Embryonic Axis Induction by the Armadillo Repeat Domain of β-catenin: Evidence for Intracellular Signaling

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Abstract. β-catenin was identified as a cytoplasmic cadherin-associated protein required for cadherin adhesive function (Nagafuchi, A., and M. Takeichi. 1989. *Cell Regul.* 1:37–44; Ozawa, M., H. Baribault, and R. Kemler. 1989. *EMBO [Eur. Mol. Biol. Organ.] J.* 8:1711–1717). Subsequently, it was found to be the vertebrate homologue of the *Drosophila* segment polarity gene product Armadillo (McCrea, P. D., C. W. Turck, and B. Gumbiner. 1991. *Science [Wash. DC].* 254:1359–1361; Peifer, M., and E. Wieschaus. 1990. *Cell.* 63:1167–1178). Also, antibody perturbation experiments implicated β-catenin in axial patterning of the early *Xenopus* embryo (McCrea, P. D., W. M. Brieher, and B. M. Gumbiner. 1993. *J. Cell Biol.* 123:477–484). Here we report that overexpression of β-catenin in the ventral side of the early *Xenopus* embryo, by injection of synthetic β-catenin mRNA, induces the formation of a complete secondary body axis. Furthermore, an analysis of β-catenin deletion constructs demonstrates that the internal armadillo repeat region is both necessary and sufficient to induce axis duplication. This region interacts with C-cadherin and with the APC tumor suppressor protein, but not with α-catenin, that requires the amino-terminal region of β-catenin to bind to the complex. Since α-catenin is required for cadherin-mediated adhesion, the armadillo repeat region alone probably cannot promote cell adhesion, making it unlikely that β-catenin induces axis duplication by increasing cell adhesion. We propose, rather, that β-catenin acts in this circumstance as an intracellular signaling molecule.

Subcellular fractionation demonstrated that all of the β-catenin constructs that contain the armadillo repeat domain were present in both the soluble cytosolic and the membrane fraction. Immunofluorescence staining confirmed the plasma membrane and cytoplasmic localization of the constructs containing the armadillo repeat region, but revealed that they also accumulate in the nucleus, especially the construct containing only the armadillo repeat domain. These findings and the β-catenin protein interaction data offer several intriguing possibilities for the site of action or the protein targets of β-catenin signaling activity.

β-CATENIN is a 92-kD cytoplasmic protein that was first identified through its association with the cytoplasmic tail of cadherin cell adhesion molecules (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989; McCrea and Gumbiner, 1991). Subsequent cDNA cloning revealed that β-catenin is the vertebrate homologue of the *Drosophila* segment polarity gene product Armadillo (McCrea et al., 1991). Both proteins also appear to be closely related to plakoglobin, another protein associated with adherens junctions and desmosomes (Peifer and Wieschaus, 1990). Interestingly, these proteins contain conserved repeated regions, termed armadillo repeats, that are found in a range of other proteins (Peifer et al., 1994a),

including the tumor suppressor gene product APC (Rubinfeld et al., 1993; Su et al., 1993).

The biological function of β-catenin is incompletely understood. It forms a complex with cadherins and α-catenin and is probably necessary for cadherin-mediated cell–cell adhesion. Mutant cadherin molecules that lack the catenin-binding region of their cytoplasmic tails are poorly adhesive (Nagafuchi and Takeichi, 1988). Several findings suggest that β-catenin may play a regulatory role in cadherin-mediated adhesion (Matsuyoshi et al., 1992; Bradley et al., 1993; Nagafuchi et al., 1994). β-catenin may also participate in cellular functions other than cell adhesion. For example, in *Drosophila*, Armadillo mediates a step in a signaling cascade that is initiated by Wingless, a wnt protein homologue, and leads to the establishment of the anterior-posterior polarity of the fly segments (Peifer and Wieschaus, 1990). The role of Armadillo in *wingless* signaling is genetically separable from its constitutive role in supporting adhesive

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junctions (Peifer et al., 1993). Also, β-catenin has been shown to form complexes with the APC tumor suppressor protein that do not contain cadherins (Rubinfeld et al., 1993; Su et al., 1993). The function of the APC/catenin complex and its involvement in cell signaling or cell adhesion is not yet known.

Recently we presented evidence that implicated β-catenin in embryonic axis patterning in vertebrates (McCrea et al., 1993). Microinjection of Fab fragments directed against β-catenin into early cleaving *Xenopus* embryos induced a secondary body axis. Interestingly, similar phenotypes have been obtained by expression of secreted proteins of the wnt family (Sokol et al., 1991). These results, therefore, implicate β-catenin and wnts in a signaling pathway regulating pattern formation in vertebrates just like Armadillo and Wingless in *Drosophila*. However, it was not clear how the injected antibodies acted to affect β-catenin function. In the present study we have sought to further investigate the role of β-catenin in embryonic axis patterning by microinjection of β-catenin mRNA. A set of deletion mutants was then tested to determine the region of β-catenin responsible for axis induction. These mutants have also been used to characterize the domains responsible for the molecular interactions with cadherins, α-catenin and APC. Finally, to better understand how ectopic β-catenin signals in *Xenopus* embryos, the intracellular distributions of the β-catenin variants were determined by cell fractionation and immunofluorescence microscopy.

Materials and Methods

Plasmid Construction

Plasmid pT1 was constructed by insertion of the coding region of β-catenin downstream of the SP6 promoter in the vector pSP36T, a variant of pSP64 (Amaya et al., 1991). pT2, pT3, pT4, and pT5 were derived from pT1 (Fig. 1). Two restriction sites, XhoI and XbaI were used to construct the deletion mutants. pT2 has the coding sequence between the XhoI site and the stop codon (634 amino acids, lacking the NH₂ terminus 147 amino acids). The region between the XhoI site and the XbaI site was deleted in pT3. This plasmid encodes 289 amino acids including the NH₂ terminus, half of the 1st armadillo repeat, half of the 12th armadillo repeat, and the COOH terminus. pT4 has the 647 amino acid coding sequence from the first codon to the XhoI site. pT5 has the coding sequence from the XhoI site to the XbaI site and encodes the second half of the 1st armadillo repeat, 10 armadillo repeats, and the NH2-terminal half of the 12th armadillo repeat. All constructs have a seven-amino acid hemagglutinin (HA)[1] sequence (DVPDYAS) (Wilson et al., 1984) in the COOH terminus as an epitope tag sequence.

