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β-catenin mediates the specification of endoderm cells in ascidian embryos

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SUMMARY

In the present study, we addressed the role of β-catenin in the specification of embryonic cells of the ascidians *Ciona intestinalis* and *C. savignyi* and obtained the following results: (1) During cleavages, β-catenin accumulated in the nuclei of vegetal blastomeres, suggesting that it plays a role in the specification of endoderm. (2) Mis- and/or overexpression of β-catenin induced the development of an endoderm-specific alkaline phosphatase (AP) in presumptive notochord cells and epidermis cells without affecting differentiation of primary lineage muscle cells. (3) Downregulation of β-catenin induced by the overexpression of cadherin resulted in the suppression of endoderm cell differentiation. This suppression was compensated for by the differentiation of extra epidermis cells. (4) Specification of notochord cells did not take place in the absence of endoderm differentiation. Both the overexpression of β-catenin in presumptive notochord cells and the downregulation of β-catenin in presumptive endoderm cells led to the suppression of *Brachyury* gene expression, resulting in the failure of notochord specification. These results suggest that the accumulation of β-catenin in the nuclei of endoderm progenitor cells is the first step in the process of ascidian endoderm specification.

Key words: Ascidian, β-catenin, Endoderm specification, Cadherin, Notochord differentiation

INTRODUCTION

The endoderm of an ascidian tadpole larva is a simple tissue comprising about 500 cells (reviewed by Satoh, 1994), and their lineage is almost completely documented (e.g. Nishida, 1987). Most of the endoderm cells are derived from the vegetal A4.1 and B4.1 blastomeres of the bilaterally symmetrical 8-cell stage embryo. The developmental potential to form endoderm is segregated into A5.1, A5.2 and B5.1 of the 16-cell stage embryo, and then into A6.1, A6.3 and B6.1 of the 32-cell stage embryo. As early as the 32-cell stage, A6.1 becomes restricted to generate endoderm only. At the 64-cell stage, not only A7.1 and A7.2 (A6.1 daughter cells) but also A7.5, B7.1 and B7.2 become endoderm-restricted. These primordial cells divide five or six times to form the endodermal tissue of about 500 cells.

Reflecting an early fate restriction, presumptive endoderm blastomeres show a high potential for autonomous differentiation when they are isolated from early embryos (Reverberi and Minganti, 1946; Whittaker, 1990; Nishida, 1992). This autonomy is dependent on maternal factors or determinants that are prelocalized in the endoplasm of eggs and early embryos (Nishida, 1993). In addition, it has been shown that the endoderm cells induce the differentiation of notochord cells at the 32-cell stage (Nakatani and Nishida, 1994). Although recent studies have identified developmental genes that are expressed zygotically in the endoderm tissue, including the LIM class homeobox gene *HrLim* (Wada et al., 1995), *fork head/HNF-3β* (Corbo et al., 1997b; Olsen and Jeffery, 1997; Shimauchi et al., 1997) and *alkaline phosphatase* gene (Kumano and Nishida, 1998), it remains to be elucidated how the first step of endodermal specification takes place.

Specification of embryonic axes after fertilization is the first and key event to achieve the animal body plan, and therefore has been the subject of intensive studies. Convincing evidence showing an involvement of β-catenin in the axis specification of various animal groups has recently been accumulated. β-catenin specifies the dorsoventral axis in *Xenopus* and zebrafish (Heasman et al., 1994; Funayama et al., 1995; Guger and Gumbiner, 1995; Kelly et al., 1995; Schneider et al., 1996; Larabell et al., 1997) and the animal-vegetal axis in sea urchin (Wikramanayake et al., 1998; Logan et al., 1999). β-catenin also specifies the endoderm during *C. elegans* embryogenesis (Thorpe et al., 1997; Rocheleau et al., 1997). To exert its function, β-catenin enters the nucleus and activates downstream genes together with TCF/LEF1, which is a transcriptional factor with an HMG box (reviewed by Willert and Nusse, 1998). β-catenin is a downstream component of the Wnt signaling pathway. The Wnt signal is received by a frizzled (fz)-like receptor, and the receptor activates the cytoplasmic protein dishevelled (Dsh). Dsh negatively regulates glycogen synthase kinase-3β (GSK3β), which works as a negative regulator of β-catenin (reviewed by Cadigan and Nusse, 1997). It has been shown that GSK3β is involved in the axis specifications of the above-mentioned animals (Dominguez et al., 1995; He et al., 1995; Pierce and Kimelman, 1995; Yost et al., 1996, 1998; Sumoy et al., 1999).

