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β-Catenin Signaling Activity Dissected in the Early *Xenopus* Embryo: A Novel Antisense Approach

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Xenopus **embryos develop dorsal/ventral and anterior/posterior axes as a result of the activity of a maternal Xwnt pathway, in which β-catenin is an essential component, acting as a transactivator of transcription of zygotic genes. However, the questions of where and when β-catenin is required in early embryogenesis have not been addressed directly, because no loss-of-function method has been available. Here we report the use of a novel antisense approach that allows us to target depletion of protein to individual blastomeres. When a "morpholino" oligo complementary to *β-catenin* mRNA is injected into early embryos, it depletes β-catenin protein effectively through the neurula stage. By targeting the oligo to different cleavage blastomeres, we block β-catenin activity in different areas and at different times. Dorsal vegetal injection at the 2- and 4-cell stages blocks dorsal axis formation and at the 8-cell stage blocks head formation, while A-tier injection at the 32-cell stage causes abnormal cement gland formation. This approach shows the complex involvement of Xwnt pathways in embryonic patterning and offers a rapid method for the functional analysis of both maternal and early zygotic gene products in *Xenopus*.** © 2000 Academic Press

Key Words: **β-catenin; antisense; Xwnt pathway; dorsal signaling; morpholino.**

INTRODUCTION

A classical question in vertebrate developmental biology is the degree to which early development is controlled by maternally stored mRNAs and proteins or is regulated predominantly by zygotic genes. In *Xenopus* embryos, there are several examples of maternal RNAs and proteins that have been shown to be essential for the establishment of embryonic cell lineages. These include the maternally localized transcription factor VegT, which regulates the formation of the endoderm and mesoderm germ layers (Zhang *et al.*, 1998; Kofron *et al.*, 1999); the protein Xdazl, which is necessary for primordial germ cell differentiation (Houston *et al.*, 2000); and the cytoplasmic protein β-catenin, required for dorsal tissue formation (Heasman *et al.*, 1994). In the last case, embryos lacking maternal β-catenin develop without dorsal structures, including the notochord, somites, and neural tube, and also lack heads and tails. Evidence from studies on several systems shows that β-catenin is a transducer of Wnt signals and acts by binding to TCF/LEF family transcription factors, transporting them to nuclei and activating target genes (Van de Wetering *et al.*, 1997; Vonica *et al.*, 2000). In *Xenopus*, immunostaining studies indicate that β-catenin accumulates in nuclei on the dorsal side of the embryo during the cleavage and blastula stages (Schneider *et al.*, 1996; Larabell *et al.*, 1997). Yeast two-hybrid studies further show that β-catenin binds to the *Xenopus* TCF family member XTcf3 and activates zygotic transcription at the midblastula transition. Two direct target genes have been described, *siamois* and *Xnr3* (Carnac *et al.*, 1996; Brannon *et al.*, 1997; Kessler, 1997), and other likely targets include *gsc* (Watabe *et al.*, 1995).

Although these results suggest a rather simple dorsal signaling pathway dependent on dorsally nuclearly localized β-catenin, several problems remain unsolved. First, depleting β-catenin causes a loss of both dorsoventral and anterior–posterior axes, and it is not clear whether this represents one or more β-catenin-containing pathways. Second, the simple model does not explain in which cells of the embryo β-catenin–XTcf3 acts. When zygotic transcription begins, genes downstream of the Xwnt pathway are expressed in specific and different patterns, not easily

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TABLE 1
Primers Used and Light Cycling Conditions for Their Use with Light Cycler PCR

PCR primer pair	Origin	Sequence	Melt temp (°C)	Annealing temp (°C)/ time (s)	Extension temp (°C)/ time (s)	Acquisition temp (°C)/ time (s)
Xwnt8	Ding *et al.*, 1998	U: 5-CTG ATG CCT TCA GTT CTG TGG-3 D: 5-CTA CCT GTT TGC ATT GCT CGC-3	95	58/6	72/14	85/3
Chordin	XMMR	U: 5-AAC TGC CAG GAC TGG ATG GT-3 D: 5-GGC AGG ATT TAG AGT TGC TTC-3	95	55/5	72/12	81/3
Cerberus	New	U: 5-GCT TGC AAA ACC TTG CCC TT-3 D: 5-CTG ATG GAA CAG AGA TCT TG-3	95	60/5	72/20	81/3
XHex	Chang *et al.*, 2000	U: 5-AAC AGC GCA TCT AAT GGG AC-3 D: 5-CCT TTC CGC TTG TGC AGA GG-3	95	60/5	72/13	87/3
Xnr-3	Kofron *et al.*, 1999	U: 5-CTT CTG CAC TAG ATT CTG-3 D: 5-CAG CTT CTG GCC AAG ACT-3	95	57/5	72/10	79/3
Siamois	New	U: 5-CTG TCC TAC AAG AGA CTC TG-3 D: 5-TGT TGA CTG CAG ACT GTT GA-3	95	55/5	72/16	81/3
ODC	New	U: 5-GCC ATT GTG AAG ACT CTC TCC ATT C-3 D: 5-TTC GGG TGA TTC CTT GCC AC-3	95	55/5	72/12	83/3
Otx-2	New	U: 5-CGG GAT GGA TTT GTT GCA-3 D: 5-TTG AAC CAG ACC TGG ACT-3	95	59/5	72/8	81/3
Xag-1	New	U: 5-CTG ACT GTC CGA TCA GAC-3 D: 5-GAG TTG CTT CTC TGG CAT-3	95	59/5	72/9	83/3
Xnrp-1	Lamb *et al.*, 1995	U: 5-GGG TTT CTT GGA ACA AGC-3 D: 5-ACT GTG CAG GAA CAC AAG-3	95	55/5	72/14	84/3

explained by the hypothesis of a single dorsal signaling event. Third, it is not clear how Xwnt signals that pass during the cleavage stages are "remembered" until zygotic transcription starts.