mRNA Injection and Treatment of Embryos

Capped RNAs were synthesized *in vitro* using SP6 RNA polymerase (Promega Corp., Madison, WI). mRNAs encoding full-length or deleted β-catenin were dissolved in DPEC treated water at a concentration of 0.15 mg/ml for injection. The equatorial region of a single prospective ventral blastomere of the 4-cell stage embryo was injected with mRNA in volumes of ~20 nl (~3 ng). Embryos were allowed to develop at room temperature in 0.1 × Marc's modified ringers (MMR). MMR was 100 mM NaCl, 5 mM Hepes-NaOH, pH 7.8, 2 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂, and 0.1 mM EDTA. Embryos were scored for duplicated axis induction according to the criteria of the presence of two neural folds at stage 18. Stages were determined according to Nieuwkoop and Faber (1967).
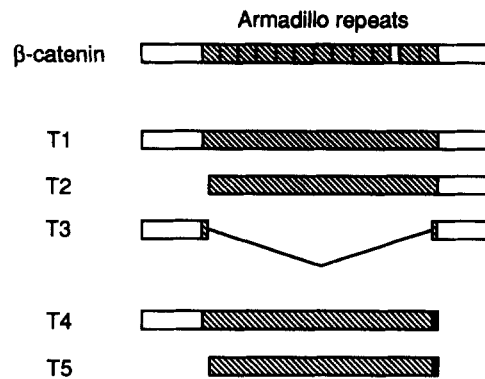
Figure 1. Structure of mutant β-catenin polypeptides. β-catenin has 12 armadillo repeats (*crosshatched region*) in the middle of the coding region. T1 is full-length β-catenin. T2, T3, and T4 encode mutant β-catenins with deletions of the NH₂-terminal 147 amino acids, 493 amino acids of the repeat region, and the COOH-terminal 134 amino acids, respectively. T5 encodes a mutant lacking both the NH₂-terminal 147 amino acids and the COOH-terminal 134 amino acids. T1-T5 also contain the 7-amino acid hemagglutinin epitope tag sequence (*solid square*) at the COOH terminus.

Immunoprecipitation and Western Blot

3 ng of β-catenin mRNAs were injected into 4- to 8-cell stage embryos as described above. To analyze the interaction between β-catenin and APC, 1.5 ng of mRNA encoding a deletion mutant of APC found in human tumors (Rubinfeld et al., 1993) was coinjected with 1.5 ng β-catenin mRNA. Cell lysates were prepared at stages 7-10. For immunoprecipitation of C-cadherin/catenin complexes, 15 embryos were extracted in 500 μl solution A (150 mM NaCl, 10 mM Hepes-NaOH, pH 7.4, 2 mM EDTA, 0.02% NaN₃) containing 1% NP-40. For coimmunoprecipitation of APC/catenin complexes, 25 embryos were extracted in 500 μl 0.5% NP-40 solution A. Immunoprecipitations were performed as described (Choi et al., 1990) using the anti-HA mAb 12CA5 (Babco, Richmond, CA). Immunoprecipitates were separated by SDS-PAGE (5% acrylamide for APC detection, 7.5% acrylamide for detection of catenins and cadherins), and the various proteins were detected by immunoblot, using the ECL Western blot development protocol (Amersham Corp., Arlington Heights, IL). The antibodies were: anti-α-catenin polyclonal antibody (pAb) raised against synthetic peptides corresponding to the residues 890 to 906 of the mouse α-catenin; anti-NH₂ terminus β-catenin pAb (McCrea et al., 1993); anti-COOH terminus β-catenin pAb; anti-C-cadherin pAb (Brieher and Gumbiner, 1994); anti-human APC pAb α2 (Rubinfeld et al., 1993); and anti-HA mAb 12CA5. The anti-COOH terminus β-catenin polyclonal antibody was raised against a synthetic peptide corresponding to the residues 701 to 721 of *Xenopus* β-catenin. Unless specified, anti-β-catenin pAb indicates the antibody recognizing the NH₂ terminus.

Cell Fractionation

1.5 ng of mRNA encoding one of the β-catenin mutants were injected into both blastomeres at the 2-cell stage. At stage 6, 20 embryos were homogenized in 1 ml ice-cold 250 mM sucrose, 10 mM Hepes-NaOH, 2 mM MgCl₂, 1 mM EGTA, 0.5 mM EDTA, pH 7.4, and spun at 750 g for 5 min at 4°C in an Eppendorf centrifuge (Netheler & Hinz GmbH, Hamburg, Germany) to remove most of the yolk platelets, pigment granules and nuclei. The supernatants were then spun at 100,000 g for 1 h at 4°C in a tabletop ultracentrifuge TL-100 (Beckman Instruments Inc., Fullerton, CA) using a 100.3 rotor. Soluble and sedimentable fractions were collected as the supernatant and pellet fractions, respectively.

Immunofluorescence Microscopy

Indirect immunofluorescence on frozen sections was performed as previously described (Fagotto and Gumbiner, 1994). The anti-HA mouse ascites fluid 12CA5, the anti-β-catenin pAb and a control nonimmune rabbit serum

were used at a 1/1,000 dilution. FITC-conjugated goat anti–mouse and anti–rabbit secondary antibodies (Molecular Probes, Inc., Eugene, OR) were diluted to 25 and 50 mg/ml, respectively. Sections were also stained with 20 mg/ml of the DNA binding dye, Hoechst 33342 (Molecular Probes) to stain nuclei. All sections were counterstained with 0.1% Eriochrome Black (Aldrich Chemical Co., Milwaukee, WI) to mask the autofluorescence of the yolk. The sections were examined under an Axioplan microscope (Carl Zeiss, Inc., Thornwood, NY) using a 40× objective (NA 0.9) and a fluorescein filter block with a long pass emission filter, that visualized both the fluorescein signal (green) and the yolk staining (red). Photomicrophotographs were taken using Ektachrome 400 ASA films (Eastman Kodak Co., Rochester, NY, USA). Double exposures of the same fields were taken to identify the Hoechst nuclear staining, using a UV filter block, and the red yolk counterstaining, visualized with a rhodamine filter block.

Confocal Microscopy

The sections were observed with a laser confocal microscope (MRC 600; Bio-Rad/Analytical Instr. Group, Cambridge, MA) on an inverted microscope (Zeiss Axiovert; Zeiss, Oberkochen, Germany), using a 63× Zeiss apo-plan oil immersion objective (NA 1.4). The slit apertures were set to obtain an optical thickness of 1–2 μm. The optical set was composed of two filter blocks, the first containing a 514-nm narrow band (10 nm wide) excitation filter and a 527-nm dichroic reflector; the second containing a 565-nm dichroic reflector and two emission filters, respectively, a 540-nm band (30 nm wide) pass filter and a 600-nm long pass filter, allowing collection simultaneously of a green and a red fluorescence image on two different channels. Images obtained on the green channel were photographed from the computer screen using TMax 100 ASA film (Eastman Kodak Co.).

