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# *Xenopus* Gastrulation But Does Not Elicit Wnt-Directed Axis Specification

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**Modulators of cadherin function are of great interest given that the cadherin complex actively contributes to the morphogenesis of virtually all tissues. The catenin p120$^{ctn}$ (formerly p120cas) was first identified as a src- and receptor-protein tyrosine kinase substrate and later shown to interact directly with cadherins. In common with β-catenin and plakoglobin (γ-catenin), p120$^{ctn}$ contains a central Armadillo repeat region by which it binds cadherin cytoplasmic domains. However, little is known about the function of p120$^{ctn}$ within the cadherin complex. We examined the role of p120$^{ctn}$1A in early vertebrate development via its exogenous expression in *Xenopus*. Ventral overexpression of p120$^{ctn}$1A, in contrast to β-catenin, did not induce the formation of duplicate axial structures resulting from the activation of the Wnt signaling pathway, nor did p120$^{ctn}$ affect mesoderm induction. Rather, dorsal misexpression of p120$^{ctn}$ specifically perturbed gastrulation. Lineage tracing of cells expressing exogenous p120$^{ctn}$ indicated that cell movements were disrupted, while *in vitro* studies suggested that this may have been a consequence of reduced adhesion between blastomeres. Thus, while cadherin-binding proteins β-catenin, plakoglobin, and p120$^{ctn}$ are members of the Armadillo protein family, it is clear that these proteins have distinct biological functions in early vertebrate development. This work indicates that p120$^{ctn}$ has a role in cadherin function and that heightened expression of p120$^{ctn}$ interferes with appropriate cell–cell interactions necessary for morphogenesis.** **© 1999 Academic Press**

## INTRODUCTION

Embryonic gastrulation is a complex process in which cell–cell interactions must be modulated to effect defined cell motility and adhesion functions (Takeichi, 1991; Huber *et al.*, 1996). Central in this regard is the cadherin–catenin complex, which facilitates morphogenetic cell movements in addition to providing junctional integrity to differentiated tissues (Brieher and Gumbiner, 1994; Lee and Gumbiner, 1995; for review see Gumbiner, 1996). Cadherins are transmembrane glycoproteins that participate in calcium-dependent cell–cell interactions via the homophilic associations of their extracellular domains. The cadherins share strikingly high conservation within their cytoplasmic domains, which have been revealed to bind distinct, but likewise well-conserved, groups of proteins named catenins. While catenins exhibit multiple cellular roles, it is generally accepted that one function of β-catenin and α-catenin is to link cadherins to the cortical actin cytoskeleton. These interactions appear to be functionally important as work from various laboratories has suggested that they have a direct impact upon cadherin-mediated adhesion and motility (Nagafuchi and Takeichi, 1988; Ozawa *et al.*, 1990; Nagafuchi *et al.*, 1994; Kofron *et al.*, 1997) and likely comprise a locus from which cadherin functions are physiologically modulated *in vivo*.

In addition to binding cadherin cytoplasmic domains, catenins associate with additional cellular partners. For example, plakoglobin (γ-catenin) binds desmogleins (Korman *et al.*, 1989) and APC (Hülsken *et al.*, 1994). β-Catenin is well known to act within the powerful Wnt signaling pathway, in which, complexed with the LEF-1/TCF-3 family of nuclear transcription factors, β-catenin participates in

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cell fate specification and embryonic patterning via the downstream transcriptional activation of gene products that remain largely unknown (Behrens *et al.*, 1996; Molenaar *et al.*, 1996; He *et al.*, 1998). In *Xenopus*, β-catenin accumulates within the nuclei of a localized group of cells comprising the dorsal organizer of the embryo (Larabell *et al.*, 1997), where the β-catenin–TCF complex activates the transcription of Siamois, an upstream transcriptional activator of the molecular cascade specifying the dorsoanterior axis (Fan and Sokol, 1997, Brannon *et al.*, 1997). Correspondingly, the antisense depletion of maternal β-catenin results in the generation of embryos lacking axial structures (Heasman *et al.*, 1994a), while the experimental elevation of β-catenin within the ventral marginal zone results in ectopic Wnt-pathway activation and thereby the generation of secondary body axes (McCrea *et al.*, 1993; Funayama *et al.*, 1995).

Another catenin, p120$^{ctn}$, has more recently been identified as a component of the cadherin adhesion complex (Reynolds *et al.*, 1994; Aghib and McCrea, 1995; Shibamoto *et al.*, 1995; Staddon *et al.*, 1995). Like β-catenin and plakoglobin, p120$^{ctn}$ harbors a central Armadillo domain, composed of 10 imperfect repeats of approximately 40 amino acids, by which it binds directly to cadherins (Daniel and Reynolds, 1995) and an uncharacterized nuclear transcription factor (Daniel and Reynolds, 1996). p120$^{ctn}$ was originally identified in extracts of mammalian cells as a major substrate of the src tyrosine kinase(Reynolds *et al.*, 1989) and of EGF, PDGF, and CSF-1 receptor tyrosine kinases, permitting speculation that p120$^{ctn}$ may participate in signaling events relevant to functions of the cadherin–catenin complex (Downing and Reynolds, 1991; Kanner *et al.*, 1991; Shibamoto *et al.*, 1995).

p120$^{ctn}$ is likely to serve unique functions within the cadherin–catenin complex. For example, it does not bind α-catenin and thus does not link cadherins to the actin cytoskeleton in a manner similar to β-catenin and plakoglobin (Daniel and Reynolds, 1995). Alternative RNA splicing within the amino- and carboxy-coding sequences outside of p120$^{ctn}$'s central Armadillo domain comprises another mechanism thought to govern p120$^{ctn}$'s *in vivo* functions. These regions are divergent from those of β-catenin and plakoglobin and probably bind other as yet unknown factors, which when identified will aid in assessing the function of p120$^{ctn}$. Multiple p120$^{ctn}$ isoforms result from transcriptional initiation at several alternative ATG start sites and the utilization of alternative exons within p120$^{ctn}$'s carboxy region (Mo and Reynolds, 1996; Keirsebilck *et al.*, 1998). While the p120$^{ctn}$ isoforms appear to bind cadherins equally well (Reynolds *et al.*, 1996), their differing amino- and carboxy-termini may confer functional diversity within the cadherin–catenin (and/or other protein) complex (Mo and Reynolds, 1996; Keirsebilck *et al.*, 1998).

p120$^{ctn}$'s distinct biological role is further indicated by work demonstrating that its binding site within the cadherin cytoplasmic tail is more membrane-proximal relative to that of β-catenin and plakoglobin (Finnemann *et al.*, 1997; Yap *et al.*, 1998, Thoreson and Reynolds, unpublished results). Work conducted in mammalian cell lines suggests that this region is required in repressing cadherin-mediated cell motility (Riehl *et al.*, 1996; Chen *et al.*, 1997) and in cadherin clustering and adhesive strengthening, functions that conceivably may be mediated via p120$^{ctn}$ (Yap *et al.*, 1998). Additional attempts to assess p120$^{ctn}$'s cellular function have included its transient overexpression in NIH3T3 fibroblasts, resulting in the production of a graphic dendritic phenotype. While the underlying mechanism is unclear, the effect appears to be specific to p120$^{ctn}$'s association with cadherin, given that the expression of p120$^{ctn}$ constructs incapable of binding cadherins displayed no effect, and the expression of β-catenin, which binds cadherin but at a more carboxy-terminal site, had little effect on cell morphology (Reynolds *et al.*, 1996).

While hints regarding p120$^{ctn}$'s biological functions are beginning to emerge, little is known regarding p120$^{ctn}$'s role in developmental processes. We have investigated the function of one of the longest isoforms of p120$^{ctn}$ (p120$^{ctn}$1A) in cadherin-based adhesion and motility, employing the developmental system *Xenopus laevis.* C-cadherin (also referred to as EP-cadherin) is maternally encoded in *Xenopus* and its homophilic extracellular contacts are believed to constitute the principal elements mediating cell–cell (blastomere) adhesion within cleavage and blastula embryos (Choi *et al.*, 1990; Heasman *et al.*, 1994b; for review see Kuhl and Wedlich, 1996). Because the juxtamembrane region to which p120$^{ctn}$ binds (Yap *et al.*, 1998) is highly conserved across species, we reasoned that the introduction of murine p120$^{ctn}$ would functionally complement *Xenopus* p120$^{ctn}$. Indeed, it has previously been shown that *Xenopus* XB/U-cadherin expressed in murine L-TK$^{-}$ fibroblasts interacts normally with murine p120$^{ctn}$, α-catenin, and β-catenin (Finnemann *et al.*, 1997).