posterior end mark (*pem*) is a gene whose maternal transcripts are localized in the posterior-vegetal cytoplasm of fertilized eggs and the posterior-vegetal blastomeres of early

embryos of the ascidian *Ciona savignyi* (Yoshida et al., 1996). Overexpression of PEM causes a loss of the anterior and dorsal structures of the larva (Yoshida et al., 1996). By contrast, lithium treatment of *C. savignyi* early embryos causes tail reduction and the anterior translocation of A4.1-line notochord cells into the trunk region and their subsequent development into endoderm cells (Yoshida et al., 1998). Interestingly, the loss of the anterior and dorsal structures in *pem*-overexpressing larvae could be rescued by lithium treatment (Yoshida et al., 1998). Lithium treatment is known to inhibit GSK3β (Klein and Melton, 1996; Stambolic et al., 1996; Hedgepeth et al., 1997), suggesting that β-catenin may be involved in these changes in cell fates of ascidian embryos. Overexpression of β-catenin in A4.1 blastomeres has effects similar to those of lithium treatment, namely that A4.1-line notochord cells are transformed into endoderm cells within the trunk region (Yoshida et al., 1998). However, it remains to be answered whether the role of β-catenin is restricted to this notochord-endoderm transformation. The aim of the present study was therefore to determine the role of β-catenin in the specification of ascidian embryonic cells. We first examined the accumulation of β-catenin in the nuclei of vegetal blastomeres of early embryos, then we examined whether or not mis- and/or overexpression of β-catenin would induce the differentiation of endoderm cells. Finally, we examined whether the downregulation of β-catenin by overexpressing cadherin would cause the suppression of endoderm cell differentiation.

MATERIALS AND METHODS

Ascidian eggs and embryos

Adults of *Ciona intestinalis* and *C. savignyi* were maintained under constant light to induce oocyte maturation. Eggs and sperm were obtained surgically from the gonoduct. After insemination, embryos were reared at 18°C in Millipore-filtered sea water (MFSW) containing 50 μg/ml streptomycin sulfate. Only batches in which 90% or more of the eggs developed normally were used for experiments.

Isolation and characterization of cDNA clones for ascidian genes

In the present study we isolated cDNA clones for the alkaline phosphatase (*AP*) gene from *C. intestinalis* (*Ci-AP*) and *C. savignyi* (*Cs-AP*), for the *Brachyury* (*Cs-Bra*) gene of *C. savignyi*, for the *C. intestinalis β-catenin* gene (*Ci-β-catenin*) and for the *C. intestinalis cadherin* gene (*Ci-cadherin*).

Because an approach using low-stringency hybridization using *Halocynthia roretzi AP* (*HrES-AP*) cDNA (Kumano and Nishida, 1998) as a probe did not succeed, we isolated fragments of *Ci-AP* and *Cs-AP* cDNAs by polymerase chain reaction (PCR) with degenerate primers. Full-length cDNA clones were isolated from the tailbud-stage cDNA libraries of both species. *Cs-Bra* cDNA of *C. savignyi* was isolated by low-stringency hybridization using *C. intestinalis Ci-Bra* cDNA as a probe. A cDNA clone for *C. intestinalis β-catenin* (*Ci-β-catenin*) was isolated in the process of identification of the genes downstream *Ci-Bra* (Takahashi et al., 1999). *C. intestinalis cadherin* (*Ci-cadherin*) cDNA was isolated by low-stringency hybridization using mouse E-cadherin cDNA as a probe (kindly provided by Dr M. Takeichi).

Nucleotide sequences were determined for both strands with a Big-Dye Primer Cycle Sequencing Ready Reaction kit and ABI PRISM 377 DNA sequencer (Perkin Elmer).

Antibody staining

The protocol for antibody staining was a modification of the method previously described by Miller and McClay (1997). *C. savignyi* embryos were fixed in 2% paraformaldehyde sea-water for 1 hour followed by a brief rinse with 100% methanol to permeabilize the embryos. Embryos were incubated with primary antibody in sea water containing 5% fetal bovine serum and 0.05% Tween-20 for 1 hour at room temperature. Affinity-purified β-catenin polyclonal antibody, which was kindly provided by Dr David McClay of the Duke University, was used at 1:100 dilution. Following four washes in sea water containing 0.05% Tween-20, embryos were incubated with HRP-conjugated rabbit anti-guinea pig secondary antibody (Sigma) at 1:400 dilution for 1 hour at room temperature. After washing as before, embryos were reacted with DAB substrate (0.06% w/v diaminobenzidine, 0.015% v/v hydrogen peroxide in PBST: phosphate buffer saline containing 0.1% Tween-20). After clearing with benzylalchol and benzylbenzoate (1:2 v/v; BABB), embryos were observed with a light microscope. Staining was not observed when embryos were treated only with the secondary antibody.