Results

β-catenin mRNA Injection Induces Axis Duplication in the Xenopus Embryo

Synthetic mRNA encoding β-catenin with a COOH-terminal HA epitope tag was injected into early cleaving embryos (Fig. 1, T1) and protein expression confirmed by Western blotting (see Fig. 4). When 3 ng of synthetic full-length β-catenin mRNA were injected into the ventral marginal area of a presumptive ventral blastomere at the 4-cell stage, the embryos developed into two-headed tadpoles. The two heads were joined to one trunk and tail. They were frequently perfectly symmetrical and complete, including two separate cement glands and two pairs of eyes (Fig. 2 b). During neurulation, the phenotype was easily observed as a duplication of the darkly pigmented lines of the forming neural tubes (Fig. 2 a). Duplicated neural tubes were observed in ~80% of the injected embryos (Table I). The same result was obtained, with a similar efficiency, using mRNA coding β-catenin without the epitope tag (data not shown). In contrast, less than 30% of the embryos injected dorsally formed duplicated neural tubes, and of these very few gave rise to double-headed tadpoles. Given the degree of uncertainty in assessing prospective ventral and dorsal cells, it is possible
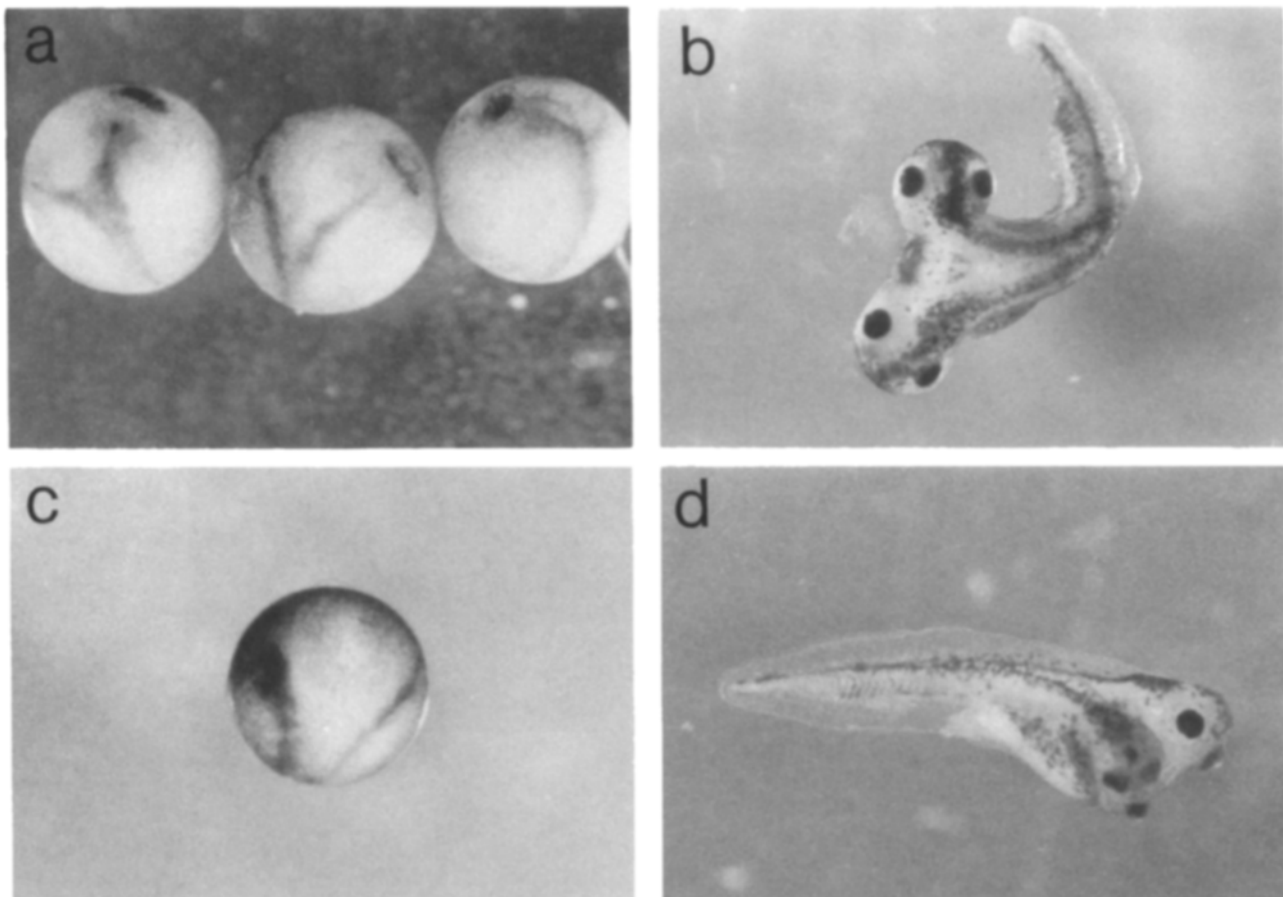
Figure 2. Double-axis phenotype induced by β-catenin and armadillo repeat region. Embryos were injected at the 4-cell stage with full-length β-catenin T1 (*a* and *b*) or with the armadillo repeat region alone T5 (*c* and *d*) as described in Materials and Methods. At neurula stage (*a* and *c*), two neural tubes fused posteriorly are visible. Tadpoles exhibit perfect axis duplication, including two eye pairs and two cement glands (*b* and *d*).

Table I. Frequency of Axis Duplication by β-catenin and Deletion Mutants

	Percentage duplicated axis	Total injected	Duplicated axis embryos
	%		
T1	78	197	154
T2	81	229	187
T3	0	217	0
T4	83	247	204
T5	85	218	186
β-gal	0	30	0

3 ng of mRNA were injected into a presumptive ventral blastomere of 4-cell stage embryos. The presence of duplicated axis was determined visually at neurula stage (stage 18). Embryos from 4 or 5 independent experiments were scored and combined to calculate the efficiency of duplicated axis induction. β-galactosidase (β-gal) mRNA injection data shown is from one experiment.

Figure 3. Interaction of endogenous β-catenin and APC. Immunoprecipitates using anti-APC pAb and anti-β-catenin pAb were blotted for either β-catenin or APC. β-catenin/APC complexes were detected in both immunoprecipitation experiments.

that the dorsally injected β-catenin sometimes acts outside of the normal dorsal region. Control injection of β-galactosidase mRNA did not induce a secondary axis (Table I). These data show that increasing the level of β-catenin polypeptide in the ventral side of the early cleaving embryo induces the formation of a secondary body axis.