One reason for our lack of understanding of patterning at the cleavage and blastula stage is that it has been impossible to carry out direct loss-of-function studies on individual areas of the embryo. The phosphorothioate oligos used in β-catenin, VegT and Xdazl antisense experiments are toxic in fertilized eggs, and so the experiments involved injecting oligos into defolliculated oocytes. These were typically cultured 24 h after injection, to allow the oligo and target mRNA to break down, and then fertilized using the host-transfer technique (Zuck *et al.*, 1998). This technique has not been widely used because it is difficult and because it is not applicable to zygotic mRNAs. Here we have tested an alternative loss-of-function approach using a "morpholino" oligo (Summerton and Weller, 1997) directed against *β-catenin* mRNA. This acts, not by degrading RNA, but by preventing translation of protein. When such a morpholino oligo (MO) complementary to *β-catenin* mRNA is injected into early embryos, it is not toxic, it depletes β-catenin protein in the embryo and is effective until the neurula stage. By targeting the oligo to different cleavage blastomeres, we block β-catenin activity in different areas and at different times. Dorsal vegetal injection at the 2- and 4-cell stage blocks dorsal axis formation, at the 8-cell stage it blocks head formation, and A-tier injection at the 32-cell stage causes abnormal cement gland formation. This provides evidence for three separable roles for β-catenin in regulating head formation (via *Xhex* and *cerberus*), dorsoventral axis formation (via *siamois* and *Xnr3* and negatively regulating *Xwnt 8*), and suppressing neural tissue formation (repressing *Otx2*, *XAG1*, and *Xnr3*). This approach shows the complex involvement of Xwnt pathways in embryonic patterning and offers a rapid loss-of-function approach for the analysis of both maternal and early zygotic gene function in specific areas of the *Xenopus* embryo.

MATERIALS AND METHODS

Embryos

Eggs were stripped and fertilized using a sperm suspension and embryos were maintained in 0.1× MMR. For injections of MO, embryos were dejellied and transferred to 1% Ficoll in 0.5× MMR at the 1-cell stage. MO was injected into blastomeres as described in the text. The dorsoventral axis was recognized at the 4-cell stage, by the pigmentation differences of dorsal animal and ventral animal cells. Only those with obvious dorsoventral differences and regular cleavage planes were selected for injection. For 32-cell-stage injections, such embryos were selected at the 4-cell stage and carefully examined until the 32-cell stage, so that the four tiers of cells could be accurately recognized. Individual embryos in which this was clear were selected for 32-cell stage injection. Embryos were washed thoroughly and returned to 0.1× MMR during the blastula stage.