In this study, we identify *Xenopus* p120$^{ctn}$ (cDNA, mRNA, and protein), and demonstrate that exogenously introduced murine p120$^{ctn}$ binds *Xenopus* C-cadherin as expected. We report that the misexpression of p120$^{ctn}$ during early *Xenopus* development results in specific failures in gastrulation, which may result from the reduced capacity of C-cadherin to engage in directed and highly orchestrated cell movements required in morphogenesis. We further report that despite p120$^{ctn}$'s structural similarity to β-catenin, p120$^{ctn}$ does not engage in Wnt signaling.

## MATERIALS AND METHODS

### *Embryos*

*Xenopus* eggs were obtained and fertilized by standard methods (Newport and Kirschner, 1982), dejellied with 2% cysteine HCl (pH 8.0), rinsed, and incubated in 0.1× MMR (10 mM NaCl, 0.2 mM KCl, 0.1 mM $MgSO_4$, 0.2 mM $CaCl_2$. 0.5 mM Hepes, pH 7.4). Embryos were placed in 1× MMR with 4% Ficoll and injected with mRNA at the two-cell or four-cell embryo stage. Injected embryos

were transferred to 0.1× MMR with antibiotics and cultured between 14 and 18°C. Embryos were staged according to the normal table of *X. laevis* development (Nieuwkoop and Faber, 1967).

β-Catenin cDNA was in the pSP36T vector (Funayama *et al.*, 1995). Murine p120$^{ctn}$ and ΔR8-10 (Daniel and Reynolds, 1995) were cloned into the expression vector pCS2+ (Rupp *et al.*, 1994; Turner and Weintraub, 1994) by introduction of a 5′ *Cla*I restriction site and a 3′ *Stu*I restriction site using PCR amplification. The frameshift mutant of myc-tagged p120$^{ctn}$ was generated by restriction with *Eco*RI, fill-in of the overhanging sequence, and blunt-end religation to introduce a premature stop codon just before the p120$^{ctn}$ coding sequence. Capped mRNAs were synthesized *in vitro* from linearized plasmids using the SP6 mMessage mMachine kit (Ambion). For phenotypic analysis, 0.2 ng of p120$^{ctn}$ or ΔR8-10 mRNA was injected into the equatorial/vegetal region of a ventral or dorsal blastomere at the four-cell stage. For the adhesion assay, 0.2 ng of mRNA was injected into the animal pole region of both blastomeres at the two-cell stage. For RT-PCR analysis of Siamois induction and *in situ* hybridization for goosecoid (gsc) expression, 0.2 ng of p120$^{ctn}$ or 2 ng of β-catenin mRNA was injected into the vegetal/equatorial region of a ventral blastomere at the four-cell stage. For lineage tracing, 0.2 ng of p120$^{ctn}$ and 1 ng of β-galactosidase or β-galactosidase alone were injected into two dorsal blastomeres at the four-cell stage. The dorsal blastomeres were distinguished from the ventral blastomeres by lighter pigmentation and smaller size. Micropipettes were formed from borosilicate glass capillaries (o.d. 1.0 mm, i.d. 0.75 mm) (Sutter) using a Sutter P-30 puller and beveled with K. T. Brown type micropipette beveler. Injections were performed using the oil-based NA-1 microinjector (Sutter).

## Western Blotting and Immunoprecipitations

For whole-embryo Western blotting, the embryos were homogenized in 15 mM Tris, pH 6.8, and extracted with 1,1,2-trichlorotrifluoroethane (Freon) to remove yolk protein from the lysates (Evans and Kay, 1991). For immunoprecipitation of p120$^{ctn}$ or C-cadherin, stage 12–13 embryos were extracted with 0.5% Triton X-100, 10 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM EDTA, and 0.02% $NaN_3$, supplemented with protease inhibitors. The protease inhibitor cocktail consisted of working concentrations of 1 mM phenylmethylsulfonyl fluoride, 4 μg/ml aprotinin, 1 μg/ml pepstatin A, 2 μg/ml leupeptin, 10 μg/ml antipain, 50 μg/ml benzamidine, 10 μg/ml soybean trypsin inhibitor, 100 μg/ml iodoacetamide, and 40 μg/ml TLCK. The extract was cleared by centrifugation. The supernatant was removed and precleared with protein G–Sepharose (Sigma). Immunoprecipitation and detection of murine p120$^{ctn}$ used the polyclonal antibody, F1, generated against the amino-terminal third of murine p120$^{ctn}$ (Mo and Reynolds, 1996). Immunoprecipitation and detection of *Xenopus* p120$^{ctn}$ used a pool of monoclonal antibodies generated against murine p120$^{ctn}$ (Wu *et al.*, 1998). C-cadherin was immunoprecipitated with a polyclonal antibody generated against the extracellular domain of *Xenopus* C-cadherin (kind gift from W. Brieher and B. Gumbiner, Memorial Sloan-Kettering Cancer Center).

Proteins were separated on 8% SDS–polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. For standardization between samples, each immunoprecipitation sample lane contained 2.5 embryos and whole-cell lysate lanes contained the equivalent of 0.5 embryos. p120$^{ctn}$ was detected by Western blotting using the p120$^{ctn}$ polyclonal antibody F1 at 0.25 μg/ml. C-cadherin was Western blotted with the *Xenopus* C-cadherin antibody used at a dilution of 1:10,000. Bands were detected with enhanced chemiluminescence (ECL, Amersham) using goat anti-rabbit IgG (1:3000) coupled to horseradish peroxidase (Bio-Rad). For quantitation of band intensities in Fig. 2B, the integrative volume of each band area was calculated with the software program NIH Image, available at http://rsb.info.nih.gov/nih-image/.

## RT-PCR

*Xenopus* p120$^{ctn}$ expression from the four-cell stage to stage 33 was detected using primers derived from an Xp120$^{ctn}$ clone isolated from a stage 17 *Xenopus* cDNA library. Siamois expression was detected by amplification of cDNA generated from total RNA isolated at stage 10–10.25 from ventral and dorsal halves of embryos injected with p120$^{ctn}$ (0.2 ng) or β-catenin (2 ng) into a ventral blastomere at the four-cell stage. Total RNA was extracted by homogenization of the embryos in Trizol (Gibco BRL) according to the manufacturer's instructions. The Siamois primer sequences used were from Fagotto *et al.* (1997), and the histone H4 primer sequences were from Niehrs *et al.* (1994). Histone H4 amplification served as a control. DNA contamination of the total RNA preparation was tested by conducting PCR amplification of reverse transcriptase (−) cDNA reactions. Primers were Siamois, 5′ CGC GGA TCC ATG GCC TAT GAG GCT GAA ATG GAG, 3′ GCT CTA GAG AAG TCA GTT TGG GTA GGG CT; histone H4, 5′ CGG GAT AAC ATT CAG GGT A, 3′ TCC ATG GCG GTA ACT GTC; and Xp120, 5′ GCT TGT GGA GCA CTG CGG AAC A, 3′ GCA AAG CAT CCA CTA GCC CGT C.

## Whole-Mount in Situ Hybridization

Whole-mount *in situ* hybridizations for gsc, Xbra, and Xnot1 were performed according to Harland (1991) with minor modifications as described by El-Hodiri *et al.* (1997). The antisense RNA probes were labeled with digoxigenin according to the manufacturer's instructions (Boehringer Mannheim) using the following constructs: pBS(SK)-gsc (Cho *et al.*, 1991), pBS(SK)-pXnot10 (Von Dassow *et al.*, 1993), and pSP73-Xbra (Smith *et al.*, 1991).