The Armadillo Repeat Region of β-catenin Is Essential for the Induction of a Secondary Axis

To determine which region of β-catenin is essential for the induction of a secondary axis, we constructed plasmids encoding a series of β-catenin deletion mutants all containing the COOH-terminal HA epitope tag sequence (Fig. 1, T2–T5). β-catenin consists of three domains: an NH$_2$-terminal domain, a COOH-terminal domain, and a middle portion that is composed of the 12 armadillo repeats. T2, T3, and T4 encode mutant β-catenins with deletions of the NH$_2$-terminal, of the repeat region, and of the COOH-terminal domains, respectively. T5 encodes only the repeat region and lacks both the NH$_2$-terminal and the COOH-terminal domains.

Embryos injected with each of the β-catenin mutants containing the internal armadillo repeat region (T2, T4, or T5 mRNAs) developed secondary axes (Table I). These double-axis embryos were identical to those obtained with the full-length β-catenin mRNA, T1. On the other hand, embryos injected with T3 mRNA, that contains the NH$_2$-terminal and COOH-terminal domains but lacks the repeats, developed normally and never showed axis duplications. Remarkably, the T5 mRNA encoding only 10 complete armadillo repeats induced secondary axes with a similar efficiency as the full-length β-catenin mRNA (Table I). These embryos displayed two symmetrical neural tubes at neurula stage, and developed into two-headed embryos identical to the twin embryos caused by the injection of T1 mRNA (Fig. 2, c and d). Thus, the armadillo repeat domain of β-catenin alone appears to be able to induce axis duplication in Xenopus.

Interactions of β-catenin Mutants with C-cadherin, α-catenin, and APC

In addition to the cadherins and α-catenin, β-catenin can interact with the tumor suppressor protein APC (Rubinfeld et
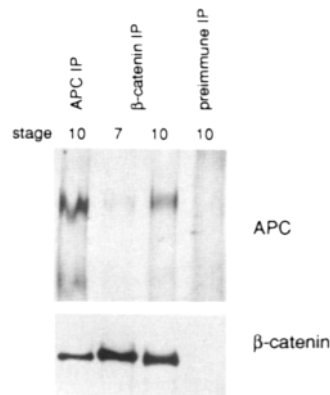
al., 1993; Su et al., 1993). C-cadherin/β-catenin/α-catenin complexes have already been characterized in the cleaving Xenopus embryo (McCrea and Gumbiner, 1993). To examine whether β-catenin is also associated with APC in early Xenopus embryos, a coimmunoprecipitation analysis of stage 7 (early blastula) and stage 10 (early gastrula) lysates was performed using an anti-β-catenin polyclonal antibody. Immunoprecipitates were blotted with anti-human APC pAb and anti-β-catenin pAb. At both stages, APC was coimmunoprecipitated with β-catenin (Fig. 3). The coimmunoprecipitation was specific, because no signal was detected using the preimmune serum. The signal was low at stage 7, but stronger at stage 10. Therefore, similar to cultured cells, the early Xenopus embryo also contains β-catenin/APC complexes.

The association of the various β-catenin deletion mutants with C-cadherin, α-catenin, and APC was analyzed at stage 8 by immunoprecipitation using the anti-HA epitope tag. Fig. 4 a shows that all the β-catenin variants were expressed in the injected embryos, and that they migrate on SDS-PAGE in agreement with their expected size. In early Xenopus embryos (blastulae and gastrulae), it is difficult to detect endogenous APC/β-catenin complexes (see Fig. 3). Therefore, in order to obtain unambiguous results on the ability of β-catenin mutants to associate with APC, the β-catenin mutant mRNAs were coinjected with an mRNA coding for the NH$_2$-terminal half of human APC, which includes the β-catenin association domain (Rubinfeld et al., 1993; Su et al., 1993). Full-length β-catenin (T1) and all the mutants containing the armadillo repeat region (T2, T4, and T5) coimmunoprecipitated with both C-cadherin and the APC fragment (Fig. 4 b). In contrast, T3, lacking the armadillo repeat region, did not associate with either of these molecules. Therefore, the armadillo repeat region is required for the association of β-catenin with both APC and C-cadherin. Interestingly, only those mutants that could interact with APC and C-cadherin induced axis duplication in the embryo.

All of the β-catenin constructs containing an intact NH$_2$ terminus (T1, T3, and T4) were found to associate with α-catenin (Fig. 4 b). Conversely, T2 and T5, which both lack the NH$_2$ terminus, did not bind to α-catenin. Nevertheless, T2 and T5 form complexes with C-cadherin that lacked α-catenin. These results show: (a) that the interaction of β-catenin with α-catenin occurs via the NH$_2$-terminal region of β-catenin, and (b) that β-catenin is required for the association of α-catenin with cadherins.

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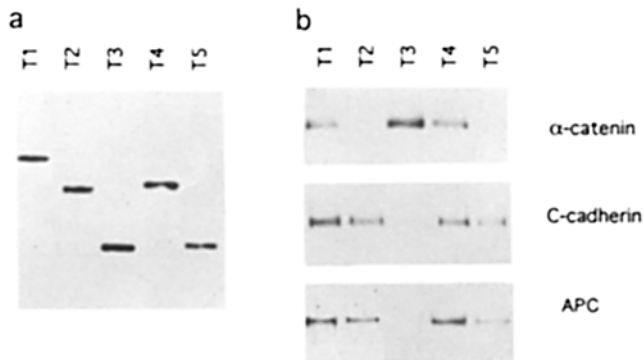
Figure 4. Protein interaction of the β-catenin variants. (a) Proteins expressed from injected mRNAs were detected at stage 7 by the anti-HA mAb, 12CA5. (b) HA-tagged β-catenin mutant proteins were immunoprecipitated at stage 8 with the 12CA5 mAb, and coimmunoprecipitating proteins were detected by immunoblotting with anti-α-catenin pAb, anti-C-cadherin pAb and anti-human APC pAb.

