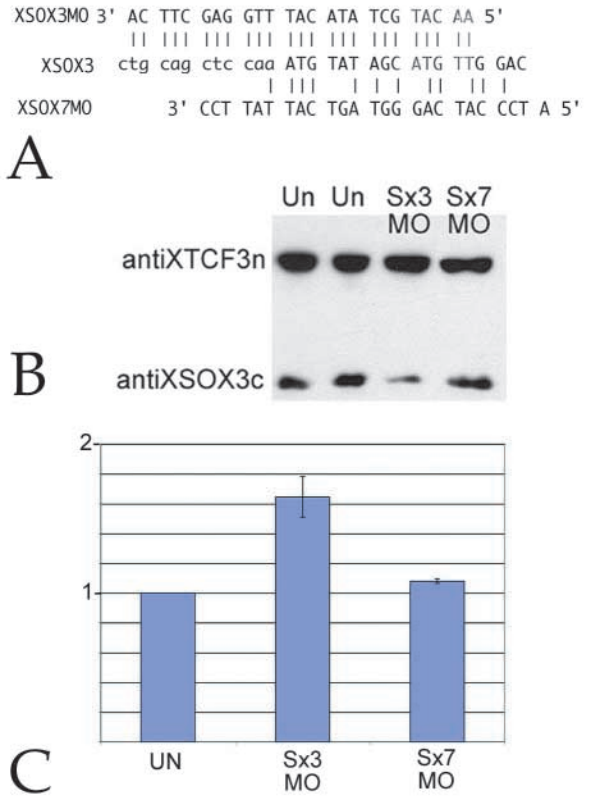

Fig. 9. Manipulating XSOX3 accumulation. (A) Morpholinos against the 5′ untranslated region (5′ UTR) and coding sequence of *XSOX3* and *XSOX7* were generated and compared with the *XSOX3* RNA sequence. (B) Upon injection into fertilized eggs, the *XSOX3* morpholino led to a decrease in XSOX3 polypeptide level in embryos analysed at stage 9; the injection of the *XSOX7* morpholino had no apparent effect on XSOX3 accumulation. Neither morpholino effected the accumulation of XTCF3 (recognized by the anti-XTCF3n antibody). (C) Embryo lysates were prepared and subject to quantitative RT-PCR analysis. Depletion of *XSOX3* led to an increase in *Xnr5* RNA compared with uninjected and *XSOX7*-morpholino-injected embryos.

mRNA (Fig. 9A) show a decrease in XSOX3 but not XTCF3 polypeptide levels, as determined by western blot (Fig. 9B). A morpholino directed against the analogous region of *XSOX7* mRNA (Fig. 9A) had no effect on XSOX3 protein levels when injected into fertilized eggs (Fig. 9B). *Xnr5* RNA levels were found to increase modestly in XSOX3 morpholino-injected embryos and were unaltered by the injection of XSOX7 morpholino (Fig. 9C).

Yang et al. (Yang et al., 2002) reported that *Xnr5* transcription begins early in *Xenopus* development, well before the mid-blastula transition, whereas Onuma et al. (Onuma et al., 2002) found evidence for interactions between the six Nodal-related genes expressed in the early *Xenopus* embryo. Given that a morpholino would not be expected to effect the maternal component of XSOX3 protein present in early stage embryos, when Xnr5 is first expressed, we turned to an alternative method to disrupt XSOX3 function: the injection of anti-XSOX3c antibody. When added directly to embryo extracts (Fig. 10A) or injected into fertilized eggs from which embryo extracts were subsequently prepared (Fig. 10B), the antiSOX3c antibody inhibited the binding of XSOX3 polypeptide to the DC5 oligonucleotide. Addition or injection of similar amounts of the anti-XTCF3n antibody had no effect on XSOX3 DNA binding (Fig. 10A,B). Injection of the anti-XSOX3c antibody produced a complex defective gastrulation phenotype that will be described in detail elsewhere (C.Z. et al., unpublished). Nevertheless, injection of anti-XSOX3c antibody produced a robust (~300%) increase in *Xnr5* RNA (Fig. 10C), as measured by quantitative RT-PCR. Injection of similar amounts of anti-XTCF3n antibody produced no overt embryonic phenotype (data not shown) and had no significant effect on *Xnr5* RNA levels (Fig. 10C).