## Adhesion/Aggregation Assay

For adhesion assays, animal pole explants were isolated at stage 8, and the blastomeres were dissociated in 1× calcium/magnesium-free MMR by passing through a serum-coated Pasteur pipette several times. Calcium was added to the medium to 2 mM and the blastomeres were allowed to adhere on a horizontal rotary shaker at 70 rpm in 35-mm dishes coated with 1% agarose. Pictures were taken of random samples at 60 min. The number of cells per aggregate was quantified from 3 to 7 slides for each condition, and the numbers were combined from two separate experiments. The Student *t* test was used for statistical comparisons. *P* values less than 0.05 were accepted as indicating statistically significant differences between the two samples.

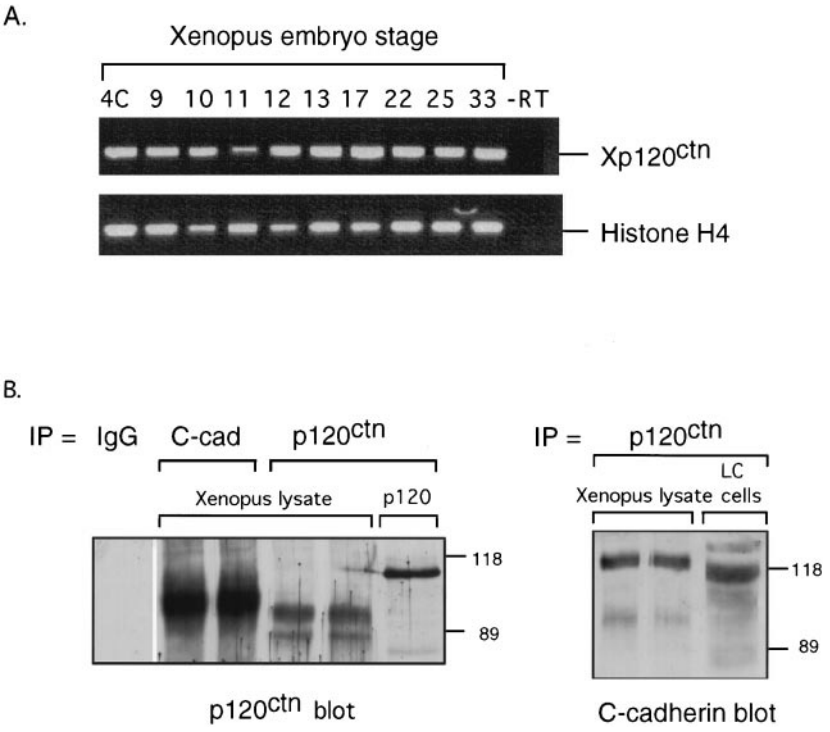


**FIG. 1.** *Xenopus* p120ctn is expressed in early development and associates with C-cadherin. (A) The expression of p120ctn in *Xenopus* embryos was detected by RT-PCR, using as template total RNA isolated from embryos at the indicated stages. The Xp120ctn-specific primers were generated from Xp120ctn sequence cloned from a stage 17 *Xenopus* cDNA library. Primers for histone H4 were used as a control for reaction conditions. (−RT indicates control reaction without reverse transcriptase.) (B) *Xenopus* embryo lysates were immunoprecipitated with a C-cadherin polyclonal antibody or a mixture of p120ctn monoclonal antibodies and blotted for p120ctn (positive control, mouse p120ctn1A-injected whole-cell lysate) or immunoprecipitated with a mixture of the p120ctn monoclonal antibodies and blotted for C-cadherin [positive control whole-cell extract of L cells expressing C-cadherin (Brieher and Gumbiner, 1994)]. (C-cad, C-cadherin, IP, immunoprecipitation.)

## RESULTS

### *Endogenous p120ctn Detected in Vivo Binds Cadherins*

To demonstrate the embryonic presence of endogenous (*Xenopus*) p120ctn mRNA and the corresponding binding of endogenous p120ctn protein to cadherin, we utilized RT-PCR and coimmunoprecipitation approaches, respectively. To detect endogenous p120ctn mRNA, we first designed nondegenerate RT-PCR primers based upon the sequence of a *Xenopus* p120 cDNA clone we have isolated. Use of these primers allowed us to determine that the endogenous p120ctn mRNA is expressed maternally and throughout early development (Fig. 1A). To evaluate the endogenous p120ctn protein, we Western-blotted anti-p120ctn and anti-C-cadherin immunoprecipitates. As anticipated from work in mammalian systems (see Introduction), we found that endogenous p120ctn protein was expressed and associated with cadherin both prior (data not shown) and following zygotic transcription (Fig. 1B). The apparent molecular weight of the endogenous p120ctn is consistent with the predicted molecular weight calculated from the full-length *Xenopus* p120ctn cDNA (unpublished data). As with all known components of the cadherin–catenin complex, our results suggest that the organization of the cadherin–catenin complex is similar in amphibian and mammalian systems with respect to p120ctn and that p120ctn is present throughout development.

### *Exogenous p120ctn Expressed in Vivo Binds Xenopus C-Cadherin*

To establish that the exogenous (mouse) p120ctn is stably expressed, p120ctn1A mRNA was transcribed *in vitro* and injected into single dorsal blastomeres of four-cell stage embryos. Western blotting of whole embryo extracts verified the expression of the mouse p120ctn protein at the expected electrophoretic mobility, over the time course tested, 1–48 h (stages 5–22) (Fig. 2A). The p120ctn polyclonal antibody (F1) used for both immunoprecipitations and Western blotting does not cross-react with endogenous p120ctn (Fig. 2A—uninjected), allowing us to selectively confirm the expression of exogenous p120ctn.

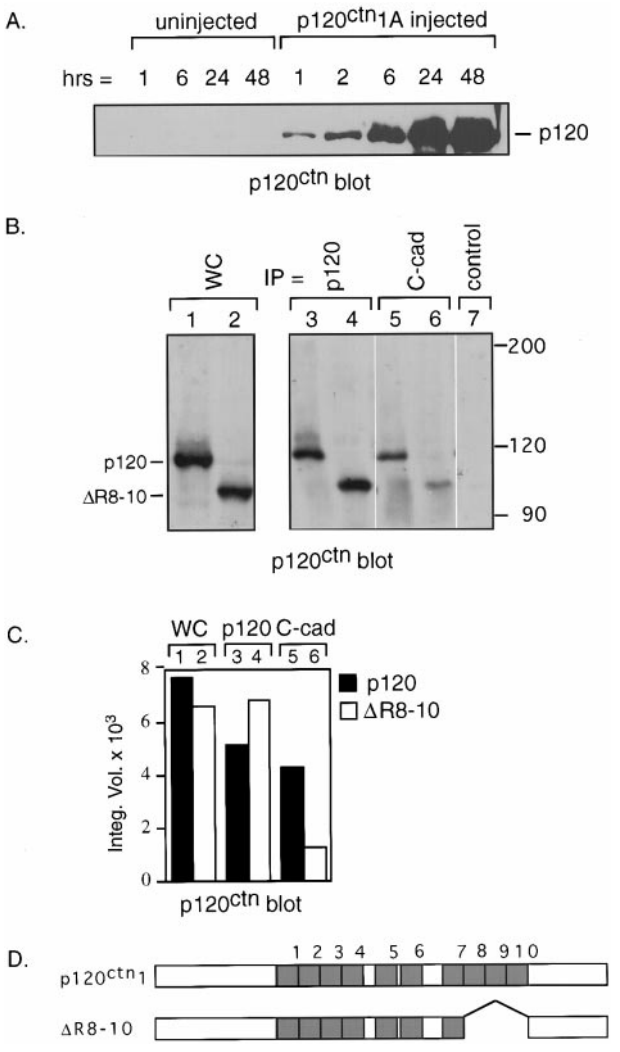


**FIG. 2.** Expression of *in vitro*-transcribed p120$^{ctn}$ mRNA injected into *Xenopus* embryos and the association of p120$^{ctn}$ with *Xenopus* C-cadherin. (A) Western blot analysis of uninjected embryos and embryos injected with 0.2 ng of p120$^{ctn}$ mRNA at the four-cell stage, extracted at 1, 2, 6, 24, and 48 h following injection, and blotted with a polyclonal antibody against p120$^{ctn}$. This p120$^{ctn}$ polyclonal antibody did not detect the endogenous p120$^{ctn}$. (B) Embryos were injected with 0.2 ng of p120$^{ctn}$ or ΔR8-10 mRNA into the animal pole of both blastomeres at the two-cell stage and extracted at stage 12–13. Lanes 1, 3, 5, and 7 are p120$^{ctn}$-injected embryos and lanes 2, 4, and 6 are ΔR8-10-injected embryos. Lanes 1 and 2 are whole embryo extracts blotted for p120. Lanes 3 and 4 are p120$^{ctn}$ IPs blotted for p120$^{ctn}$. Lanes 5 and 6 are C-cadherin IPs blotted for p120$^{ctn}$. Lane 6 is rabbit serum IgG control. (WC, whole-cell extract, C-cad, C-cadherin, IP, immunoprecipitation.) (C) Quantitation of p120$^{ctn}$ and ΔR8-10 bands in B. (D) Diagram of full-length p120$^{ctn}$1 and the deletion construct ΔR8-10.