Distribution of β-catenin Mutants into Soluble and Particulate Pools

In *Drosophila*, the accumulation of soluble cytosolic Armadillo protein, under the control of Wingless, correlates with the signaling activity of Armadillo (Peifer et al., 1991). For comparison, therefore, the distribution of exogenous β-catenin into soluble and particulate pools in embryos injected with the various mutants was analyzed by differential centrifugation. C-cadherin was used as a control for complete sedimentation of membrane proteins. In normal, uninjected embryos, most of the β-catenin is sedimentable, and the amount of soluble β-catenin is estimated to be less than 10% of the total (Fagotto and Gumbiner, 1994; and Fig. 5).
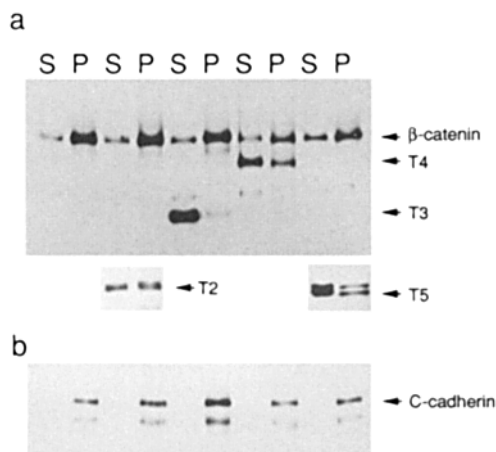
Figure 5. Subcellular distribution of the endogenous and mutant exogenous β-catenins in mRNA injected embryos. *S*, soluble fraction; *P*, pellet. Fractions from the equivalent of 1 embryo (stage 7) were blotted with the anti-NH₂ terminus β-catenin pAb (*a, top*). To determine the distribution of T2 and T5 (*a, bottom*), an anti–COOH-terminal β-catenin pAb (for *T2*) and the anti-HA mAb 12CA5 (for *T5*) were used for blotting. Fractions were also blotted with anti-C-cadherin pAb (*b*) to check for complete sedimentation of membrane proteins.

In embryos injected with mutants causing axis duplication (T2, T4, T5), about one third to one half of each of the mutant proteins was recovered in the particulate fraction (Fig. 5). T3 was entirely soluble, consistent with its lack of interaction with C-cadherin or APC. Since the exogenous β-catenin mutants that can induce axis duplication were distributed in both the soluble and the particulate fractions, these data do not allow us to conclude whether their presence in the soluble cytoplasmic pool correlates with inductive activity.

The distribution of endogenous β-catenin was also examined to determine whether it was altered by expression of the β-catenin mutants. Because a significant fraction of the exogenous proteins is sedimentable, it is conceivable that the mutants could compete with endogenous β-catenin for membrane binding. However, no consistent detectable change in the soluble/particulate ratio of endogenous β-catenin could be detected in injected embryos. These data suggest that there is an excess of β-catenin binding sites in the membrane, a finding that is confirmed by immunofluorescence microscopy (see below). Therefore the exogenous β-catenin mutants do not compete significantly with endogenous β-catenin for membrane binding.

Localization of Exogenous β-catenin and β-catenin Mutants by Indirect Immunostaining

The intracellular localization of the various mutants was further studied by immunofluorescence on frozen sections of stage 10 embryos. The exogenous β-catenin variants were detected using the anti-HA mAb 12CA5. Exogenously expressed full-length β-catenin T1 accumulated along the plasma membrane, but was also abundant throughout the cytoplasm (Fig. 6). Cytoplasmic staining was strong around the nuclei, probably because this area is yolk platelet-free. However, β-catenin was also regularly detected at high levels inside the nuclei (Fig. 6, *arrowheads*). Colocalization with chromatin (Fig. 6, Hoechst staining) allowed us to clearly distinguish nuclear from perinuclear staining. The staining was specific, because it was not detected in uninjected blastomeres (Fig. 6, *arrows*), or in embryos injected with β-galactosidase mRNA. T4, which differs from the full-length protein by the lack of the COOH terminus, but still is able to bind C-cadherin, α-catenin, and APC, displayed a very similar pattern, except that the plasma membrane was somewhat less intensely stained compared to the cytoplasm. Nuclei were again clearly stained. The signal for T2 was much lower than for the other mutants. However, this mutant, which lacks the NH₂ terminus, was clearly present both at the plasma membrane and in the nucleus (not shown).