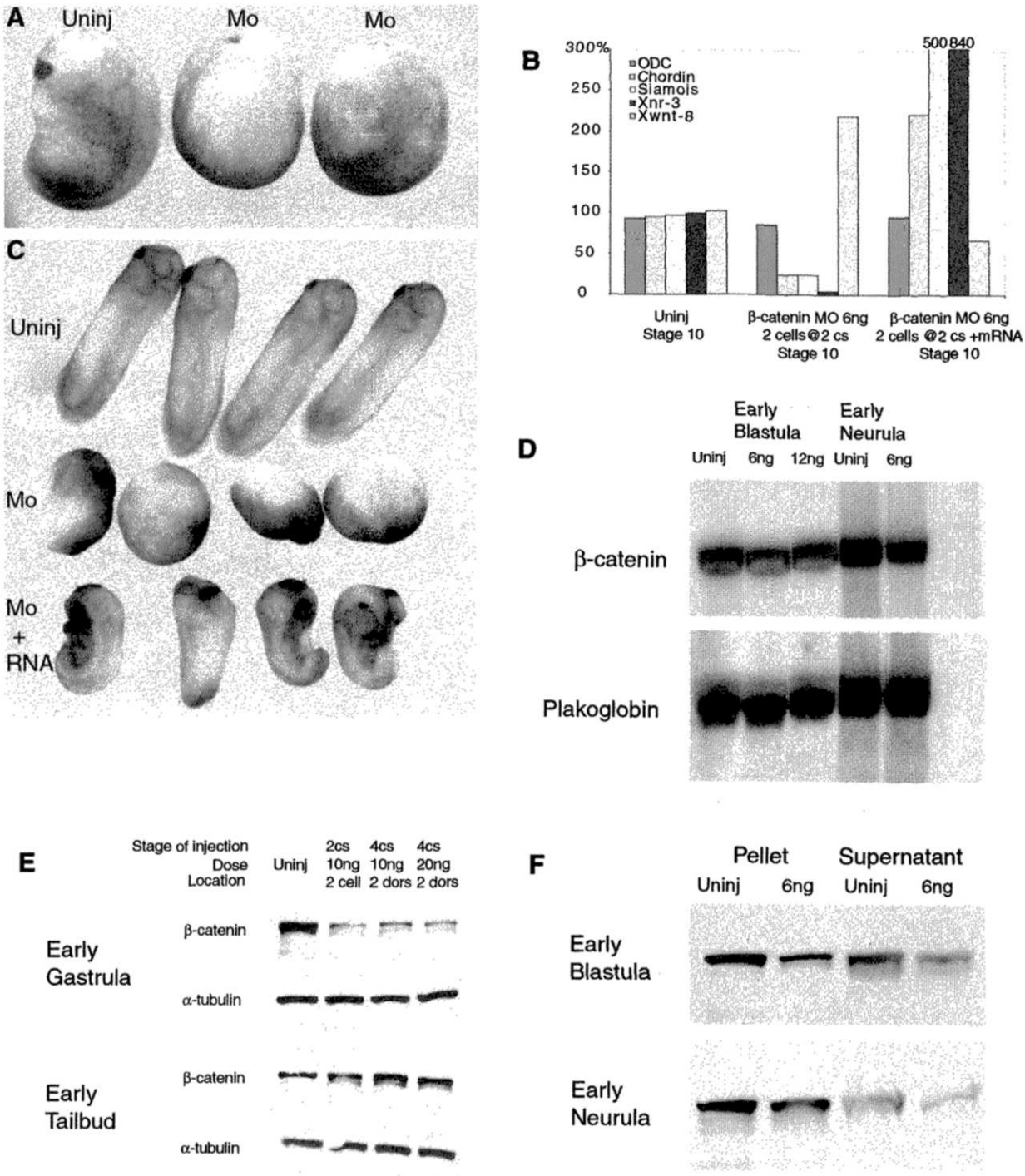


FIG. 1. The effectiveness of β-catenin MO in causing ventralization and reducing β-catenin protein levels. 6 ng of morpholino oligo (Mo) was injected into two cells (3 ng each) at the two-cell stage. This caused a ventralized phenotype compared to uninjected (Uninj) controls. (B) Real-time RT PCR analysis of sibling embryos of those in A, for molecular markers of dorsal development *siamois*, *Xnr3*, and *chordin* and the ventral marker *Xwnt 8*. The level of expression of these markers is compared in control, MO injected, and MO + *β-catenin* mRNA at the early gastrula stage (see Materials and Methods for details). While dorsal markers were reduced by MO injection, the ventral marker was increased. When MO was injected together with *β-catenin* mRNA, the ventralized phenotype was reversed and dorsal markers were hyperexpressed. (C) When MO was injected together with 500 pg *β-catenin* mRNA, the ventralized phenotype was reversed and dorsalized embryos resulted (Mo + RNA). (D) Northern blotting shows that the morpholino oligo injected at 6 and 12 ng did not degrade *β-catenin*

Oligos and mRNAs

The antisense oligodeoxynucleotide used was a 25-mer morpholino oligo (Gene Tools LLC) with the base composition 5′TTTCAACCGTTTCCAAAGAACCAGG 3′. Morpholino oligos have substitutions of the riboside moieties with nitrogen-containing morpholine moieties and are phosphorodiamidate linked (Summerton and Weller, 1997). Oligos were resuspended in sterile, filtered water and injected in doses of 3–20 ng per embryo. Capped *β-catenin* mRNA was synthesized using the mMessage mMachine kit (Ambion) from pSP36T vector linearized with *EcoRI* and transcribed by SP6, ethanol precipitated, and resuspended in sterile distilled water for injection.

Northern Blot Analysis

Embryo RNA was extracted as described (Gurdon *et al.*, 1985). Electrophoresis and Northern blotting were performed as described (Hopwood *et al.*, 1989) using two embryo equivalents per lane. The probe was synthesized by random priming of the excised insert of *β-catenin* (*Nco*I and *Spe*I). Blots were stripped and rehybridized with a probe for *plakoglobin* as a loading control.

Analysis of Gene Expression Using Real Time RT-PCR

Total RNA was prepared from oocytes and embryos using the proteinase K method and treated with RNase-free DNase 1 (10 μg/μl; Boehringer Mannheim) prior to cDNA synthesis. cDNA was synthesized from 0.5 to 1.0 μg RNA according to Zhang *et al.* (1998) in a volume of 20 μl. After reverse transcription, 1 μl 0.5 M EDTA, 30 μl H_2O, 1 μl glycogen (20 μg/μl), and 10 μl 5 M ammonium acetate were added to each RT reaction. Each sample was extracted once with phenol/chloroform/isoamyl alcohol (25/24/1) and precipitated overnight at −20°C with 2.5 vol 100% EtOH. Samples were centrifuged at 4°C, 16,000*g* for 15 min, washed with 70% EtOH, dried in a speed vac, and resuspended in 150 μl H_2O per $\frac{1}{6}$ embryo equivalent of RNA used for cDNA synthesis.

RT-PCR was carried out using a LightCycler System (Roche), which allows amplification and detection (by fluorescence) in the same tube, using a kinetic approach. LightCycler PCRs were set up in microcapillary tubes using 5 μl cDNA with 5 μl of a 2× SYBR Green I (Roche Molecular Biochemicals; Wittwer *et al.*, 1997) master mix containing upstream and downstream PCR primers, $MgCl_2$, and SYBR green. The final concentrations of the reaction components were 1.0 μM each primer, 2.5 μM $MgCl_2$, and 1× SYBR green master mix. The primers used and cycling conditions are listed in Table 1. In order to compare expression levels of depleted and rescued embryos relative to controls, a dilution series of uninjected control cDNA was made and assayed in each LightCycler run. Undiluted control cDNA was taken as 100%, 1:1 cDNA:H_2O as 50%, and 1:10 cDNA:H_2O as 10%. In experiments in which multiple embryonic stages were examined, the dilution series was used from cDNA of the uninjected control stage of development predicted to give the highest expression of the gene product being amplified. These values were entered as concentration standards in the LightCycler sample input screen. Other controls included in each run were −RT and water blanks. These were negative in all cases but not included in the figures for lack of space.