To demonstrate that exogenous p120$^{ctn}$ binds endogenous cadherins, we utilized coimmunoprecipitation assays. Embryos expressing the exogenous p120$^{ctn}$ were immunoprecipitated for C-cadherin, the major cadherin of early *Xenopus* development (Choi *et al.*, 1990; Ginsberg *et al.*, 1991). As anticipated, given the remarkably high sequence conservation of p120$^{ctn}$'s binding site within cadherins (Yap *et al.*, 1998), exogenous p120$^{ctn}$ clearly coprecipitated with C-cadherin (Fig. 2B). Also apparent, but considerably more weakly bound to C-cadherin, was the negative control construct ΔR8-10, which is lacking Armadillo repeats 8–10 (Fig. 2B). Quantitation of the band intensities for the blot in Fig. 2B illustrated that the level of ΔR8-10 protein coprecipitating with C-cadherin was about 30% of that for full-length p120$^{ctn}$ (Fig. 2C). In NIH3T3 cells, the removal of repeats 8–10 prevented p120$^{ctn}$ (ΔR8-10) from binding cadherin, and exogenous expression of ΔR8-10 did not generate the branching phenotype induced by full-length p120$^{ctn}$ (Reynolds *et al.*, 1996). These results indicate that the exogenous p120$^{ctn}$ properly binds cadherin, while negative control construct ΔR8-10 exhibits a deficiency in cadherin association even when expressed at readily detectable levels in whole-embryo extracts and direct anti-p120$^{ctn}$ immunoprecipitations.

### *p120$^{ctn}$ Is Not Active in Wnt Signaling*

To examine whether p120$^{ctn}$ participates in Wnt signaling in a manner analogous to β-catenin, we examined the effect of exogenous p120$^{ctn}$ expression upon two established downstream targets of the Wnt pathway, Siamois and goosecoid (Brannon and Kimelman, 1996; Carnac *et al.*, 1996; Laurent *et al.*, 1997). Siamois is normally expressed within the Nieuwkoop center (Lemaire *et al.*, 1995), while gsc is largely restricted to the Spemann organizer (Cho *et al.*, 1991). Each marker may be induced elsewhere following the ectopic activation of Wnt/β-catenin signaling (Fagotto *et al.*, 1997). In contrast to ectopic β-catenin expression (positive control), we observed that the ectopic expression of p120$^{ctn}$ was incapable of inducing the ventral expression of these markers in injected embryos, as assayed using RT-PCR (Siamois) (Fig. 3A) and whole-mount *in situ* hybridization (gsc) (Fig. 3B). The inability of p120$^{ctn}$ to transduce Wnt signals was further made graphically evident in that ventral injections did not result in the generation of ectopic (secondary) body axes, which are clearly apparent following the ventral overexpression of β-catenin (data not shown) (Funayama *et al.*, 1995). Thus, despite their similar structures and the association of both proteins with cadherins, p120$^{ctn}$ and β-catenin differ in their capacity to transduce Wnt signals.

### *Increased Expression of p120$^{ctn}$ Results in Failed Gastrulation*

To address the function of p120$^{ctn}$ in early vertebrate development, we employed a proven experimental approach within the *Xenopus* system, specifically increasing p120$^{ctn}$'s expression via the microinjection of exogenous p120$^{ctn}$ constructs. *In vitro*-transcribed p120$^{ctn}$ mRNA, and

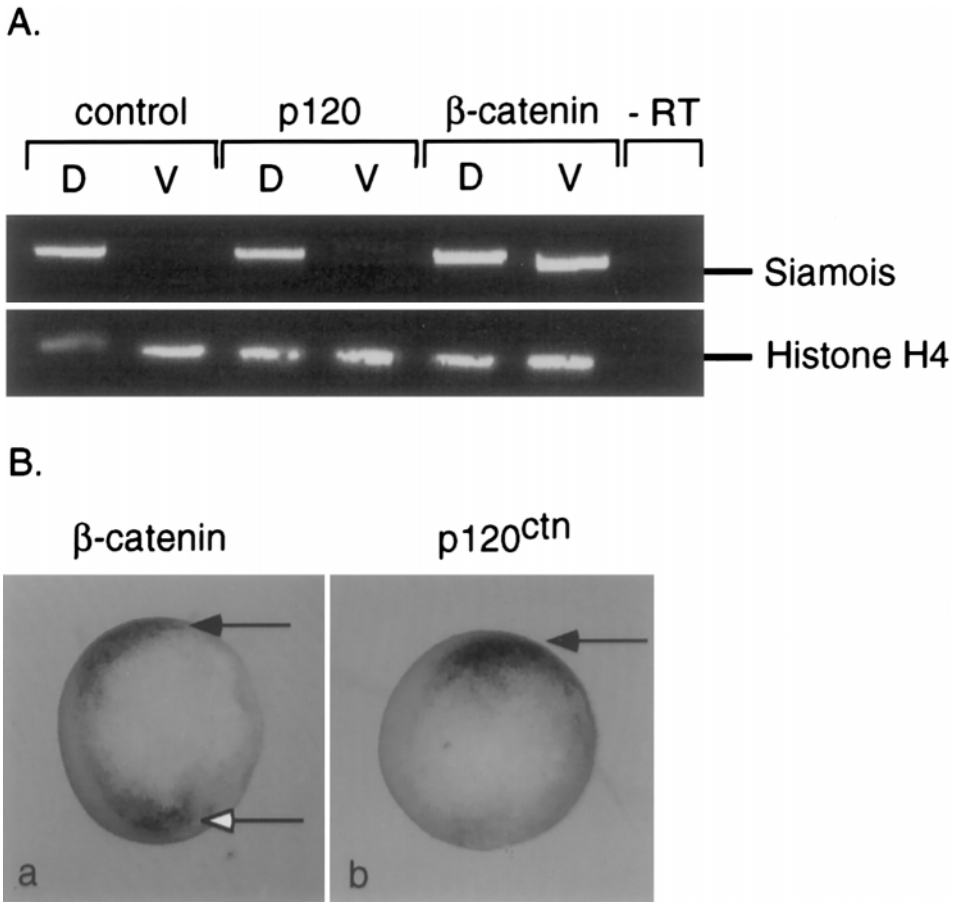


**FIG. 3.** p120$^{ctn}$ does not induce expression of Siamois or goosecoid. (A) Detection of Siamois expression in dorsal and ventral halves of stage 10 embryos with RT-PCR. Embryos were injected vegetally with 0.2 ng of p120$^{ctn}$ or 2 ng of *β*-catenin mRNA into one ventral blastomere at the four-cell stage. The embryos were dissected into ventral and dorsal halves at stage 10 and analyzed for Siamois expression by RT-PCR. Control embryos are uninjected. (−RT is control reaction without reverse transcriptase.) (B) Embryos were injected with 0.2 ng of p120$^{ctn}$ (A) or 2 ng of *β*-catenin (B) mRNA into the marginal zone of one ventral blastomere at the four-cell stage. Whole-mount *in situ* hybridization was undertaken using digoxigenin-labeled cRNA probes to detect gsc expression in stage 10 embryos. The embryos are viewed from the vegetal side, with the dorsal lip at top. The solid arrows refer to endogenous gsc expression found in the organizer region, while the white arrow refers to exogenous ventral expression of gsc.

that of various negative control constructs including ΔR8-10 and a frame-shifted p120$^{ctn}$, were injected into the marginal zone of single dorsal or ventral blastomeres at the four-cell stage, and subsequent development was evaluated.