Rabbit anti-*Xenopus*-β-catenin polyclonal antibody was kindly provided by Dr McCrea, University of Texas, Houston. β-catenin-myc was detected with mouse anti-myc monoclonal antibody (Berkeley antibody company, CA, USA). The staining protocol was the same as that described above.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed using digoxigenin (DIG)-labeled antisense probes as described by Satou (1999). RNA probes were prepared with a DIG RNA labeling Kit (Boehringer-Mannheim). Control embryos hybridized with a sense probe did not show any signal above background levels.

Histochemical staining for alkaline phosphatase and acetyl cholinesterase

Differentiation of endoderm cells was monitored by histochemical detection of alkaline phosphatase (AP) activity. Embryos were fixed with 5% formaldehyde in sea water for 10 minutes at room temperature. Embryos were washed in AP staining buffer (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5) twice. For signal detection, specimens were incubated with NBT/BCIP/AP (NBT, Nitro Blue Tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate) staining buffer (2.25 μg/ml NBT, 1.75 μg/ml BCIP).

Differentiation of muscle cells was examined by histochemical reaction of acetylcholinesterase (AChE). Embryos were fixed with 5% formaldehyde in sea water for 10 minutes at room temperature. The specimens were washed in AChE staining buffer (65 mM sodium acetate, 3 mM copper sulfate, 0.5 mM potassium ferricyanide, 5 mM sodium citrate, pH 5.5) twice, and then the buffer was replaced with AChE staining buffer containing 0.2 mg/ml acetylthiocholine iodide. The reaction was performed at 4°C overnight.

RNA isolation and northern blotting

Total RNA was isolated from eggs or embryos by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was purified using Oligotex beads (Roche Japan). For northern blotting, total or poly(A)$^+$ RNA was fractionated by agarose gel electrophoresis, and transferred to Hybond-N (+) membranes (Amersham). Blots were hybridized with a [^{32}P]-random-labeled DNA probe in 6× SSPE, 0.5% SDS, 5× Denhardt's solution, 100 μg/ml salmon sperm DNA and 50% formamide at 42°C. The filters were washed twice in 2× SSC/0.1% SDS, and twice in 0.2× SSC/0.1% SDS at 60°C, and then exposed to X-ray film.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

20 embryos were lysed in 200 μl of GTC solution (4 M guanidinium thiocyanate, 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 2% sarkosyl,

1% β-mercaptoethanol) and the lysates were stored at –80°C. Following phenol-chloroform extraction and isopropanol precipitation, RNA was treated with 1 unit of RNase-free DNase (Life Technologies, Inc., Rockville, MD, USA) for 15 minutes at room temperature and then with 0.2 mg/ml proteinase K for 30 minutes at 37°C. After annealing with 10 pmol oligo(dT), the total RNA was incubated with 200 units of Superscript II reverse transcriptase (Life Technologies, Inc.) for 50 minutes at 42°C in total volume of 20 μl. One-twentieth of the reverse-transcribed cDNA was used as template for the subsequent polymerase chain reaction (PCR). PCRs were performed in total volume of 50 μl containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.05 μl [^{32}P]dCTP, 100 pmol of each primer, 1× Taq buffer, and 1.25 units of Taq DNA polymerase (Toyobo, Osaka, Japan). 15 μl of the reaction products was resolved on 6% non-denaturing polyacrylamide gels and subjected to autoradiography. The number of cycles of PCR reactions within the quantitative range was determined empirically. The primers used in the present study and the number of cycles were as follows: *CsAP*, 5′-GGAATGTTGTGC-CTTACA-3′ and 5′-TCATTATGACCAAAAGGGT-3′, 23 cycles; *CsEpi1*, 5′-TTCGAGCACCTCGAGACA-3′ and 5′-CATTGGCCGT-TCAAAGCA-3′, 20 cycles; *CsMA1*, 5′-TTGCTCCTCCTGAGA-GGA-3′ and 5′-GCCCTTGGTGAAATCAGA-3′, 23 cycles; and *CsCA1*, 5′-CCTTACCCCGAACTTTTA-3′ and 5′-GCTTTGGTC-CTTAATTCA-3′, 23 cycles.