Most notably, T5, containing the armadillo repeat region alone, accumulated primarily in the nuclei. A low signal was still detected along the cell periphery, consistent with the detection of T5 complexes with C-cadherin. In contrast, T3, which lacks most of the repeat region, accumulated mostly in the cytoplasm. This mutant appeared to be relatively excluded from the nuclei. The nuclear localization of T1, T4, and T5 was confirmed by confocal microscopy (Fig. 7, *arrowheads*). The dotted staining corresponded to a typical chromatin pattern. Again, T3 had only a very weak signal in the nuclei (*arrowheads*), lower than in the cytoplasm. Thus all forms of β-catenin that have the repeat domain and
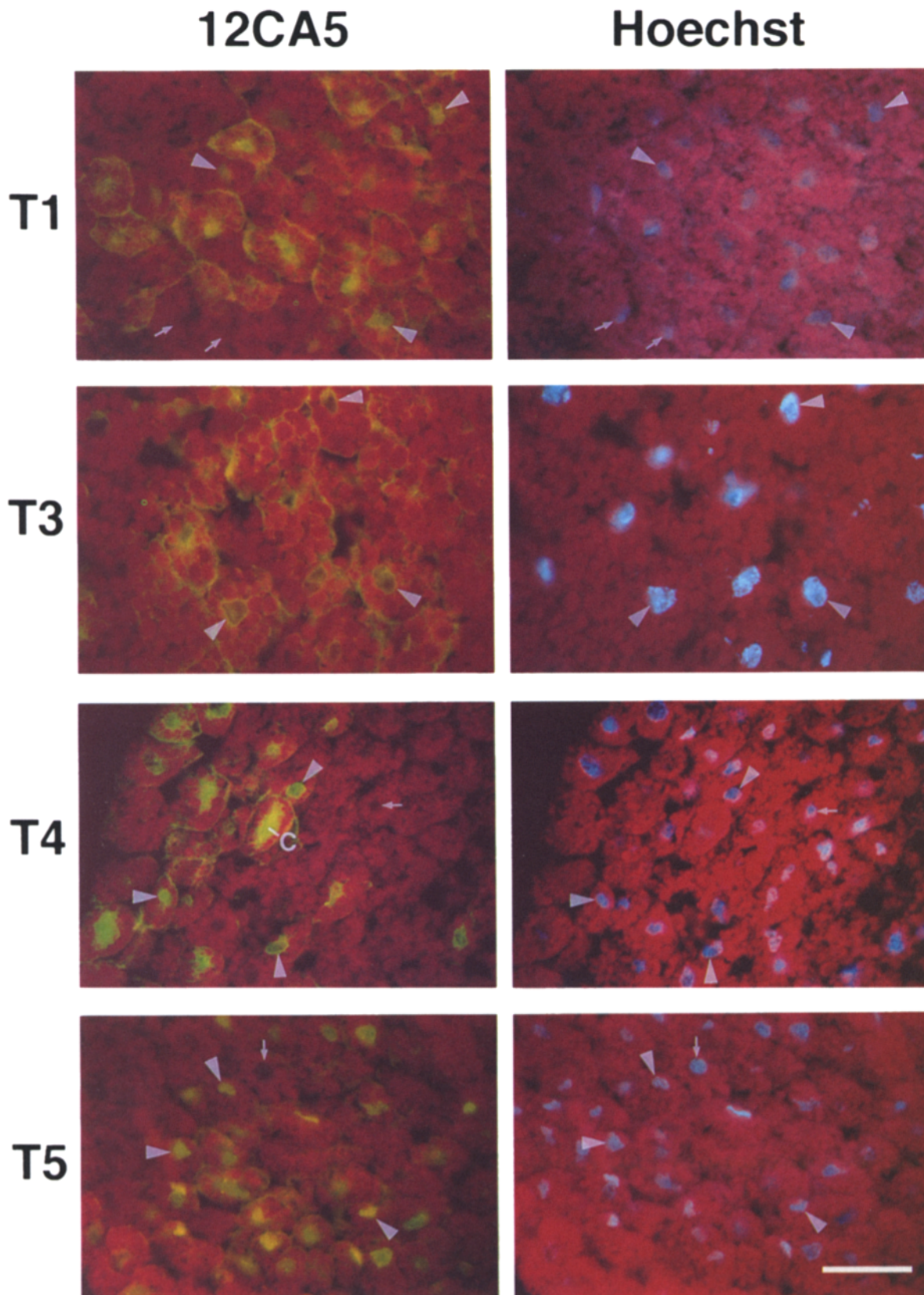
Figure 6. Immunofluorescence localization of exogenous β-catenin variants expressed in *Xenopus* embryos. Frozen sections (stage 10) of mRNA injected embryos were immunolabeled using 12CA5 and a FITC conjugate (*left column, green*). Nuclei were stained with Hoechst (*right column, blue*), and the yolk was counterstained with Eriochrome Black (*red*). *Arrowheads*, nuclei of cells expressing β-catenin mutants; *small arrows*, nuclei of unlabeled cells; *C*, bright cytoplasmic staining in mitotic cells. Note that the plasma membranes are strongly labeled for T1 and T4, but weakly for T5. Nuclear β-catenin staining is found for T1, T4, and is particularly intense for T5, but not for T3. T3 displays the strongest cytoplasmic staining. Bar, 50 μm.
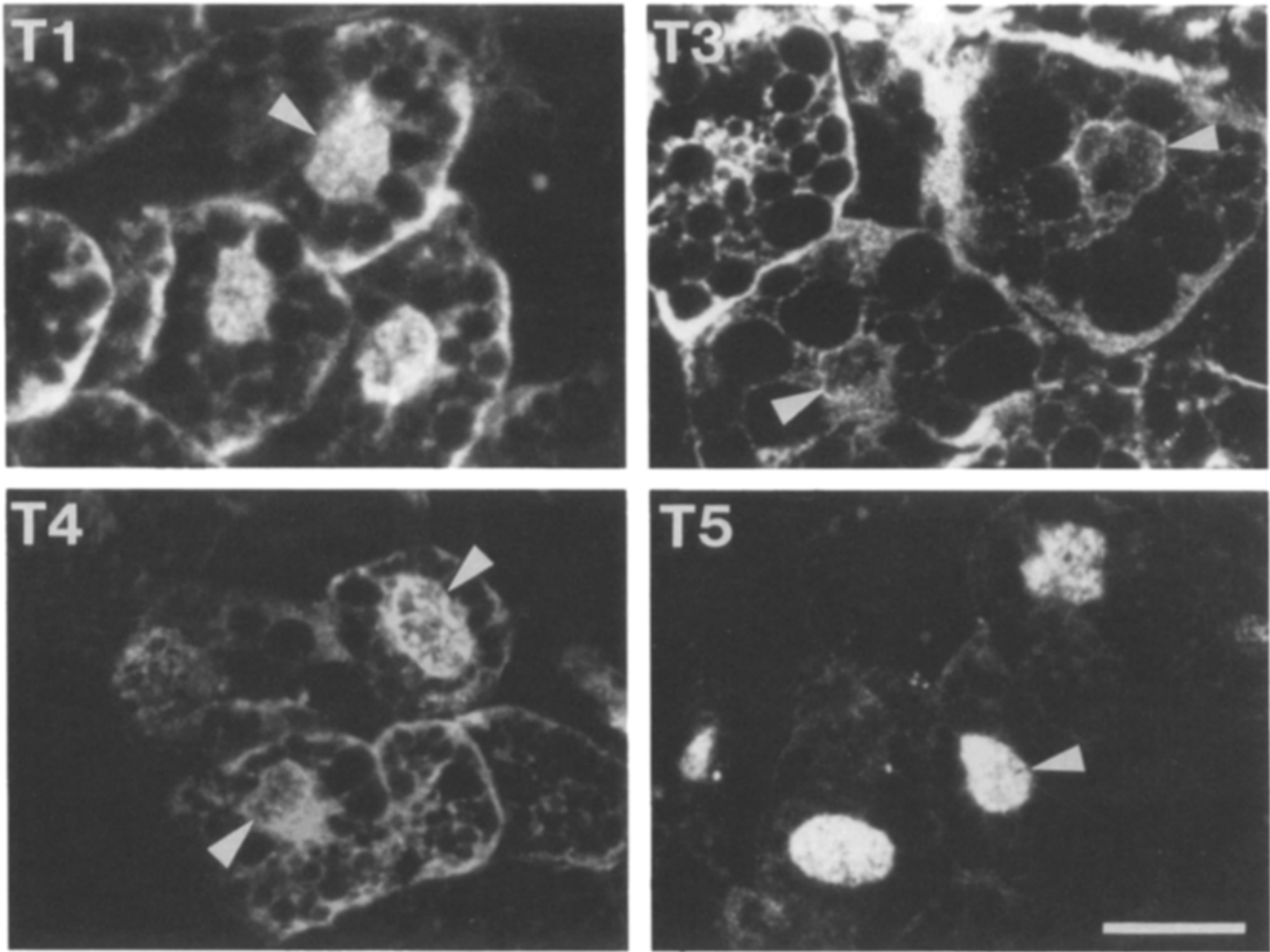
Figure 7. Confocal immunofluorescence microscopy of cells expressing β-catenin variants. Sections from stage 10 embryos were labeled with the 12CA5 mAb. *Arrowheads*, nuclei. Bar, 20 μm.

induce axis duplication accumulate in nuclei of *Xenopus* embryos. They all also accumulate in the plasma membrane and in the cytoplasm to varying extents.