The dorsal misexpression of p120$^{ctn}$ resulted in a variety of posterior defects (Fig. 4). Embryos appeared normal through the late blastula–early gastrula stages, with no apparent differences among embryos injected with p120$^{ctn}$, ΔR8-10, *β*-galactosidase, or frame-shift p120$^{ctn}$ mRNA. By mid- to late-gastrula stages, however, embryos injected dorsally with p120$^{ctn}$ exhibited the extrusion of embryonic endoderm and the corresponding failure of blastopore closure (Fig. 4A). At later neurula and tailbud stages, many of these same embryos showed failures in the closure of the dorsal midline (neural folds) (Figs. 4B and 4C). We expect that such aberrantly extruded endoderm prevents the normal progress of subsequent cell movements, ultimately generating gross posterior abnormalities, including ancillary tails (10–15% of embryos), displaced notochords, and truncated axes (Fig. 4D). Ancillary tails likely result from the failure of circumblastoporal cells, including those of the prospective tail organizer, to coalesce at the dorsal midline (Fig. 5) (Gont *et al.*, 1993).

Approximately 62% of p120$^{ctn}$ dorsally injected embryos demonstrated obvious gastrulation defects, while only 11% of ΔR8-10 negative control injected embryos revealed such deformities (Table 1). A further control of specificity was evident following dorsal injections of *β*-catenin, which, despite its structural similarity to p120$^{ctn}$, did not perturb embryogenesis (data not shown). The dorsal microinjection

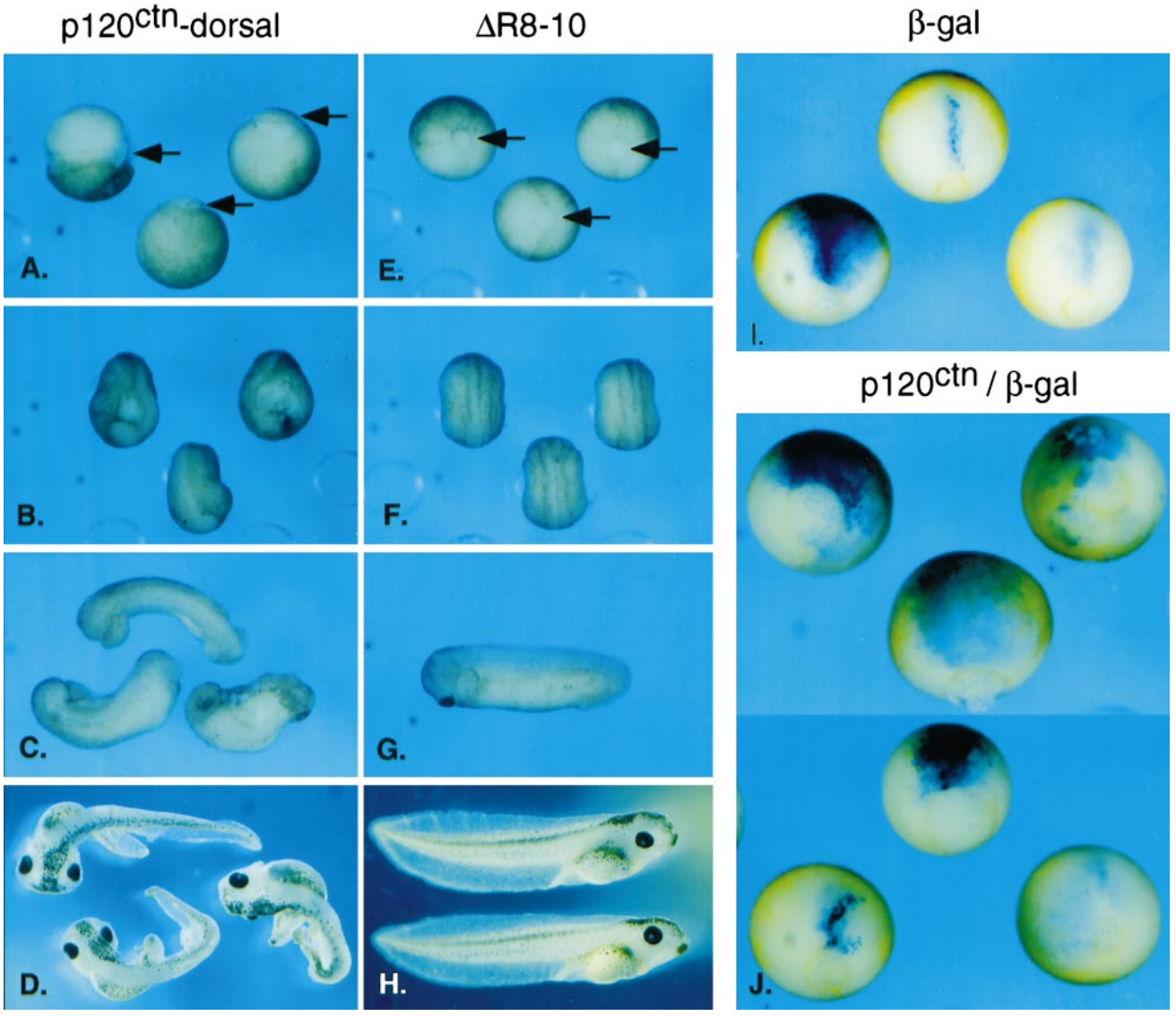


**FIG. 4.** Effect of p120$^{ctn}$ misexpression on early *Xenopus* development. Embryos were injected with 0.2 ng of p120$^{ctn}$ or ΔR8-10 mRNA into one dorsal blastomere at the four-cell stage. (A–D) Embryos injected with p120$^{ctn}$ into the dorsal marginal zone and (E–H) embryos injected with ΔR8-10 into the dorsal marginal zone. A and E are gastrula-stage embryos, B and F are neurula-stage embryos, C and G are tailbud-stage embryos, and D and H are tadpole-stage embryos. Lineage tracing of p120-expressing cells suggests that p120$^{ctn}$ perturbs convergent extension movements in the whole embryo. Embryos (I) injected with 2 ng of *β*-galactosidase mRNA or (J) co-injected with 2 ng *β*-galactosidase mRNA and 0.2 ng p120 mRNA at the four-cell stage were allowed to develop until late gastrula (12–12.5), fixed in MEMFA, and processed with X-gal to stain for *β*-galactosidase.

**FIG. 5.** Misexpression of p120$^{ctn}$ does not alter expression of mesodermal markers Xbra, Xnot1, or gsc. Embryos were injected with 0.2 ng of p120$^{ctn}$ (B, D, F, H, J, L) or ΔR8-10 (A, C, E, G, I, K) mRNA into one dorsal blastomere at the four-cell stage. Embryos were permitted to develop to the appropriate stage, fixed in MEMFA, and subjected to whole-mount *in situ* hybridization with antisense mRNA probes to Xbra (A–F), Xnot1 (G–J), and gsc (K, L). Stage 10 (K, L) and stage 10.5 embryos (A, B and G, H) are viewed vegetally with dorsal side up. Stage 12.5 embryos (C, D and I, J) are viewed dorsally with the anterior at the top and posterior at the bottom. Stage 22 embryos are viewed laterally (E) and dorsally (F) with the anterior side on the left and posterior on the right. The closed arrows refer to the tailbud, and the open arrows refer to the head.