Mis- and/or overexpression of genes with electroporation

Ci-fkh is a *fork head* gene of *C. intestinalis*, which is expressed in the endoderm, endodermal strand, notochord and later in the nerve cord of the tailbud embryo (Corbo et al., 1997b). About 1.4 kb of the *Ci/fkh* promoter of 5′ flanking region was used for mis- and/or overexpression of genes in these tissues (cf. Takahashi et al., 1999). The stability of β-catenin is regulated by GSK3β. The putative phosphorylation sites for GSK3β are located at the N terminus of the β-catenin protein. It has been shown that β-catenin without the N-terminal region of the GSK3β phosphorylation sites is more stable and behaves as an active form (Yost et al., 1996), therefore we used *Ci-β-catenin* without its N-terminal 39 residues, and thus lacking the putative GSK3β phosphorylation sites (Fig. 1A). The coding region of *Ci-β-catenin* cDNA was amplified with the high-fidelity KOD DNA polymerase (Toyobo), and cloned downstream of the promoter and the N-terminal coding sequence of *Ci-fkh*.

For mis- and/or overexpression of *Ci-cadherin*, the coding sequence of *Ci-cadherin* cDNA was amplified with KOD DNA polymerase and cloned directly downstream of the *Ci-fkh* promoter. Electroporation was performed as described (Corbo et al., 1997a).

Overexpression of genes by microinjection of synthetic capped mRNA

Synthetic capped mRNAs were synthesized from cDNAs cloned into pBluescript RN3 vector (Lemaire et al., 1995) using a Megascript T3 kit (Ambion, Austin, TX, USA). To obtain capped mRNAs, the concentration of GTP was lowered to 1.5 mM and the cap analog 7mGpppG was added at a final concentration of 6 mM. The injection of mRNAs into fertilized eggs was performed as described previously (Hikosaka et al., 1992).

RESULTS

Isolation and characterization of cDNA clones for *Ciona* genes

To investigate the role of β-catenin in the specification of ascidian embryonic cells, we isolated and characterized cDNAs for the alkaline phosphatase (*AP*) gene of *C. intestinalis* (*Ci-AP*) and *C. savignyi* (*Cs-AP*), cDNA for *C. intestinalis β-catenin* (*Ci-β-catenin*), cDNA for *C. intestinalis cadherin* (*Ci-cadherin*) and cDNA for *C. savignyi Brachyury* (*Cs-Bra*).

Alkaline phosphatase (*AP*) gene

Histochemical assays of AP activity have previously been used to monitor the endoderm differentiation of ascidian embryos (e.g. Whittaker, 1977). A cDNA clone for the *AP* gene (*HrES-AP*) of *Halocynthia roretzi*, the zygotic expression of which is specific to endoderm cells, was isolated previously (Kumano and Nishida, 1998). In the present work we isolated cDNA clones for *Ci-AP* and *Cs-AP* by PCR amplification of target fragments using degenerate primers. The cDNA for *Ci-AP* consisted of 1949 nucleotides, which predicted a polypeptide of 579 amino acids (the *Ci-AP* cDNA nucleotide sequence will appear in the DDBJ/GenBank/EMBL database under the accession number AB031539), while the cDNA for *Cs-AP* consisted of 2089 nucleotides, which predicted a polypeptide