After each elongation phase, the fluorescence of SYBR green (a dye that binds double-stranded DNA giving a fluorescent signal proportional to the DNA concentration) was measured at a temperature 1°C below the determined melting point for the PCR product being analyzed. This excluded primer dimers, which melt at a lower temperature, from the measurement. The fluorescence level is thus quantitated in real time, allowing the detection and display of the log-linear phase of amplification as it happens. LightCycler quantification software v1.2 was used to compare amplification in experimental samples during the log-linear phase to the standard curve from the dilution series of control cDNA. The comparisons are displayed as histograms. For each primer pair used, we optimized conditions so that melting curve analysis showed a single melting peak after amplification, indicating a specific product.

Western Blot Analysis

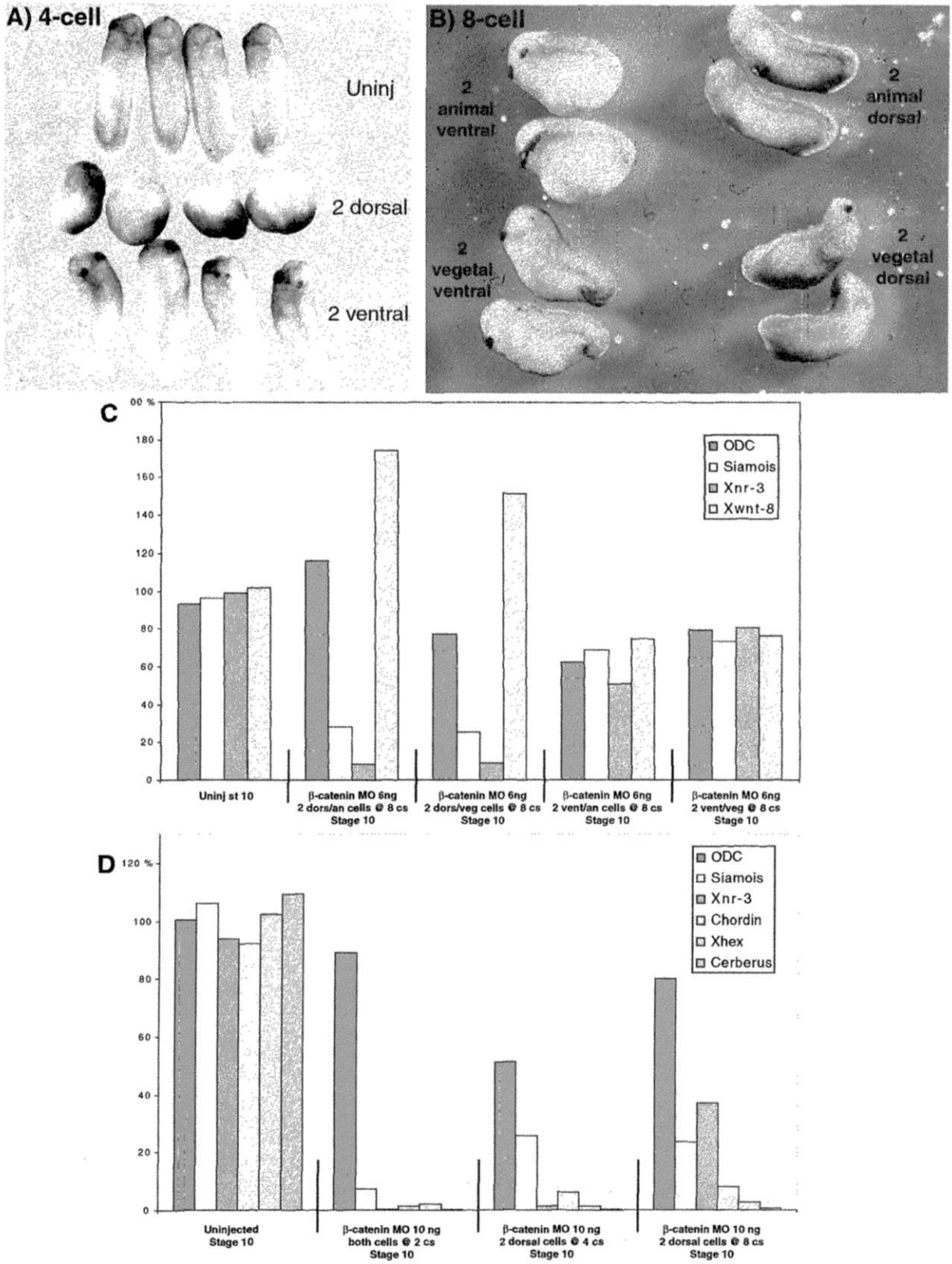
Protein extractions were carried out by the freon extraction method for total protein, to remove yolk from protein lysates (Heasman *et al.*, 1994), or as described previously for membrane and supernatant fractions (Kofron *et al.*, 1997). Polyclonal β-catenin antibody was used at a dilution of 1:1000 and was a gift from Dr. P. McCrea. Blots were stripped by standard protocols and probed with anti-α-tubulin antibody (N356 Amersham).

RESULTS AND DISCUSSION

A Morpholino Oligo against β-Catenin Is Effective When Injected into Cleaving Embryos

We studied the effectiveness and toxicity of the β-catenin MO by injecting it into embryos (two cells at the two-cell stage). Six to twenty nanograms of MO caused the same ventralized phenotype as seen previously using 1 ng of chimeric phosphorothioate oligo injected into oocytes (Heasman *et al.*, 1994) and had no toxic effects on development. Ventralized embryos typically lacked heads, tails, and dorsoventral axes (Fig. 1A; 41/41 cases). This was confirmed using real-time PCR for molecular markers of dorsal development, *siamois* and *Xnr3*, both of which have

mRNA at the blastula or neurula stages. A probe for *plakoglobin* mRNA was used as a loading control. (E) The degree of depletion of β-catenin protein at early gastrula (stage 10) and tailbud (stage 24) stages is compared in a Western blot. When the oligo was injected into both cells of the two-cell stage embryo, or into the two dorsal cells of the four-cell stage embryo, it caused a significant reduction in total β-catenin protein at the early gastrula stage but not at the tailbud stage. α-Tubulin is used as a loading control. cs, cell stage; dors, dorsal injection. (F) MO caused a greater depletion of β-catenin protein at the neurula stage than at the blastula stage and reduced protein levels in both membrane and cytosolic fractions. Sibling embryos of those shown in A were Western blotted in pellet and supernatant fractions at the stages shown. Significant reduction of protein levels occurred in both fractions compared to the uninjected control.