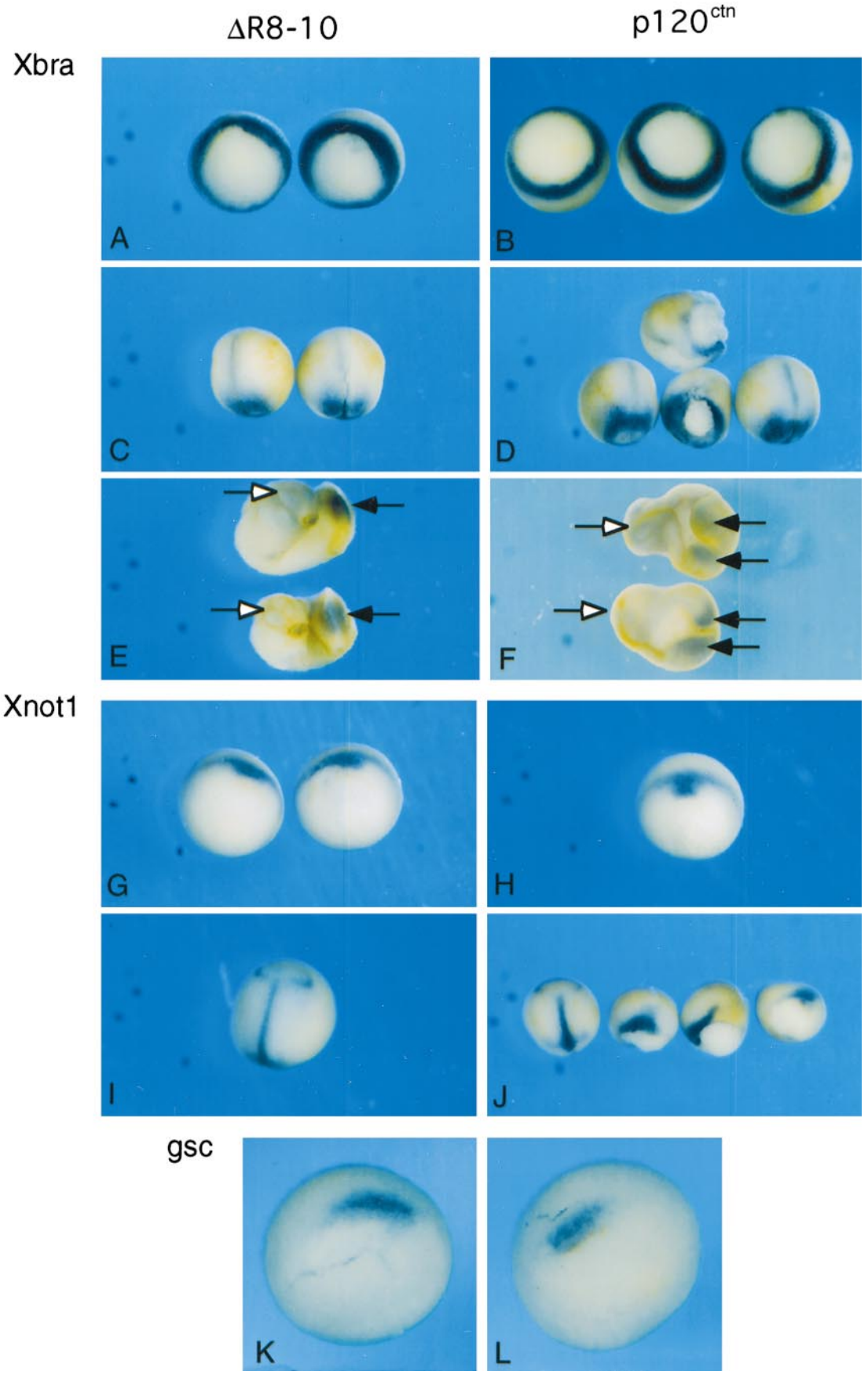
ΔR8-10
p120ctn
Xbra
A
B
C
D
E
F
Xnot1
G
H
I
J
gsc
K
L

**TABLE 1**
Dorsal Injection of p120$^{ctn}$ mRNA or DNA Produces Gastrulation Defects

| mRNA | Position | Abnormal gastrulation |
|---|---|---|
| p120$^{ctn}$ | Dorsal | 435/699 (62%) |
| | Ventral | 50/152 (33%) |
| ΔR8-10 | Dorsal | 53/475 (11%) |
| | Ventral | 20/140 (14%) |
| Frame-shift p120$^{ctn}$ | Dorsal | 29/163 (18%) |
| | Ventral | 17/109 (16%) |
| **DNA** | **Position** | **Abnormal gastrulation** |
| p120$^{ctn}$ | Dorsal | 68/103 (66%) |
| | Ventral | 27/92 (25%) |
| ΔR8-10 | Dorsal | 2/43 (5%) |
| | Ventral | 3/48 (6%) |
| Frame-shift p120$^{ctn}$ | Dorsal | 1/50 (2%) |
| | Ventral | 3/53 (6%) |

*Note.* 0.2 ng of p120$^{ctn}$ ΔR8-10, or frame-shift p120$^{ctn}$ mRNA or 35 pg of the corresponding cDNA was injected into one dorsal or one ventral blastomere of the *Xenopus* embryo at the four-cell stage. Embryos were evaluated for gastrulation defects at stage 12.5.

of exogenous p120$^{ctn}$ cDNA, which in contrast to exogenous mRNA is not expressed until the initiation of zygotic transcription (stage 8.5 or midblastula transition), produced gastrulation phenotypes which appeared indistinguishable from those evident following the introduction of p120$^{ctn}$ mRNA (Table 1). Thus, it appears that the developmental events perturbed as a result of p120$^{ctn}$ expression are likely to transpire following the initiation of zygotic transcription (about stage 8.5), which supports our observation that no embryonic defects are apparent prior to this developmental stage (midblastula transition). Further, the readily apparent gastrulation defects observed following wildtype p120$^{ctn}$ expression, versus the significantly weaker effects following the microinjection of negative control construct ΔR8-10, suggest that the capacity of p120$^{ctn}$ to bind cadherin is a significant factor in generating the aberrant gastrulation phenotypes.

Given that widely disparate outcomes are evident following dorsal β-catenin overexpression (normal phenotype) versus ventral overexpression (duplicate axis phenotype) (Funayama *et al.*, 1995), we compared phenotypes arising following the dorsal versus ventral expression of exogenous p120$^{ctn}$. We observed that while gastrulation defects were considerably more prominent resulting from the heightened expression of p120$^{ctn}$ in the embryo's dorsal hemisphere (62%), failures in gastrulation were also evident following p120$^{ctn}$'s ventral expression (33%) (Table 1). The greater dorsal penetrance of the phenotype could be attributed to the fact that dorsal cells normally undergo more extensive cell movements during embryonic development (Keller and Danilchik, 1988)—movements that are mediated via cadherin cell–cell contacts (Brieher and Gumbiner, 1994; Lee and Gumbiner, 1995).

### *Lineage Tracing of Cells Expressing Exogenous p120$^{ctn}$ Indicates That Directed Cell Movements Are Perturbed*

To explicitly address the impact of p120$^{ctn}$ expression upon properly directed cell movements occurring during gastrulation, we utilized an *in vivo* lineage tracing approach. β-Galactosidase mRNA was co-injected with p120$^{ctn}$ and progeny cells were visualized via X-gal staining of whole embryos at later points during development. We found that while p120$^{ctn}$-expressing cells remained capable of undergoing cell movements, they appeared largely incapable of converging toward the dorsal midline in a directed fashion (Figs. 4I and 4J) (see also Fig. 5). With animal pole explants, overexpression of p120$^{ctn}$ did not inhibit activin-