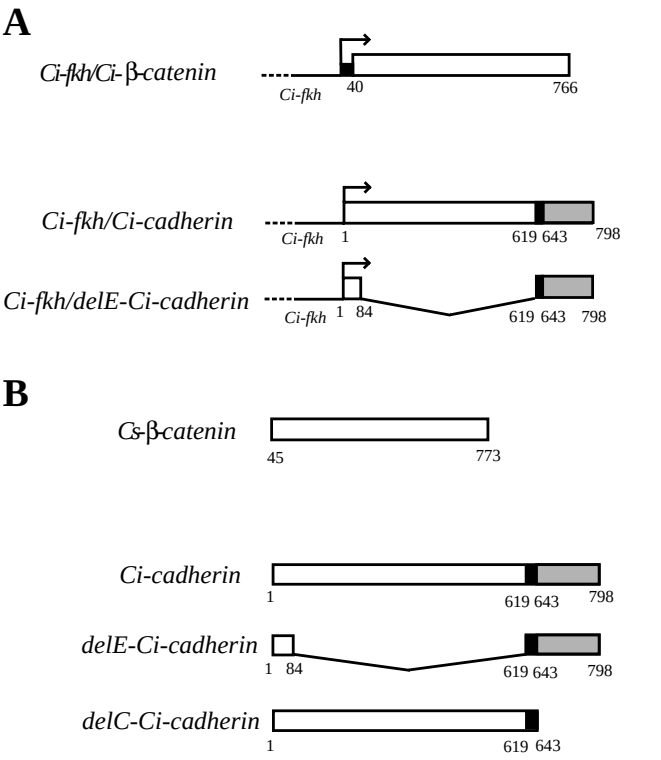

Fig. 1. (A) DNA constructs used for misexpression of genes in *C. intestinalis* embryos by electroporation. For misexpression of *Ci-β-catenin*, the promoter and the N-terminal 87 coding residues (small black box) of *Ci-fkh* (*fork head* gene of *C. intestinalis*) were connected to *Ci-β-catenin* cDNA lacking the N-terminal 39 residues (white box). For misexpression of *Ci-cadherin*, two kinds of construct were prepared: *Ci-fkh/Ci-cadherin* contained the entire coding sequence including the extracellular domain (white box), transmembrane domain (black box) and cytoplasmic domain (gray box), while *Ci-fkh/delE-Ci-cadherin* lacked the extracellular domain except for the signal peptide at the N terminus. (B) mRNAs used for overexpression of genes in *C. savignyi* embryos by microinjection. *Cs-β-catenin* mRNA lacked the N-terminal 44 residues that encode putative GSK3β phosphorylation sites. *delE-Ci-cadherin* contained only the transmembrane domain (black box) and cytoplasmic domain (gray box) but not the extracellular domain (except for the N-terminal signal peptide), while *delC-Ci-cadherin* lacked the cytoplasmic domain.

of 570 amino acids (the database accession number, AB031538).

In situ hybridization and quantitative reverse transcription-polymerase chain reaction (RT-PCR) showed that *Ciona AP* is expressed both maternally and zygotically and that the zygotic expression is restricted to endoderm cells of tailbud embryos (Fig. 2). Maternal mRNA of *Cs-AP* was distributed evenly in the egg cytoplasm, suggesting that the mRNA is not anchored by special cytoplasmic components (Fig. 2A). However, the mRNA gradually disappeared in blastomeres other than the

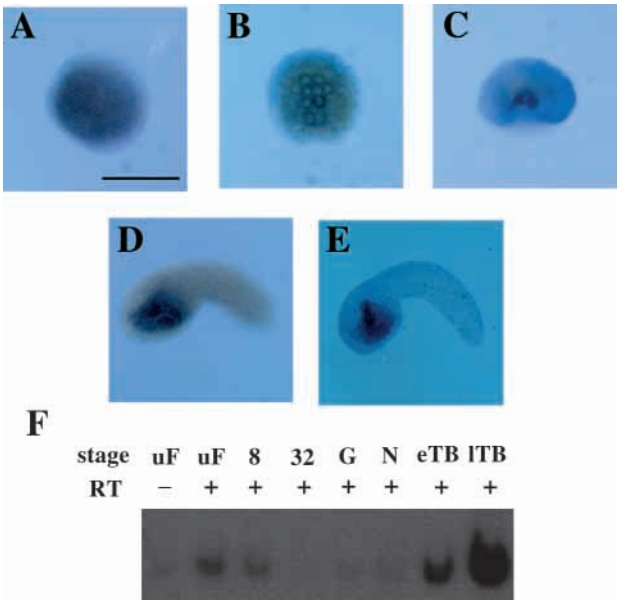

Fig. 2. Expression of *Ciona AP* gene. (A-C) Whole-mount in situ hybridization showing maternal *Cs-AP* mRNA in a fertilized egg (A) and in a 110-cell embryo viewed from the vegetal side (B) and posterior side (C). Bar, 100 μm. (D,E) Whole-mount in situ hybridization showing zygotic *AP* mRNA in the tailbud embryo of *C. savignyi* (D) and *C. intestinalis* (E). Hybridization signal is detected exclusively in the endoderm. (F) Temporal expression pattern of *Cs-AP* detected by quantitative RT-PCR. Each PCR reaction was performed using cDNA that was reverse-transcribed from RNA. The amount of mRNA corresponds to that extracted from one egg or one embryo. Stages indicated at the top are: uF, an unfertilized egg; 8, an 8-cell embryo; 32, a 32-cell embryo; G, a gastrula; N, a neurula; eTB, an early tailbud embryo; lTB, a late tailbud embryo. A negative control without reverse transcriptase is shown in the left lane (RT–).