To compare the exogenous protein with the distribution of the endogenous protein, consecutive sections of embryos injected with the full-length β-catenin were stained with the anti-HA mAb, 12CA5 (Fig. 8 *a*) and with the anti-β-catenin pAb (Fig. 8 *b*). Cells overexpressing β-catenin were readily detected with the anti-β-catenin pAb because of a very high signal compared to the endogenous staining in neighboring cells. As expected, β-catenin was detected in the nuclei of cells overexpressing it. In particular, the plasma membrane appeared much brighter than in control, uninjected cells. This observation confirms that the plasma membrane of blastomeres contains many free binding sites for β-catenin, as suggested by the cell fractionation experiments.

As previously observed in our studies on the distribution of endogenous β-catenin in *Xenopus* embryos (Fagotto and Gumbiner 1994), we could not reliably detect endogenous β-catenin in the nuclei of uninjected embryos above background levels (Fig. 8 *b*). If endogenous β-catenin ever accumulates in nuclei, it either does at low levels below the limit of detection, or else it does so in a regulated or restricted way.

Discussion

The present study confirms and extends our discovery of the involvement of β-catenin in the induction of dorsoanterior structures in the *Xenopus* embryo, which originally resulted from microinjecting anti-β-catenin Fab fragments into the cleaving embryo (McCrea et al., 1993). We now find that simple overexpression of β-catenin in the ventral side of the embryo, by mRNA injection, consistently leads to a duplication of the embryonic axis. Axis duplication resulting from expression of certain proteins, like the growth factors wnt and noggin, in the ventral region of the embryo probably mimics an endogenous inductive signal normally present in the dorsal region of the early embryo (McMahon and Moon, 1989; Smith and Harland, 1992). β-catenin also seems to mimic an inductive signal, because β-catenin mRNA injections can rescue the primary dorso-ventral axis in embryos that have been ventralized by UV-irradiation (Guger and Gumbiner, manuscript in preparation). Furthermore, reducing the levels of maternal β-catenin protein in the early embryo by antisense oligonucleotide directed degradation of β-catenin mRNA in the oocyte produces ventralized embryos lacking dorsal tissues (Heasman et al., 1994). Together these findings demonstrate that β-catenin is an essen-
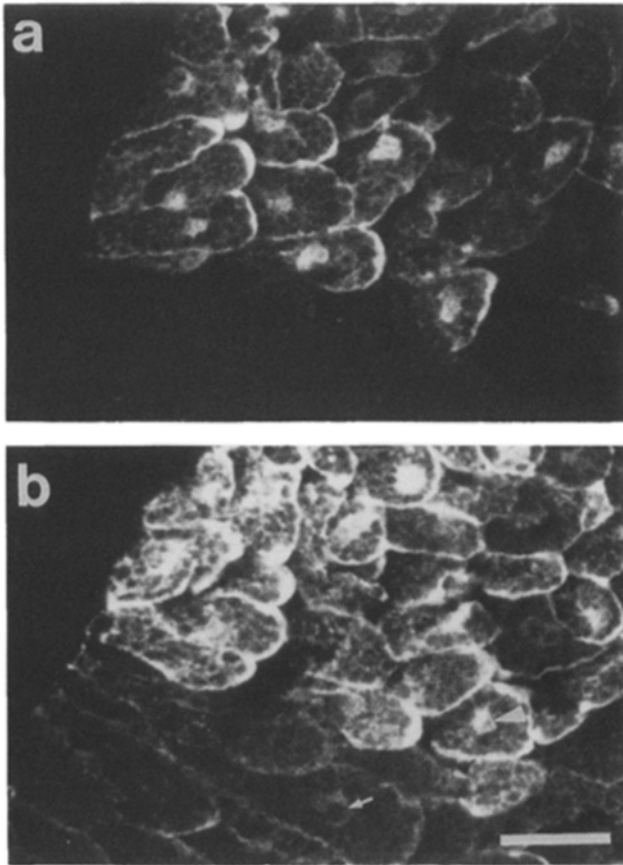
Figure 8. Comparison of the distributions of endogenous and exogenous β-catenin in mRNA injected embryo by immunofluorescence microscopy. Confocal microscope images of two consecutive sections (stage 10) of T1 mRNA injected embryos that were labeled with either the anti-HA mAb 12CA5 (*a*) or with the anti-β-catenin pAb (*b*). Note that the cells expressing T1 have a much stronger signal for total β-catenin, including staining along the plasma membrane. Labeling could be detected in nuclei of injected cells (*arrowhead*) but not of uninjected cells (*arrow*). Bar, 50 μm.

tial component of an inductive signaling pathway that leads to the establishment of the body axis in *Xenopus*.

In a previous study (McCrea et al., 1993) injection of anti-β-catenin Fab fragments into early *Xenopus* embryos caused a similar, although less complete, axis duplication phenotype as the β-catenin mRNA injections described here. At that time it was unclear whether the Fab fragments acted to inhibit or to activate β-catenin with respect to its function in axis induction. No perturbation of the cadherin–catenin complex was detected. In light of the present findings, the simplest (but not the only) interpretation is that the Fab fragments acted to activate the β-catenin in the axis induction pathway. The mechanism of activation by Fab binding is not known, but one possibility is that the antibody binding induces an activating conformation in the β-catenin protein. Alternatively, the Fab fragments could cause a redistribution of the β-catenin into different cellular pools, making it available to express its inducing activity. Nevertheless, overexpression by synthetic mRNA injection provides a more powerful and more easily interpretable approach to analyzing the function of β-catenin in axis induction, because it

provides an opportunity to directly manipulate its structure and level of expression.

Our present analysis of β-catenin deletion constructs demonstrates that the internal armadillo repeat region of β-catenin is both necessary and sufficient for induction of a secondary axis in *Xenopus*. This is the most highly conserved region between β-catenin and Armadillo, and the two proteins may act in *Xenopus* and *Drosophila* pattern formation through similar mechanisms. The function of the repeat region of *Drosophila* Armadillo is not completely clear. The originally isolated zygotic alleles that gave rise to mutant segment polarity phenotypes were all found to have truncations in the internal repeat domain, with the strongest alleles having the largest truncations (Peifer and Wieschaus, 1990). However, when mutants of both the maternal and zygotic *armadillo* alleles were analyzed, the COOH-terminal region alone was found to be required for wingless-dependent signaling (Peifer et al., 1991). The function of the Armadillo COOH terminus is not yet known, but it may be different from the signaling activity of the repeat domain that we have detected in *Xenopus*. In the *Drosophila* embryo the Armadillo protein accumulates in response to the *wingless* signal (Peifer et al., 1991; van Leeuwen et al., 1994) and it is possible that the COOH terminus is required for this early upstream step in the pathway. The repeat domain could then mediate signaling in a subsequent step. In our experiments, overexpression of β-catenin or the repeat domain of β-catenin as a result of mRNA injection into the embryo might bypass the early steps in a signaling pathway and act directly on a downstream target.