If wild-type XSOX3 acts directly to repress *Xnr5* expression, as the DNA-binding, morpholino and antibody injection experiments suggest, then a constitutively activating form of XSOX3 should lead to an increase in *Xnr5* RNA, whereas a constitutively repressing form of XSOX3 should behave like the wild-type protein. We deleted the C-terminal 20 amino acids of XSOX3 and replaced them with either the VP16 transcription activation domain or the transcriptional repression domain of Engrailed, both within the pCS2mt plasmid. As described above, injection of RNA encoding wild-type XSOX3-V5H$_6$ led to a decrease in *Xnr5* RNA. Injection of Myc-tagged *XSOX3ΔC-VP16* RNA lead to an increase in *Xnr5* RNA, and injection of Myc-tagged *XSOX3ΔC-EnR* RNA lead to a decrease in *Xnr5* RNA (Fig. 10C). Both activator and repressor chimeras accumulated to similar levels (data not shown). Based on these data, we conclude that maternally supplied XSOX3 protein normally represses the accumulation of *Xnr5* RNA, presumably by directly binding to the *Xnr5* promoter.

Discussion

The SOXs are a large, diverse family of polypeptides expressed in complex patterns during embryonic development and in the adult (Bowles et al., 2000; Prior and Walter, 1996; Wilson and Koopman, 2002). Like the β-catenin-regulated LEF/TCF proteins, SOXs bind to specific DNA sequences. Binding leads to the intercalation of amino acid side chains between DNA base pairs and subsequent DNA bending, typically by 80-130°. As such, they are capable of acting as both conventional and architectural transcription factors modulating the juxtaposition of other factors involved in gene regulation.

The SOX/LEF/TCF protein family is phylogenetically ancient and appears to have been present in the last common ancestor of the metazoans (Bowles et al., 2000). There are four known LEF/TCFs in vertebrates – TCF1, LEF1, TCF3 and TCF4 – three of which (TCF1, TCF3 and TCF4) are present as maternal RNAs in *Xenopus*. *LEF1* (Hovanes et al., 2000), *TCF1* (van de Wetering et al., 1996) and *TCF4* (Duval et al., 2000; Young et al., 2002) transcripts occur in alternatively spliced variants that produce polypeptide variants. The activity of LEF/TCF appears to be determined by promoter context and associations with accessory factors. For example, all LEF/TCFs associate with Groucho-related co-repressors (Brantjes et al., 2001), as well as with β-catenin, whose C-terminal domain appears to act as a co-activator (Vleminckx et al., 1999; Williams et al., 2000).

The SOX proteins are grouped based on the similarity of their HMG-box DNA binding domain to that of the *sex-related*