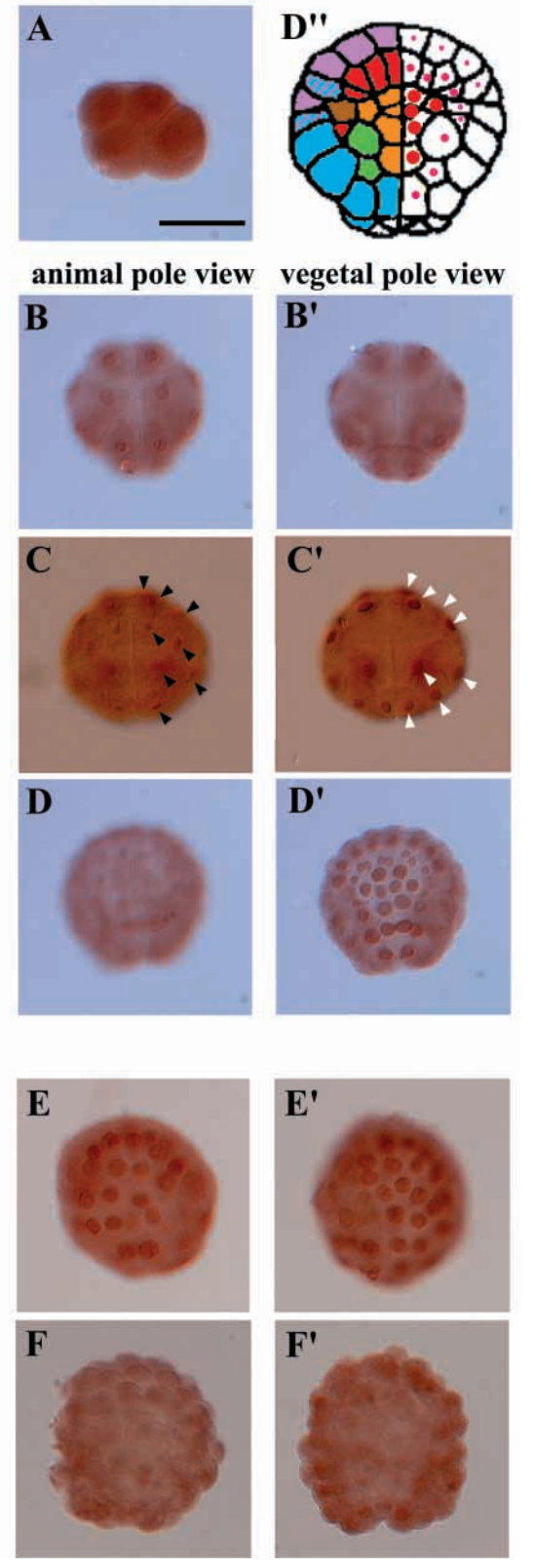

Fig. 3. Patterns of nuclear β-catenin accumulation in normal embryos (A-D) and in manipulated embryos (E,F). (A) An 8-cell embryo, side view. Bar, 100 μm for all panels. (B) A 16-cell embryo, animal pole view (B) and vegetal pole view (B′), showing the uniform distribution of β-catenin in the nucleus and the cytoplasm. (C) A 32-cell embryo showing the nuclear accumulation of β-catenin in vegetal blastomeres (C′, white arrowheads), compared to the cytoplasmic staining of animal blastomeres (C; black arrowheads indicate the nuclei). (D) A 110-cell embryo showing elevated levels of nuclear β-catenin in vegetal blastomeres (D′) compared to levels observed in animal blastomeres (D). (D″) A schematic drawing showing the fates of blastomeres that accumulate β-catenin in their nuclei. The size of the nucleus corresponds to the relative quantity of nuclear β-catenin accumulation. Yellow, endoderm; red, notochord; green, mesenchyme; blue, muscle; brown, trunk lateral cells; and pink, nerve cord. (E) A 110-cell embryo that developed from an egg injected with β-catenin mRNA, showing nuclear β-catenin accumulation in animal (E) and vegetal blastomeres (E′). (F) A 110-cell embryo that developed from an egg injected with cadherin mRNA, showing the loss of nuclear β-catenin accumulation in animal (F) and vegetal blastomeres (F′).