Similar armadillo-like tandem repeats have been found in a number of cytoplasmic proteins, including APC, p120, SRP-1, and B6P and it has been proposed that the armadillo repeat region is a protein-protein interaction motif, analogous to SH2 or ankyrin repeat motifs (Hatzfeld et al., 1994; Peifer et al., 1994a). In support of this idea, we find that this region of β-catenin is the binding domain for both C-cadherin and APC. Our findings support the idea that the armadillo repeats participate directly in a signaling process.

Two general kinds of models have been proposed to explain how Armadillo and β-catenin participate in developmental signaling in *Drosophila* and *Xenopus* embryos (Peifer et al., 1991, 1993; Gumbiner and McCrea, 1991; McCrea et al., 1993). One possibility is that β-catenin or Armadillo acts directly in an intracellular signal transduction pathway in response to a wnt or *wingless* signal. Alternatively, regulation of β-catenin could increase cadherin-dependent adhesion, for example in response to the reception of a wnt signal, and thereby facilitate juxtacrine signaling between adjacent cells, i.e., signaling when both the ligand and receptor are cell surface-bound. Consistent with the latter hypothesis are the recently reported findings that elevated expression levels of β-catenin or plakoglobin in response to wnt expression can increase cadherin-mediated adhesion in certain cultured cell lines (Bradley et al., 1993; Hinck et al., 1994).

Although it is possible that elevation of β-catenin levels enhances cell adhesion in the *Xenopus* embryo, several findings make it unlikely that enhanced cell adhesion accounts for the axis induction by β-catenin. In the present study, we found that all of the NH₂-terminal deletion mutants of β-catenin, that fail to bind α-catenin, still induce axis duplication. Yet,

α-catenin association with the cadherin complex is known to be required for cadherin-dependent adhesion (Hirano et al., 1992; Breen et al., 1993; Shiozaki et al., 1994). Thus, cadherin complexes containing NH₂-terminally deleted β-catenin and lacking α-catenin probably cannot promote cell adhesion in the *Xenopus* embryo. Second, increasing cell adhesion by ectopic expression of various cadherins in *Xenopus* does not induce axis duplication (Detrick et al., 1990; Fujimori et al., 1990; Levine et al., 1994; Lee and Gumbiner, manuscript submitted for publication). In fact, overexpression of cadherins at the oocyte stage inhibits dorsal axis development in *Xenopus* embryos, probably because the excess cadherin binds up the free β-catenin and prevents it from participating in the signaling pathway (Heasman et al., 1994). Finally, in *Drosophila* the adhesion and signaling functions of Armadillo appear to be genetically separable (Peifer et al., 1993). Together these findings argue strongly that the activity of β-catenin in axis induction in early *Xenopus* embryos is independent of its role in promoting cadherin-dependent adhesion. Therefore, we believe that β-catenin participates directly in an intracellular signaling pathway.

There are several potential targets for the armadillo repeat region of β-catenin in the signaling pathway that leads to axis induction in *Xenopus*. One interesting possibility is the APC tumor suppressor protein, that has been reported to form protein complexes with β-catenin in colon cells (Rubinfeld et al., 1993; Su et al., 1993). We have confirmed that β-catenin interacts with APC in the *Xenopus* embryo and found that the armadillo repeat region alone is capable of interacting with APC. The β-catenin repeat domain also retains the capacity to interact with C-cadherin, which formally remains a potential target. α-catenin is unlikely to be a target for axis duplicating activity, because it does not bind to the repeat domain. Another intriguing potential target is some component of the cell nucleus, because overexpressed β-catenin accumulates in the nuclei of embryonic *Xenopus* cells. Similarly, it has recently been observed that plakoglobin, which is related to β-catenin and contains the highly conserved armadillo repeat region, causes axis duplication and accumulates in nuclei when it is overexpressed in *Xenopus* (Karnovsky and Klymkowsky, manuscript submitted for publication). Notably, nuclear accumulation of the armadillo repeat domain of β-catenin (T5), that retains potent signaling activity, is especially impressive. Of course, it is also possible that some other unknown protein is the relevant target for β-catenin signaling. Further studies will be required to determine whether any one of these potential targets or another unknown protein is functionally involved in the axis inducing activity of β-catenin.

The possibility that β-catenin interacts with a nuclear target to produce its signaling activity is very intriguing, but the physiological significance of its nuclear accumulation is still uncertain. So far there is no evidence that either endogenous β-catenin or Armadillo are present in nuclei. It is possible, however, that they are present in nuclei of some cells at low levels not easily detectable by immunofluorescence microscopy. Although β-catenin does not seem to have an identifiable nuclear localization sequence, it is possible that it can be imported in association with another protein that has an import signal. In our experiments with the early *Xenopus* embryo the nuclear envelope breaks down repeatedly during the rapid early cleavage divisions, and β-catenin could gain access to the nucleoplasm without requiring specific import. Even so, the substantial accumulation of the armadillo repeat domain T5 in the nucleus could be best explained if it binds with reasonable affinity to some nuclear component; the question would then be whether this interaction is important for β-catenin signaling activity. Further studies are needed to determine whether nuclear localization of β-catenin is required for its signaling activity.

We propose that β-catenin participates directly in an intracellular signaling pathway, similar to the one proposed for Armadillo in *Drosophila* (Noordermeer et al., 1994; Peifer et al., 1994b; Siegfried et al., 1994). Findings from other laboratories suggest that β-catenin might first accumulate (and perhaps also be modified) in response to an extracellular wnt signal (Bradley et al., 1993; Hinck et al., 1994; van Leeuwen et al., 1994). This active intracellular β-catenin probably then interacts, via its armadillo repeat domain, with some downstream protein target(s), that leads ultimately to a change in gene expression. The determination of the relevant downstream target(s) for β-catenin will be a challenging and important problem. Clearly, β-catenin also functions to regulate cadherin-dependent adhesion. It will also be important, therefore, to learn how the adhesive function and signaling activity of β-catenin are related and coordinated in the same cell.

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