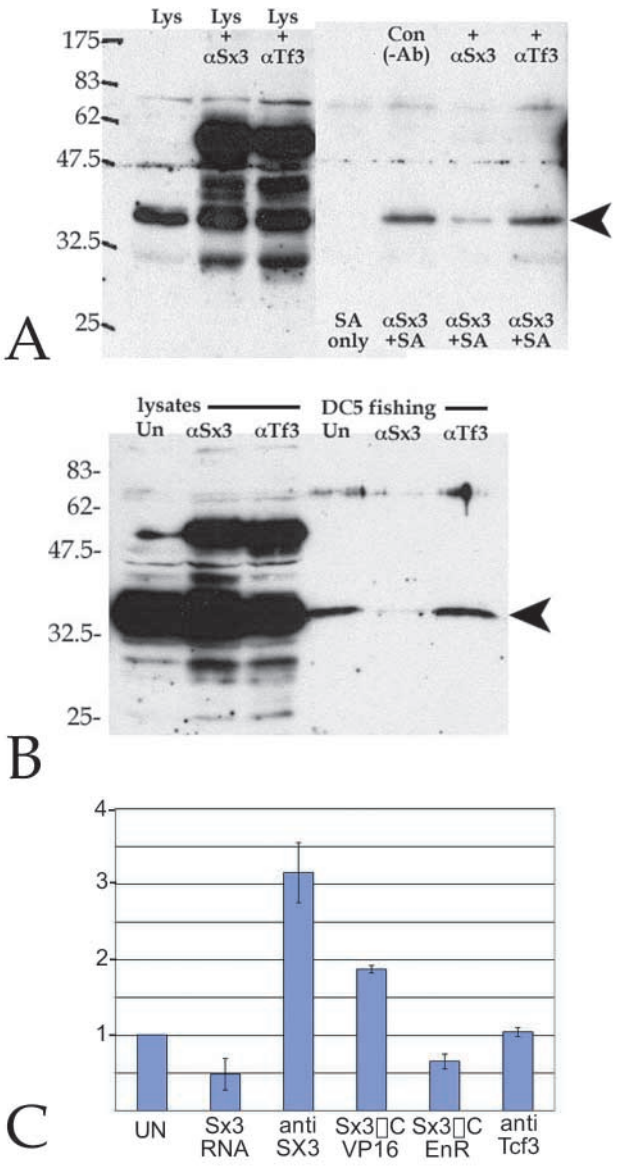

Fig. 10. Manipulating maternal XSOX3 activity. (A) Stage 8 embryo lysates (300 μl) were incubated alone (Lys) or together with 0.5 μg anti-XSOX3c (Lys + αSx3′) or anti-XTCF3n (Lys + αTf3′) antibodies. Lysates were then incubated with either streptavidin-agarose beads (SA alone) or biotinylated DC5-streptavidin-agarose beads (DC5-SA). Both lysates and DC5-bound proteins were analysed by immunoblot with anti-XSOX3c. XSOX3 (arrowhead) was bound to DC5 in control and anti-XTCF3n-containing lysates, but its binding was greatly reduced by the addition of the anti-XSOX3c antibody; no binding was seen in the absence of DC5 DNA. (B) Fertilized eggs were injected with anti-XSOX3c antibody (10 nl of a 7.5 mg ml^{-1} solution) or anti-XTCF3n antibody (7.8 mg ml^{-1} solution). At stage 9, the embryos were homogenized and analysed by DNA fishing with DC5-streptavidin-agarose. Anti-XSOX3c-injected (αSx3), anti-XTCF3n-injected (αTf3) and uninjected (Un) lysates were analysed by immunoblot using anti-XSOX3c. The total amount of XSOX3 was unchanged upon antibody injection, but anti-XSOX3c dramatically inhibited the binding of XSOX3 to DC5-streptavidin-agarose (DC5 fishing). (C) Fertilized eggs injected with either anti-XSOX3c antibody (10 nl of a 7.5 mg ml^{-1} solution) (antiSX3), anti-XTCF3n antibody (10 nl of a 7.8 mg ml^{-1} solution) (anti-TCF3), *XSOX3* RNA (1 ng) (Sx3 RNA), *XSOX3ΔC-VP16* RNA (1 ng) (Sx3ΔCVP16) or *XSOX3ΔC-EnR* RNA (1 ng) (Sx3Δ-EnR) were allowed to develop to stage 9 and then homogenized and analysed by quantitative RT-PCR. *XSOX3* and *XSOX3ΔC-EnR* RNA injection reduced *Xnr5* RNA levels; injection of either anti-XSOX3c and *XSOX3ΔC-VP16* RNA increased *Xnr5* RNA levels; injection of anti-XTCF3n did not alter *Xnr5* RNA accumulation.

on the Y (*SRY*) testis-determining gene of mammals (Graves, 2001). Of these, the B-type SOX proteins appear to be the most phylogenetically ancient and highly conserved (Bowles et al., 2000). SRY has been suggested to have evolved from SOX3 (Foster and Graves, 1994; Stevanovic et al., 1993). The B-type SOXs have been divided into two subtypes, B1 and B2, which can be distinguished by conserved amino acids at positions 2 and 79 of the 80 amino acid long HMG box – both types are present the arthropod *Drosophila melanogaster*, the nematode *Caenorhabditis elegans*, the hemichordate *Ptychodera flava* and the chordate *Ciona intestinalis* (Bowles et al., 2000; Kirby et al., 2002; Taguchi et al., 2002; Yamada et al., 2003).