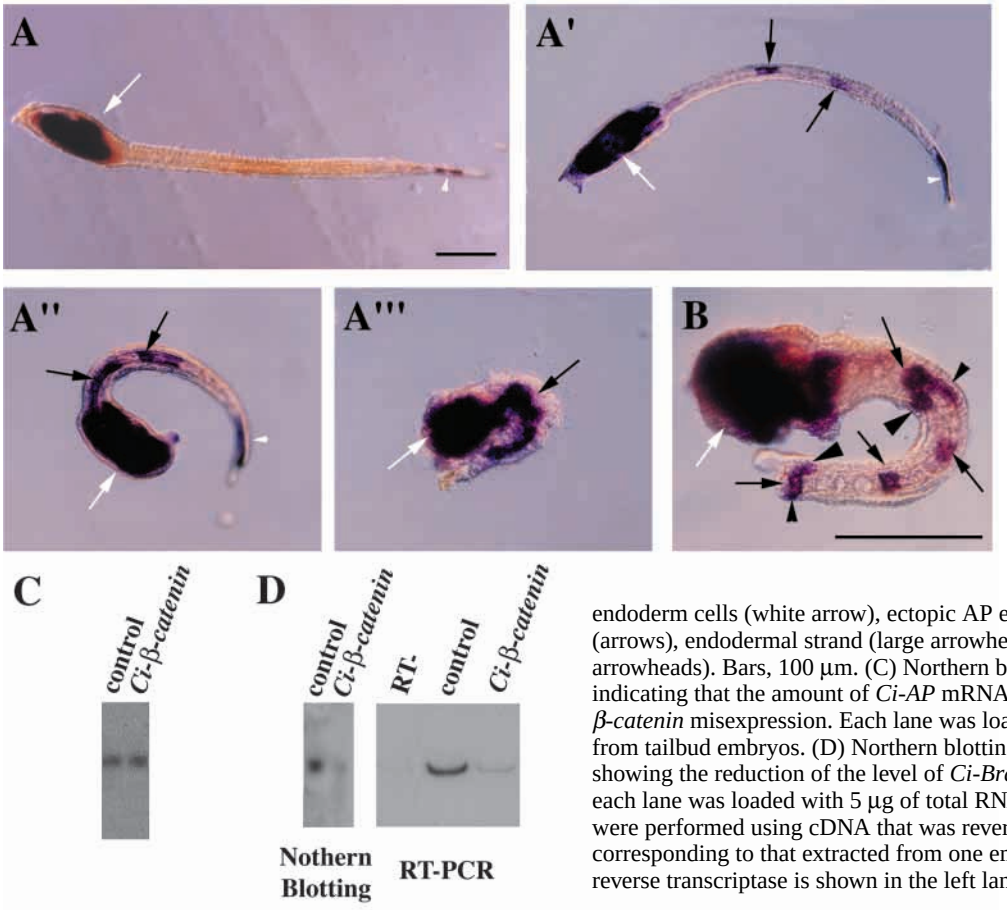

Fig. 4. Mis- and/or overexpression of *Ci-β-catenin* in notochord, endodermal strand and nerve cord of *C. intestinalis* embryos. (A-A‴) Histochemical staining of AP with a mock-electroporated larva (A) and larvae electroporated with *Ci-fkh/Ci-β-catenin* (A′-B). In the mock-electroporated larva (A), only endoderm cells (white arrow) and cells at the tip of the tail (white arrowhead) are stained. In contrast, ectopic AP expression is detected in notochord cells (arrows) of larvae electroporated with *Ci-fkh/Ci-β-catenin* (A′,A″,A‴). (B) High magnification of a *Ci-fkh/Ci-β-catenin* electroporated larva. In addition to expression of AP in endoderm cells (white arrow), ectopic AP expression is evident in notochord (arrows), endodermal strand (large arrowheads) and nerve cord (small arrowheads). Bars, 100 μm. (C) Northern blotting with *Ci-AP* cDNA probe, indicating that the amount of *Ci-AP* mRNA was not increased in embryos with *Ci-β-catenin* misexpression. Each lane was loaded with 0.45 μg of poly(A)$^+$ RNA from tailbud embryos. (D) Northern blotting and quantitative RT-PCR analysis showing the reduction of the level of *Ci-Bra* expression. In the northern blots, each lane was loaded with 5 μg of total RNA of gastrulae. In RT-PCR, reactions were performed using cDNA that was reverse-transcribed from an amount of RNA corresponding to that extracted from one embryo. A negative control without reverse transcriptase is shown in the left lane (RT−).

presumptive endoderm cells until the 110-cell stage (Fig. 2B,C; refer to Fig. 3D″). After the neurula stage, *Cs-AP* zygotic transcript became evident in endoderm cells (Fig. 2D). The amount of mRNA increased dramatically at the late-tailbud stage (Fig. 2F). *Ci-AP* exhibited an expression pattern similar to that of *Cs-AP* (Fig. 2E).