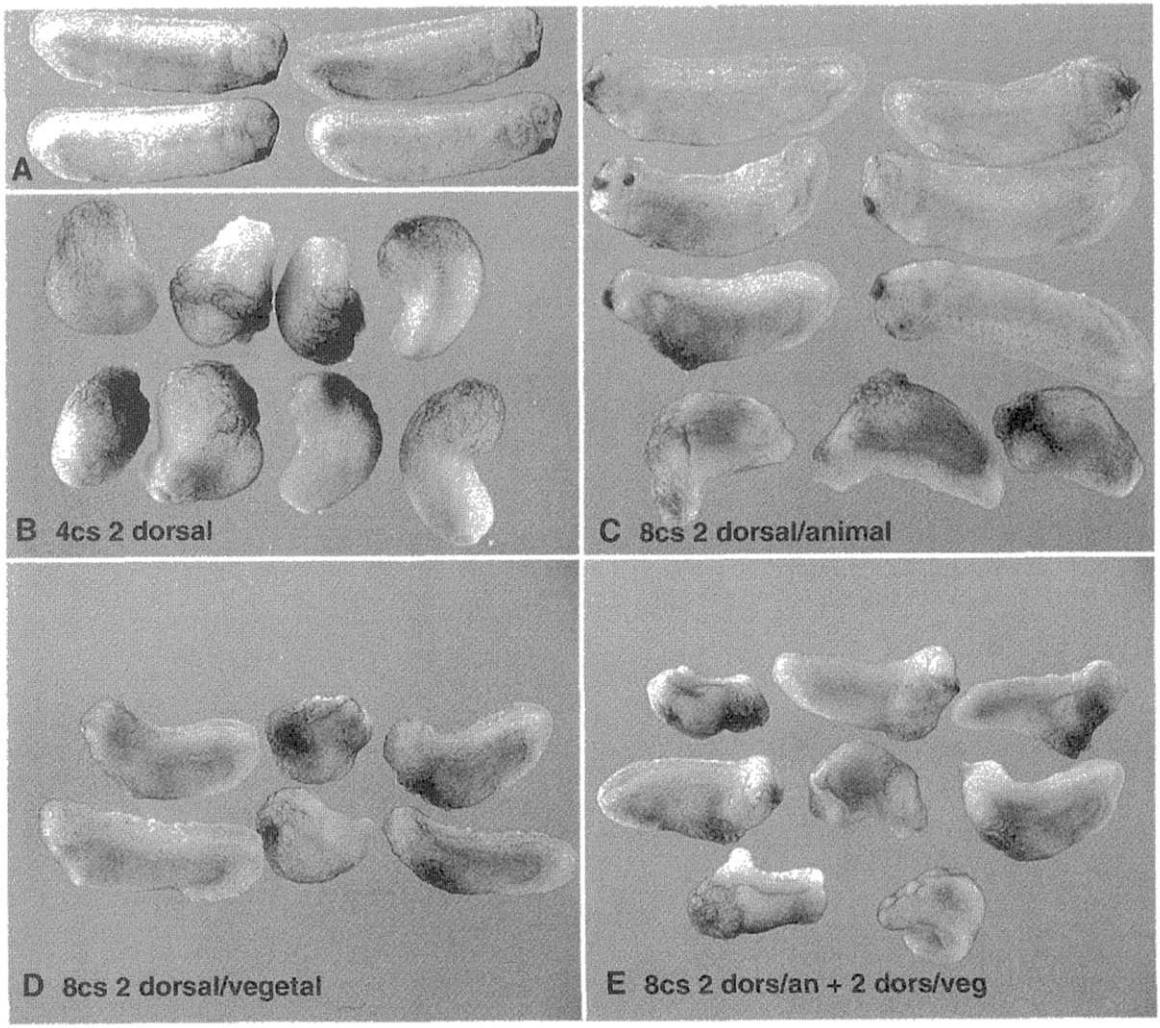


FIG. 3. The phenotype caused by β-catenin depletion at the eight-cell stage is less extreme than that caused by injection of the same dose in the same volume at the four-cell stage. 10 ng of oligo was injected either into two dorsal cells at the four-cell stage or into four dorsal cells at the eight-cell stage. Injection into two dorsal cells at the four-cell stage caused complete ventralization in all cases (B); injections into two dorsal animal (C), two dorsal vegetal (D), or two dorsal animal + two dorsal vegetal cells (E), at the eight-cell stage, caused mainly reduced heads. cs, cell stage; dors/an, dorsal animal cells; dors/veg, dorsal vegetal cells.