B-type SOX proteins are expressed early in the development of many organisms. In the mouse, maternal SOX2 protein persists into the early blastocyst; it is located cytoplasmically in cells of the trophectoderm and is primarily nuclear in the cells of the inner cell mass. *SOX2* mRNA appears in morula stage embryos (2.5 days post-coitum) and is restricted to cells of the inner cell mass (Avilion et al., 2003). SOX3 is expressed together with SOX2 in the cell of the epiblast at day 5.5 (Avilion et al., 2003; Wood and Episkopou, 1999). SOX3 is expressed early during embryonic development in the chick (Rex et al., 1997). Although Koyano et al. (Koyano et al., 1997) originally reported that XSOX3 was expressed during oogenesis but was absent from eggs and early embryos, it is now clear that the gene is expressed maternally and is present throughout early blastula stages (Penzel et al., 1997) (Fig. 1F, Fig. 2A-C). Preliminary studies indicate that SOX3-like polypeptides are also supplied maternally in the zebrafish (M.W.K. and K. B. Artinger, unpublished). In all vertebrates examined to date, SOX3 is expressed zygotically in the developing neural tube (Graves, 2001).

Whole-mount immunocytochemistry reveals that the XSOX3 polypeptide is initially cytoplasmic and becomes restricted to nuclei as development proceeds (Fig. 2B,C). Cytoplasmic XSOX3 can be seen in cells captured in the process of mitosis (Fig. 2F). Whether XSOX3 actively shuttles between cytoplasm and nuclei, as has recently been described for mouse SOX10 (Rehberg et al., 2002), remains to be seen, although we have seen evidence for cytoplasmic XSOX3 in neurula stage embryos (data not shown). *XSOX3* mRNA appears to be largely restricted to the animal hemisphere (Fig. 2A) (Penzel et al., 1997). However, the nuclei generated during the first three cleavages lie within animal hemisphere and all blastula stage nuclei, including the most vegetal cells of the yolk plug, appear to contain SOX3 polypeptide (Fig. 2G). We have not quantified the amounts of XSOX3 per nuclei in animal and vegetal blastomeres, although a superficial examination suggests that XSOX3 concentrations are higher in the animal hemisphere. Immunocytochemical analyses with antibodies directed against XTCF3 reveals a similar pattern of distribution through the blastula stages of development (Fig. 2D,E).

Based on DNA and protein binding studies, Zorn et al. (Zorn et al., 1999) concluded that SOX exerted its ability to inhibit β-catenin-mediated dorsal axis formation by competing with endogenous TCFs for binding to β-catenin. We choose to extend those studies by formally eliminating the possibility the XSOX3 was acting through its ability to bind to DNA. We generated a series of six mutations in the XSOX3 HMG box domain (Fig. 3A-C). We saw no obvious effect of these mutations on the interaction between XSOX3 and β-catenin (data not shown) (see Fig. S1 at http://dev.biologists.org/supplemental/). When tested by conventional electrophoretic mobility gel shift assay, DNA binding to the DC5 SOX consensus sequence was abolished by five of six mutations and was reduced in the sixth (m55) (Fig. 3E).

The TOPFLASH reporter is widely used as an assay for β-catenin-regulated TCF-mediated gene expression; for example, it was used by Takash et al. (Takash et al., 2001) as evidence for the ability of human SOX7 to modulate β-catenin activity. In the course of our studies of SOX/catenin interactions, we have found several examples in which activity in the TOPFLASH assay does not correlate with activity in the *Xenopus* embryonic ventralization assay. For example, even though XSOXD inhibits TOPFLASH activation by β-catenin in cultured mammalian cells, it does not suppress β-catenin-induced axis duplication, nor can it ventralize *Xenopus* embryos (Fig. 8H) (Klymkowsky, 2004), suggesting that the two assays measure distinct facets of the interaction between β-catenin, SOXs and target genes.