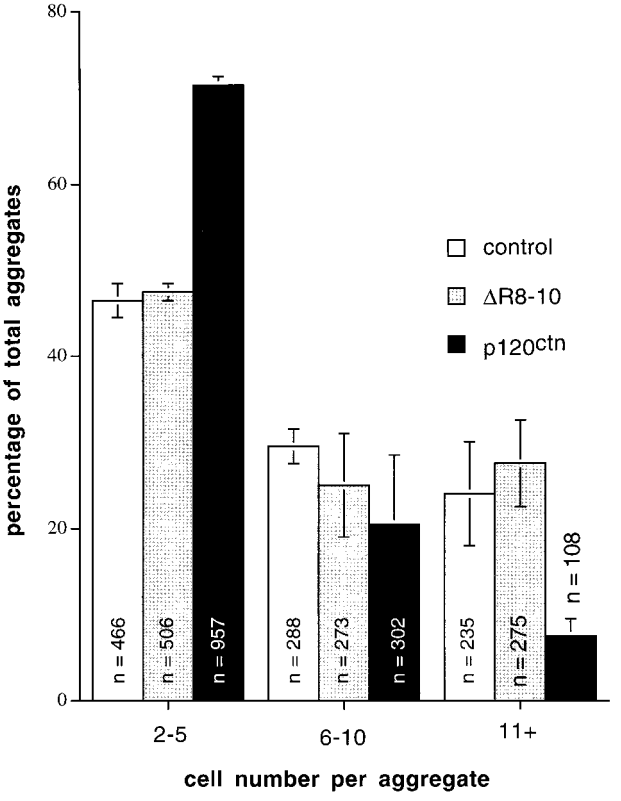


**FIG. 6.** p120$^{ctn}$ decreases adhesion between reaggregated animal cap blastomeres. Embryos were injected with 0.2 ng of p120$^{ctn}$ or ΔR8-10 mRNA into the animal hemisphere of each blastomere at the two-cell stage. Animal pole explants were isolated from stage 8 embryos and dissociated in 1× CMF-MMR. Calcium was added to the medium to 2 mM, and pictures were taken of random samples at 60 min of aggregation. The number of cells per aggregate was quantified for each condition.

induced elongation (data not shown), illustrating that these blastomeres retain the ability for cell movements and that the *in vivo* situation is different from the *in vitro* assay. These data suggest that the heightened expression of p120$^{ctn}$ perturbs directed cell movements, which are required for normal embryogenesis. Given that p120$^{ctn}$ binds cadherin (Fig. 1), it is likely that such perturbations occur via altered cadherin function, as is indicated further below (Fig. 6).

### *p120$^{ctn}$ Misexpression Does Not Perturb Mesoderm Induction*

Because normal mesoderm induction is necessary for the involution of the marginal zone during gastrulation (Symes and Smith, 1987), we employed whole-mount *in situ* staining of the mesodermal markers Xbra, Xnot1, and gsc to evaluate the impact of heightened p120$^{ctn}$ expression upon mesoderm induction and early embryonic patterning. Gsc is a marker of the presumptive anterior mesoderm (Cho *et al.*, 1991), Xbra is an early panmesodermal marker later expressed in the notochord and tail organizer (Smith *et al.*, 1991), and Xnot1 is a marker of the dorsal mesoderm, notochord, converging cells of the dorsal midline, and the tailbud (Von Dassow *et al.*, 1993). A comparison of *in situ* staining for gsc at stage 10 (Figs. 5K and 5L), Xbra at stage 10.5 (Figs. 5A and 5B), and Xnot1 at stage 10.5 (Figs. 5G and 5H), illustrates that heightened expression of p120$^{ctn}$ does not noticeably perturb the expression of these mesodermal markers relative to patterns evident following injection of negative control construct ΔR8-10 or no injection. Such results are consistent with the observed absence of an aberrant morphological phenotype during early gastrula stages of development.

However, the detection of the Xbra and Xnot1 markers at the late gastrula/early neurula stage (Figs. 5C, 5D, 5I, and 5J), and of the Xbra marker at the tailbud stage (Figs. 5E and 5F), shows that their expression is properly timed in p120$^{ctn}$-injected embryos, yet their spatial localization is displaced relative to negative control (normal) embryos. We surmise that while p120$^{ctn}$ misexpression does not alter cell fate or mesoderm specification, it does result in alterations of directed cell movements as was indicated previously via lineage tracing analysis (Figs. 4I and 4J).

### *p120$^{ctn}$ Misexpression Decreases Blastomere Adhesion*

To directly establish the impact of heightened p120$^{ctn}$ expression upon cadherin function, we employed an established *in vitro* reaggregation assay, known to be driven via cadherin-mediated cell–cell interactions. Embryos were microinjected with p120$^{ctn}$ mRNA, versus negative control ΔR8-10 mRNA, in the animal pole region of both cells at the two-cell stage. Animal caps were subsequently dissected (stage 8) and the blastomeres dissociated in low calcium media and then permitted to reaggregate over time following the reintroduction of calcium. By simple visual inspection, p120$^{ctn}$ overexpression did not induce graphic changes in cell reaggregation relative to the negative control ΔR8-10. This result is consistent with the integrity of embryos overexpressing p120$^{ctn}$ at blastula and early gastrula stages (data not shown). However, quantitation of the number of cells per aggregate readily revealed that the size of cell aggregates formed from p120$^{ctn}$ -injected embryos was decreased relative to ΔR8-10-injected (or uninjected) embryos (Fig. 6). At the 60-min time point, for example, heightened p120$^{ctn}$ expression shifted the percentage of aggregates containing between two and five cells from 47 and 48% (uninjected and ΔR8-10-injected embryos) to 70% (p120$^{ctn}$-injected) ($P < 0.02$ for uninjected and ΔR8-10-injected), while shifting the percentage of aggregates with 11 or more cells from 24 and 26% (uninjected and ΔR8-10-injected embryos) to 8% (p120$^{ctn}$-injected) ($P < 0.01$ for uninjected and $P < 0.04$ for ΔR8-10-injected). Expression of β-catenin at the same concentration (0.2 ng) did not produce decreases in blastomere adhesion (not shown), while in another study, injection of 1 ng of β-catenin mRNA induced a small increase in the rate of adhesion (Guger and Gumbiner, 1995). These results suggest that the heightened expression of p120$^{ctn}$ during embryonic development results in a subtle but specific decrease in cadherin-mediated cell–cell adhesion, which may be a contributing and/or principle factor underlying the observed embryonic phenotypes of misdirected cell movements and failed gastrulation.

## DISCUSSION

To investigate p120$^{ctn}$'s role in early vertebrate development, we elevated its expression in *Xenopus* embryos via the microinjection of p120$^{ctn}$1A mRNA and cDNA constructs and analyzed the resulting molecular, cellular, and developmental phenotypes. p120$^{ctn}$ shares a number of similarities with β-catenin and plakoglobin, including the presence of a central Armadillo domain, direct association with cadherins, and its designation as a prominent substrate of src and receptor protein tyrosine kinases (Downing and Reynolds, 1991; Reynolds *et al.*, 1994). In mammalian cells, evidence suggests that p120$^{ctn}$ may participate in transducing signals having subsequent effects upon cadherin function. For example, reports suggest that increased p120$^{ctn}$ phosphorylation following ras transformation or protein kinase C activation correlates with p120$^{ctn}$'s increased association with cadherin and the decreased association of the cadherin–catenin complex with cortical actin cytoskeleton (Kinch *et al.*, 1995; Skoudy *et al.*, 1996). Likewise, a greater amount of p120$^{ctn}$ was found to be associated with E-cadherin in MDCK cells transfected with a temperature-sensitive v-src compared to normal MDCK cells (Aghib and McCrea, 1995).

We report that p120$^{ctn}$ has a distinct role in *Xenopus* development relative to β-catenin. In contrast to β-catenin, the ventral microinjection of p120$^{ctn}$ did not cause the

morphological generation of an ectopic (duplicate) dorsoanterior axis, nor on a molecular level did it induce the expression of known target genes of the Wnt pathway such as Siamois. The overexpression of p120$^{ctn}$ within dorsal embryonic tissues further indicated that p120$^{ctn}$'s developmental roles are distinctive. Whereas the dorsal introduction of exogenous β-catenin results in embryos displaying few defects, the dorsal overexpression of p120$^{ctn}$ had graphic developmental consequences, including decreased cell–cell adhesion, failure of blastopore closure, misdirected convergence of the axial mesoderm toward the dorsal midline and defects in neural-fold closure. Thus, p120$^{ctn}$'s developmental functions clearly differ from those of β-catenin. With additional Armadillo proteins being identified and sequenced, it is becoming apparent that p120$^{ctn}$, together with p0071, ARVCF, plakophilins 1 and 2, and δ-catenin, form a subfamily of the Armadillo proteins that is distinct from β-catenin and plakoglobin (Hatzfeld and Nachtsheim, 1996; Reynolds and Daniel, 1997; Keirsebilck *et al.*, 1998).