FIG. 2. β-Catenin requirement differs for dorsoventral and anterior patterning. 6 ng of MO was injected at the four-cell stage either into two dorsal or into two ventral blastomeres. While injections into dorsal blastomeres caused ventralization, injection into ventral blastomeres caused ventralization in few cases. (B) 6 ng of MO was injected into two cells of each of the four quadrants of the embryo. Injection into dorsal but not ventral quadrants caused embryos to develop with reduced heads but did not prevent the formation of dorsoventral axes and tail structures. (C) Sibling embryos of those shown in B were analyzed by RT-PCR and compared for the degree of expression of dorsal markers at the early gastrula stage (stage 10). *Siamois* and *Xnr3* were unaffected when ventral animal or vegetal cells were targeted, but reduced after dorsal injections of MO. *Xwnt8* was increased after dorsal MO injection. ODC, *ornithine decarboxylase* used as a control for the efficiency of RNA synthesis in each sample. (D) One batch of embryos was injected with 10 ng of MO at the two-, four-, or eight-cell stage, targeting the dorsal vegetal area. Embryos were analyzed at the early gastrula stage for dorsal markers *siamois, Xnr3,* and *chordin* and head markers *Xhex* and *cerberus*. ODC was used as control for the efficiency of RNA synthesis in each sample. Embryos injected with MO at the two-cell stage have both dorsal and head markers depleted to below 10% of the uninjected control level at the gastrula stage. Eight-cell stage injections result in head marker *Xhex* and *cerberus* depletion, while *siamois, chordin,* and *Xnr3* are partially expressed.

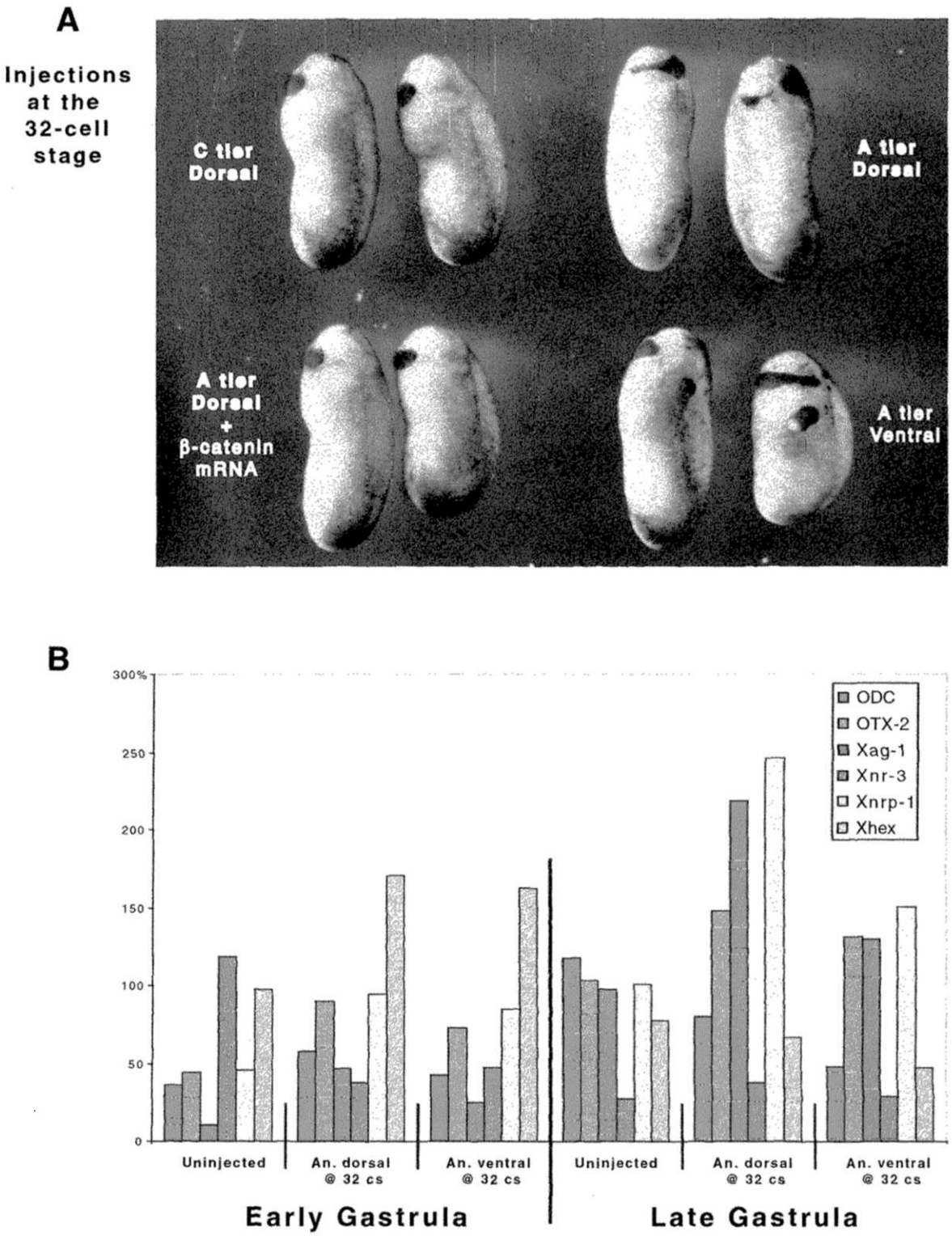


FIG. 4. Depletion of β-catenin in animal cells at the 32-cell stage causes excessive anterior neural development. (A) Pairs of embryos at the tailbud stage that received 15–20 ng of MO. MO was injected into 32-cell stage embryos, targeting either four dorsal (upper right) or ventral (lower right) animal cells (A tier) or four C-tier cells (upper left). The lower left pair received 15 ng of oligo plus 100 pg of *β-catenin* mRNA. Injection of the C tier has no effect on cement gland development, while dorsal or ventral animal cell injection results in embryos