Although all of the mutated forms of XSOX3 we analysed in this study inhibited the β-catenin activation of the TOPFLASH reporter (Fig. 4A), they differed dramatically in their ability to ventralize embryos (Fig. 4D; Table 2). Because they lie adjacent to one another, we focused our analysis on the m7 and m8 mutations – m7 behaves very much like the wild-type XSOX3 polypeptide, whereas m8 appears to be inactive, although it accumulates to levels similar to that seen for wild-type and m7 polypeptides in embryos, cultured mammalian cells and in vitro protein synthesis extracts. The XSOX3 m7 and m8 mutations are analogous to mutations made in mouse SOX2 by Scaffidi and Bianchi (Scaffidi and Bianchi, 2001). Their m7-like M47I mutation had little effect on DNA binding affinity or bending, whereas the m8-like N48G mutation reduced DNA binding affinity more than tenfold and DNA bending by ~40°. When tested for binding to target sequences in embryonic lysates, a similar difference in apparent DNA-binding affinity was seen for the m7 and m8 mutant forms of XSOX3 (Fig. 7A). Based on these differences, we conclude that differences in their DNA binding affinity are responsible for the differences in the ventralizing activities of the two polypeptides.

Dorsal-determination system and XSOX3

We began our analysis of the mode of XSOX3 action with the knowledge that its overexpression ventralized embryos (Fig. 4C,D) and inhibited β-catenin dorsal axis duplication (Zorn et al., 1999). However, where along the dorsalization pathway XSOX3 acts was unclear. It is known that the cortical rotation establishes a cytoplasmic asymmetry within the fertilized eggs that manifests itself in the blastula-stage embryo as asymmetries in gene expression that underlie the initial dorsal-ventral/organizer-contraorganizer axis. The best established of these rotation-induced cytoplasmic asymmetries is the asymmetry in β-catenin. Over the past few years, several target genes regulated by β-catenin asymmetry have been identified. In the case of *Siamois*, *Twin*, *Xnr3* and *Xbra*, the initial expression of these genes begins following the mid-blastula transition, when embryos consist of ~4000 cells. We found no evidence, however, for the binding of XSOX3 to sites within the *Siamois* promoter (Fig. 5D). Because the sequences of the TCF binding sites in *Siamois* are similar to those found in *Twin*, *Xnr3* and *Xbra*, and are distinct from the sequences recognized by SOX3 (Klymkowsky, 2004) (Fig. 6E), we were unable to explain the difference between the activity of m7 and m8 forms of XSOX3 in terms of DNA binding to this specific set of target genes. We therefore suspect that these effects are indirect, but it remains a formal possibility that, in the context of intact chromatin, XSOX3 is more promiscuous in its DNA binding than it is on the naked DNA probes used in our studies. We are currently exploring this possibility using chromatin immunoprecipitation.

It was in this light that the observation that *Xnr5* and *Xnr6* are expressed in a β-catenin/TCF dependent manner as early as the 256-cell stage (Yang et al., 2002) was particularly resonant. *Xnr5* and *Xnr6* encode Nodal-related proteins, members of the TGFβ family of secreted signaling molecules (Agius et al., 2000; Jones et al., 1995; Whitman, 2001; Zhou et al., 1993). A network of Nodal-related proteins is involved in the patterning of the early embryo and the determination of left-right asymmetry (Branford and Yost, 2002; Levin and Mercola, 1998; Onuma et al., 2002; Osada and Wright, 1999; Rex et al., 2002; Takahashi et al., 2000).

Our immunochemical studies (Figs 1, 2) indicate that XSOX3 is abundant in 256-cell embryos. The connection between XSOX3 and *Xnr5* was made possible by the isolation of a minimal promoter fragment of the *Xnr5* gene (Hilton et al., 2003). The TCF/LEF sites within this promoter fragment differ from the conventional consensus TCF/LEF sequence (see above) and we originally hypothesized that XSOX3 might bind to these sites. However, a closer look at the *Xnr5* promoter sequence (Fig. 6E) revealed the presence of two consensus SOX binding sites, which we termed SOXa and SOXb. DNA fishing experiments indicate that XSOX3 can bind to either of these sites but not to the TCF/LEF site (Fig. 6F). Removing the SOX sites abolished the *Xnr5* reporter's responsiveness to exogenous XSOX3, whereas removing the TCF binding sites did not (Fig. 6G). Whether XSOX3 and XTCF3 can bind concurrently to this region of the *Xnr5* promoter is currently under study.