*Xenopus* gastrulation is driven by convergent extension movements of the mesoderm, which is induced in equatorial (marginal) cells in response to vegetal signals (Smith and Howard, 1992). Gastrulation begins with the apical constriction of the bottle cells within the dorsal marginal zone, spreading laterally and ventrally to form an encircling band of involuting mesoderm. Because disruption of the mesoderm was one possible cause of the abnormalities manifest during gastrulation, we tested whether the dorsal misexpression of p120$^{ctn}$ might alter inductive events. Interestingly, study of the spatial patterning of *Xenopus* brachyury (Xbra), a panmesodermal marker (Smith *et al.*, 1991); *Xenopus* not 1 (Xnot1), a dorsal mesodermal marker (Von Dassow *et al.*, 1993); and goosecoid (gsc), a dorsal mesodermal marker (Cho *et al.*, 1991), revealed no alteration in mesodermal induction.

However, the later examination of Xbra and Xnot1 expression in neurula-stage embryos revealed that presumptive notochord tissues were displaced in embryos having experienced incomplete blastopore closure. These same embryos exhibited failed neural-fold closure at later tailbud stages and, on occasion, the formation of ancillary tails. Such secondary tails likely resulted from the failure of the "tail organizer" to coalesce into a single tissue primordium at the posterior dorsal midline following gastrulation, resulting in the establishment of two bilaterally distinct organizers each capable of directing tail formation. Our results together suggest that p120$^{ctn}$ does not contribute to cell fate specification or axis formation during early vertebrate development, but rather that p120$^{ctn}$ is required in directing prescribed cell movements at these times. Thus, we expect that the effects of p120$^{ctn}$ overexpression were more pronounced following dorsal, rather than ventral, microinjections because cells of the dorsal marginal zone normally undergo considerably more extensive movements than cells of the ventral marginal zone (Keller and Danilchik, 1988).

Several observations indicate that the phenotypic effects we observed following heightened p120$^{ctn}$ expression resulted from the specific disruption of cadherin functions. First, exogenous p120$^{ctn}$ binds endogenous C-cadherin in a manner indistinguishable from endogenous p120$^{ctn}$, suggesting that the exogenous protein could functionally complement endogenous p120$^{ctn}$ and might competitively sequester additional endogenous factors necessary for cadherin function. Second, the negative control construct ΔR8-10, which contains a small deletion of Armadillo repeats 8–10, bound cadherin with much-reduced effectiveness, as anticipated (Reynolds *et al.*, 1996), and correspondingly did not display a discernible embryonic phenotype. Third, normal embryonic development followed the microinjection of frame-shifted p120$^{ctn}$ mRNA, or of β-galactosidase mRNA, indicating that p120$^{ctn}$'s effects were not a simple consequence of expressing exogenous mRNAs or proteins. Finally, in contrast to p120$^{ctn}$, normal embryonic development followed the heightened dorsal expression of β-catenin, which itself contains an Armadillo domain and binds cadherins. Thus, the elevated expression of p120$^{ctn}$ appears to have distinct *in vivo* functions in early vertebrate development.

It seems likely that the primary function of p120$^{ctn}$ is the modulation of cadherin adhesion, which we know from the work of others is a dominant facilitator of intercellular adhesion and motility taking place during *Xenopus* morphogenesis (Brieher and Gumbiner, 1994; Lee and Gumbiner, 1995). A previous study indicates, for example, that the elevated exogenous expression of wildtype C-cadherin or a dominant-negative construct possessing only C-cadherin's extracellular and transmembrane domains each result in defective gastrulation (Lee and Gumbiner, 1995). Interestingly, the phenotypes arising from the elevated expression of the C-cadherin dominant-negative construct are quite similar to those we observe following increased p120$^{ctn}$ expression, including failure of blastopore closure and adhesion defects. These observations suggest that the modulation of cadherin adhesive function is required for the prescribed intercellular adhesion and motility processes occurring during gastrulation, and that even modest changes in cadherin function are disruptive.

The elevated expression of p120$^{ctn}$ reported in this study resulted in decreased cadherin-mediated cell–cell adhesion as assayed *in vitro*, while that of ΔR8-10 did not, explicitly suggesting a biochemical link between the phenotypes arising from exogenous p120$^{ctn}$ expression and reduced cadherin function. In parallel, lineage tracing conducted *in vivo* revealed that normal cadherin-dependent cell–cell interactions were perturbed in p120$^{ctn}$ microinjected embryos, as evident in the poorly directed convergence of cells toward the dorsal midline. While our results support the view that p120$^{ctn}$ is important to cadherin-dependent adhesion and motility, the mechanism by which p120$^{ctn}$ exerts its effects upon cadherin function remains an important question.

In this regard, a recent report demonstrates that p120$^{ctn}$ binds to a region of cadherin that is sufficient for the

clustering and consequent "adhesive strengthening" of cadherin contacts, which occurs as cells become more intimately opposed. The juxtamembrane region of cadherins contains the p120$^{ctn}$ binding site (Finnemann *et al.*, 1997; Yap *et al.*, 1998; Thoreson and Reynolds, unpublished results), which comprises a domain that had earlier been implicated in suppressing cadherin-dependent cell motility (Riehl *et al.*, 1996; Chen *et al.*, 1997). It is possible that p120$^{ctn}$ modulates cadherin function by binding to this membrane proximal domain and that elevated expression of p120$^{ctn}$ results in the occupation of this juxtamembrane site to an increased extent. Whether p120$^{ctn}$ binding promotes or inhibits cadherin clustering remains to be clarified (Yap *et al.*, 1998; Ozawa and Kemler, 1998). By binding to this region, p120$^{ctn}$ may either mediate the interaction (clustering) of cadherin molecules or prevent the interaction of cadherin juxtamembrane domains and inhibit clustering. Furthermore, it is also possible that the exogenous p120$^{ctn}$ isoform we expressed, p120$^{ctn}$1A, displaced endogenous isoforms having greater propensities to cluster cadherins and engage in adhesive strengthening. Indeed, type 1 isoforms (for example p120$^{ctn}$1A and p120$^{ctn}$1B) are enriched in mammalian cells such as fibroblasts displaying high motility, whereas type 3 isoforms (formally referred to as p120$^{cas}$2A and 2B) are enriched in cells such as epithelia having well-developed cell–cell contacts (Reynolds *et al.*, 1994; Mo and Reynolds, 1996).

The possibility that the various p120$^{ctn}$ isoforms may have differing properties *in vivo* is further suggested by a recent report employing murine p120$^{ctn}$1B in the *Xenopus* system (Geis *et al.*, 1998). In common with our study, this isoform was exogenously expressed in developing *Xenopus* embryos and indicated that p120$^{ctn}$ does not engage in Wnt signaling. While p120$^{ctn}$1B's elevated dorsal expression (2.0 ng mRNA) similarly resulted in the generation of gastrulation defects (26.5%), a high incidence of anterior abnormalities (41%) were reported compared to our studies employing p120$^{ctn}$1A, in which posterior defects were predominant. In addition to differences arising from the choice of exogenous (murine) p120$^{ctn}$ isoforms, Geis *et al.* (1998) employed mRNA microinjections of considerably higher dose (1–2 ng versus 0.2 ng). For the p120$^{ctn}$ isoform used in this study, expression of p120$^{ctn}$1A at higher doses (0.5–1 ng) produced a small percentage of embryos displaying anterior abnormalities, while 80% of these embryos displayed abnormalities in gastrulation (data not shown). Microinjection of p120$^{ctn}$1B mRNA at a dose similar to the mRNA concentration used in this paper (0.25 ng) resulted in reduced head structures for 8% of the embryos and had no effect on blastopore closure (Geis *et al.*, 1998). Considering the number and consistency of the negative controls employed in our study, we expect that the developmental aberrations reported result from the perturbation of authentic *in vivo* functions of p120$^{ctn}$, which notably appear to include the modulation of cadherin-dependent cell–cell adhesion and motility. The fully comprehensive analysis of p120$^{ctn}$ in early *Xenopus* development awaits the cloning of the entire *Xenopus* p120$^{ctn}$ cDNA, identification of the various *Xenopus* isoforms, and the comparative evaluation of their functions using elevated expression, dominant-negative, and antisense approaches.

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