been shown to be direct targets of β-catenin/XTcf3 (Carnac *et al.*, 1996; Brannon *et al.*, 1997; Kessler, 1997; McKendry *et al.*, 1997). These markers were much reduced in MO-injected embryos, while the ventral marker *Xwnt 8* was increased compared to uninjected controls (Fig. 1B). To test for the specificity of the phenotype, *β-catenin* mRNA was injected together with MO. The synthetic mRNA used here does not contain the portion of the 5′UTR of the *β-catenin* sequence which the MO targets and thus does not act simply by binding to the MO. Injection of this mRNA together with MO resulted in a reversal of the phenotype and excessive dorsal development (Figs. 1B and 1C). We established by Northern blotting that the oligo did not degrade *β-catenin* mRNA (Fig. 1D) and compared the degree of depletion of β-catenin protein at different stages in Western blots (Figs. 1E and 1F). When the oligo was injected into both cells of the two-cell stage embryo, or into the two dorsal cells of the four-cell stage embryo, it caused a significant reduction in total β-catenin protein at the early gastrula stage but not at the tailbud stage (Fig. 1E). MO caused a greater depletion of β-catenin protein at the neurula stage than at the blastula stage and also reduced protein levels in both membrane and cytosolic fractions (Fig. 1F).

Thus MOs behave differently from phosphorothioate oligos when injected into embryos. They are nontoxic, can be injected after fertilization, and in this case prevent the translation of protein in both cytosolic and membrane-associated fractions through the neurula stage. In comparison, phosphorothioate oligos complementary to β-catenin cannot be injected postfertilization because they are toxic and unstable and do not deplete zygotic protein or membrane-associated maternal protein (Kofron *et al.*, 1997). The most problematic aspect of using MOs to target novel uncharacterized mRNAs is the problem of specificity. For antisense oligos that act by degrading mRNA, specificity can be tested directly, by reintroducing synthetic RNA, after the endogenous RNA has degraded, and testing its ability to rescue the phenotype. For MOs, the reintroduction of synthetic target mRNA is a rigorous test only if the rescuing mRNA lacks the target region of the MO (as here, where the MO targets a region in the 5′UTR which is replaced by globin flanking sequences in the pSP36T vector). An alternative control would be to use third-base modification of the rescuing mRNA, so that it is no longer recognized by the MO (Raats *et al.*, 1997).

β-Catenin Is Required for Dorsoventral and Anterior Signaling over Different Defined Periods in the Early Embryo

Many studies have suggested that the key asymmetry that establishes the dorsal axis in *Xenopus* embryos is the activity of an Xwnt pathway restricted to the dorsal cells of the cleavage to blastula stage embryo (Yuge *et al.*, 1990; Gimlich, 1996; Kikkawa *et al.*, 1996; Sakai, 1996; Schneider *et al.*, 1996; Kageura, 1997; Larabell *et al.*, 1997; Marikawa *et al.*, 1997). Precisely where in the cleavage stage embryo β-catenin is required is uncertain. Since MOs are not toxic when injected into cleaving embryos, we could ask directly, for the first time, where in the embryo β-catenin acts for dorsal axis formation. First, we injected MO at the four-cell stage either into two dorsal or into two ventral blastomeres. While injections into dorsal blastomeres caused ventralization (17/17 cases), injection into ventral blastomeres caused ventralization in few cases (3/17) (Fig. 2A). The experiment was repeated with the same result (7/7 dorsal injections ventralized; 3/20 ventral injections ventralized).

To localize the area of activity further, the experiment was repeated at the eight-cell stage, targeting two cells of each of the four quadrants of the embryo. Again injection into ventral cells had little effect on dorsal axis formation (4/30 ventral vegetal injections ventralized; Fig. 2B). This was confirmed by examining the expression of dorsal markers in early gastrula stage sibling embryos. *Siamois* and *Xnr3* were unaffected when ventral animal or vegetal cells were targeted (Fig. 2C).

Injection of MO into dorsal animal and vegetal cells affected axis formation (Fig. 2B) and reduced dorsal markers by RT-PCR analysis (Fig. 2C). However, the phenotype caused by injection at the eight-cell stage was not identical to the completely ventralized phenotype caused by injections at the two- and four-cell stages. These embryos typically had dorsoventral axes and tail structures but lacked heads (e.g., 21/24 headless and 3/24 completely ventralized by dorsal vegetal injections).

To confirm this biochemically, one batch of embryos was injected with 10 ng of MO at the two-, four-, or eight-cell stage, targeting the dorsal vegetal area. Embryos were analyzed at the early gastrula stage for dorsal and head markers. *Xhex* and *cerberus* are expressed in the dorsal vegetal area of wild-type early gastrulae and have been

with normal dorsal axes but enlarged and ectopic cement glands. The phenotype is rescued by the co-injection of *β-catenin* mRNA along with MO. (B) RT PCR analysis of sibling embryos of those shown in A analyzed at the gastrula stages for anterior neural markers *Xag1* and *OTX2* and the general neural marker, *Xnrp1*. Notice that *Xnrp1* is expressed maternally as well as zygotically and increases in level due to new zygotic transcription by the late gastrula stage, in uninjected controls. Embryos injected in dorsal or ventral animal cells with MO at the 32-cell stage overexpress all three markers compared to controls. Injections into these cells also causes a 40–50% reduction in the level of expression of *Xnr3* at the early gastrula stage and enhances *Xhex* expression. ODC, *ornithine decarboxylase* is used as a control for the efficiency of mRNA synthesis in each sample.

shown to endow this area with anterior signaling properties (Bouwmeester *et al.*, 1996; Jones *et al.*, 1999; Zorn, 1999). Embryos injected with MO at the two-cell stage had both dorsal and head markers depleted to below 10% of the uninjected control level at the gastrula stage. At the eight-cell stage, *Xhex* and *cerberus* remained off, while *siamois* and *Xnr3* were partially expressed (Fig. 2D). This indicates that β-catenin depletion at the eight-cell stage in dorsal vegetal cells affects anterior signaling more effectively than dorsoventral and tail patterning.