Although both *Siamois* and *Xnr5* reporters respond to the injection of *XSOX3* RNA, the direction of the response is the opposite of what would be predicted based on the ventralizing activity of XSOX3. We currently have no compelling explanation for this anomalous behavior except to suggest that the promoter plasmids might form configurations distinctly different from those that occur within endogenous chromatin. Given that the binding of a SOX induces a dramatic 80-130° bend in DNA, subtle differences in DNA organization and accessory proteins could lead to dramatic differences between reporters and endogenous genes. Both exogenous and endogenous XSOX3 regulate endogenous genes in a manner consistent with their ability to ventralize embryos. XSOX3

overexpression inhibits the VegT-induced expression of *Xnr5* in animal caps (Fig. 7B) and the β-catenin-induced expression of *Siamois* in UV-ventralized embryos (Fig. 5C).

The action of XSOX3 on *Xnr5* RNA accumulation is one of repression. This conclusion is supported by the effects of chimeric forms of the XSOX3 polypeptide on *Xnr5* RNA levels in the embryo (Fig. 10C). Expression of a chimeric form of XSOX3 that contains a viral transcription activation domain leads to an increase in *Xnr5* RNA accumulation, whereas a chimeric form of XSOX3 that contains a transcription repressor domain behaves like the wild-type protein (Fig. 10C). B1-type SOX proteins such as SOX3 are often assumed to be transcriptional activators (Uchikawa et al., 1999; Bowles et al., 2000). SOX3 has been proposed to act as a transcriptional repressor of SOX9 (Graves, 1998), although no direct molecular data has been supplied to support this contention.

Beginning with the observation that injection of *int-1* (*Wnt1*) RNA induced axis duplication in *Xenopus* (McMahon and Moon, 1989), RNA-based overexpression studies have been invaluable in elucidating the mechanism of axis formation in particular and signaling pathways in general. At the same time, the relationship of such studies to the normal developmental processes is not necessarily straightforward. It was in this light that the results of downregulating XSOX3 activity (Figs 9, 10) are particularly crucial. We injected a morpholino that suppressed the accumulation of XSOX3 protein (Fig. 9A,B) and the anti-XSOX3c antibody, which inhibits XSOX3 DNA binding (Fig. 10A,B) to examine the role of endogenous XSOX3. In each case, the results were consistent with the hypothesis that endogenous XSOX3 acts to suppress the accumulation of *Xnr5* RNA (Fig. 9C, Fig. 10C).

The embryonic phenotypes associated with these two reagents (XSOX3 morpholino and anti-XSOX3c antibody) are quite different. We find little if any overt effect from the injection of the XSOX3 morpholino, even though it produces a clear decrease in XSOX3 protein levels by late blastula stages (Fig. 9B). We attribute this result to the maternal nature of the SOX3 protein, the early expression of the XSOX3-regulated target genes and, later in development, to the expression of compensatory SOX proteins, particularly XSOX2 (Avilion et al., 2003) (A. A. Avilion, unpublished). By contrast, injection of the anti-XSOX3c antibody produces dramatic, complex phenotypes (C.Z. et al., unpublished). The effects of the injected anti-XSOX3c antibody appears to involve a number of distinct gene targets. XSOX3 appears to regulate *eFGF*/*FGF4* RNA levels positively (C.Z. et al., unpublished) while decreasing *Xnr5* and *Xnr6* RNAs (as reported here). Nevertheless, the data presented here clearly support a mechanism in which maternal XSOX3 inhibits the β-catenin-mediated process of dorsal axis specification by directly repressing animal hemisphere expression of very early zygotic gene *Xnr5*.

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