To determine whether the different phenotype at the eight-cell stage compared to the four-cell stage was simply the result of targeting the oligo to a smaller volume, 10 ng of oligo was injected into an equal volume at the two stages, i.e., either into two dorsal cells at the four-cell stage or into four dorsal cells at the eight-cell stage. While injection into two dorsal cells at the four-cell stage caused complete ventralization in all cases (14/14; Fig. 3), injections into two dorsal animal + two dorsal vegetal cells at the eight-cell stage caused complete ventralization in 4/14 cases and reduced heads in 9/14 cases (Fig. 3). Further increasing the concentration of MO to 20 ng into dorsal animal and vegetal cells did not cause further ventralization (2/11 cases ventralized; data not shown).

These results confirm that the dorsalizing Xwnt pathway is active on the dorsal side of the embryo and indicate that there is a sensitive period from the one- to the four-cell stage when oligo injection can completely interrupt signaling. From the eight-cell stage onward, anterior patterning is affected by MO injection. There are several possible interpretations of these data. There may be two separable pathways in the early embryo, one determining dorsoventral and posterior pattern (defined here by *siamois* and *Xnr3*) and the second regulating anterior structures (defined by *Xhex* and *cerberus*), both dependent on β-catenin activity, but acting at different times or at different concentrations of β-catenin. This view is supported by the recent finding that *Xhex*, which is expressed in animal cells of the blastula stage embryo and dorsal vegetal cells at the gastrula stage, endows vegetal cells with anterior signaling properties, independent of neural and mesoderm differentiation (Jones *et al.*, 1999). Alternatively, there may be one pathway activated by β-catenin, regulating different genes according to different nuclear concentrations or affinities of the β-catenin/XTcf3 complex. When MO is injected at the eight-cell stage there may be sufficient protein translated before it acts to ensure activation of dorsoventral and tail regulating genes, but insufficient protein to activate *Xhex*.

β-Catenin Is Required in Progeny of the A-Tier Cells for Patterning of the Nervous System

Those embryos injected with MO on the ventral side at the 4- and 8-cell stages formed heads but often showed obvious cement gland abnormalities (Fig. 2B). To localize the site responsible for this abnormality, we injected MO into 32-cell stage embryos, targeting either 4 dorsal or ventral animal cells of the A tier or 4 B-, C-, or vegetal, D-tier cells (Wylie *et al.*, 1996).

Injection of 4 cells (15–20 ng total) of B, C, or D tier at this stage had no effect on cement gland development (43/43 cases; data not shown). Embryos injected at the 32-cell stage into dorsal (A1 and 2) or ventral (A3 and 4) animal cells developed normal dorsal axes but had abnormal head development. They formed enlarged and/or ectopic cement glands (dorsal animal, 19/21 cases; ventral animal, 18/21 cases) (Fig. 4A). The phenotype was specific since it was rescued by the co-injection of *β-catenin* mRNA along with MO (12/13 cases had no cement gland abnormality). To confirm that the phenotype was one of excessive anterior neural development, the early anterior neural markers *Xag1* and *OTX2*, and the general neural marker *Xnrp1*, were examined in early gastrula stage embryos. These markers were overexpressed in embryos injected with MO at the 32-cell stage compared to uninjected controls (Fig. 4B). Injections into dorsal animal cells at the 32-cell stage did not visibly affect dorsoventral axis formation (Fig. 4), or the expression of the dorsal marker *siamois* (data not shown), but did cause a 40–50% reduction in the level of expression of *Xnr3* (Fig. 4B).

Thus blocking β-catenin activity at the 2 and 4 cell stages prevents the formation of neural tissue (Heasman *et al.*, 1994 and Fig. 1A) and prevents the expression of the head marker *Xhex* (Fig. 2D). In contrast, blocking its activity in animal cap cells at the 32 cell stage (A-tier progeny) enhances neural tissue formation and also *Xhex* expression (Fig. 4B). The simplest explanation of this is that the effect of the 32-cell stage injection is on a different pathway also dependent on β-catenin, responsible for downregulating *otx*, *XAG1*, *Xnrp1*, and *Xnr3* and upregulating *Xhex*. It will be of interest to determine whether XTcf3 or zygotic LEF1 interacts with β-catenin in this repressive pathway.

These results are in agreement with another study in which overexpression of the negative regulator of Xwnt pathways, *GSK3β*, caused ectopic cement gland formation (Itoh *et al.*, 1995). Since *Xnr3* is known to be regulated by *XTcf3*, the downregulation of *Xnr3* by the loss of β-catenin in animal cells is consistent with a role for *XTcf3* in this pathway (McKendry *et al.*, 1997). The results are not in agreement with overexpression studies in animal cells, in which *Xnr3* was shown to cause neural induction (McKendry *et al.*, 1997). The results shown here suggest the opposite, that this Xdsh/GSK/β-catenin pathway actively suppresses neural tissue formation (Hansen *et al.*, 1997).

This work establishes the usefulness of MOs for studying the roles of both maternal and zygotic gene products in *Xenopus*. Since there is a detailed 32-cell stage fate map of *Xenopus*, and individual cells can be injected with relative ease, it is possible to target MOs to specific tissues of the developing embryo and to cause a sustained loss of protein function. This provides a very simple and quick method for studying gene function in vertebrate embryos.

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