β-catenin gene

A cDNA clone for the *β-catenin* gene (*Cs-β-catenin*) of *C. savignyi* has already been characterized by Yoshida et al., (1998). A cDNA clone for the *C. intestinalis β-catenin* gene (*Ci-β-catenin*) was isolated in the process of identification of *Ci-Bra* target genes (Takahashi et al., 1999), and it was fully characterized in the present study (database accession number, AB031543). The cDNA consisted of 2702 nucleotides that encoded a polypeptide of 769 amino acids. Both Ci-β-catenin and Cs-β-catenin have putative phosphorylation sites for GSK3β in the N terminus (data not shown). Maternal transcripts of *Ci-β-catenin* and *Cs-β-catenin* are distributed throughout the entire cytoplasm of eggs and early embryos (data not shown).

cadherin gene

A cDNA clone for a *C. intestinalis* homolog of *cadherin* was isolated by low-stringency hybridization screening with mouse E-cadherin cDNA as probe (database accession number, AB031540). The cDNA consisted of 3182 nucleotides that predicted a polypeptide of 798 amino acids. The polypeptide had a transmembrane domain and a signal peptide (data not shown). Database searches suggested that Ci-cadherin resembled cadherin of the colonial ascidian *Botryllus schlosseri* (Levi et al., 1997) and human E-cadherin (Bussemakers et al., 1993).

Brachyury gene

Brachyury of *C. intestinalis* (*Ci-Bra*) has been characterized (Corbo et al., 1997a). To monitor notochord differentiation in relation to endoderm specification of *C. savignyi* embryos, a cDNA clone for the *C. savignyi Brachyury* gene (*Cs-Bra*) was isolated by low-stringency hybridization with *Ci-Bra* probe (database accession number, AB031545). The cDNA consisted of 1621 nucleotides, which predicted a polypeptide of 491 amino acids. Like *As-T* of *H. roretzi* (Yasuo and Satoh, 1993) and *Ci-Bra* of *C. intestinalis* (Corbo et al., 1997a), *Cs-Bra* was expressed exclusively in notochord cells (see Figs 5I, 7H).

Pattern of nuclear β-catenin localization during early development

The signaling activity of β-catenin is thought to be linked to its accumulation in the nucleus (Miller and Moon, 1996; Cavallo et al., 1997). If β-catenin were involved in signaling events associated with early cell-fate specification in ascidian embryos, as demonstrated in sea urchin embryos (Logan et al., 1999), β-catenin would be expected to accumulate in nuclei during the early phase of cell specification. Using an affinity-purified polyclonal antibody raised against *Lytechinus*

variegatus β-catenin (Miller and McClay, 1997), we determined the subcellular distribution of β-catenin in embryos from early cleavage stage until just prior to gastrulation.

In early embryos up to the 16-cell stage, β-catenin appeared to be uniformly distributed in the nucleus and cytoplasm in both the animal and vegetal blastomeres (Fig. 3A,B). Animal-vegetal differences in the levels of β-catenin were first detected at the 32-cell stage (Fig. 3C). In the 32-cell embryo, blastomeres of the vegetal hemisphere showed elevated levels of nuclear β-catenin relative to those observed in animal blastomeres. This quantitative difference in nuclear β-catenin levels became more evident as development proceeded. At the 110-cell stage, the nuclear accumulation of β-catenin was most evident in A- and B-line endoderm cells, then the level appeared to decrease in notochord and mesenchyme cells (Fig. 3D′), becoming less evident in the animal hemisphere blastomeres (Fig. 3D).

A similar pattern of subcellular distribution of β-catenin was obtained using anti-*Xenopus*-β-catenin (data not shown). Injection of myc-tagged β-catenin mRNA into *Ciona* eggs and the subsequent examination of embryos for nuclear β-catenin accumulation using anti-myc antibody resulted in a similar staining pattern (data not shown).

Effects of mis- and/or overexpression of *Ci-β-catenin*

First, we used electroporation to attempt mis- and/or overexpression of *β-catenin* under the control of the *Ci-fkh* promoter (Fig. 1A). A 2.6-kb genomic DNA fragment from the 5′-flanking region of *Ci-fkh* is sufficient to direct the ectopic expression of *β-catenin* in notochord and nerve cord cells after electroporation into fertilized eggs (cf. Corbo et al., 1997b). Each experiment was repeated at least three times using good batches of eggs. Manipulated embryos developed at almost the same time as control mock-electroporated embryos did. The morphology of most of the experimental larvae was affected, and in most cases, the tails of experimental larvae were shortened (Fig. 4A″-B). Larval endoderm differentiation was examined using a histochemical assay of AP activity. In control mock-electroporated larvae, AP activity was detected in endoderm cells and cells in the tip of the tail (Fig. 4A; Whittaker, 1977; the cells in the tip of the tail are part of the B-line notochord cells in *Ciona* larvae). In *Ci-β-catenin*-electroporated larvae, the domain with AP expression was expanded. Fig. 4A′ shows a larva with normal morphology, in which AP staining was detected in a few notochord cells in addition to the endogenous expression domain. In a larva with a moderately shortened tail, the number of notochord cells with AP expression increased (Fig. 4A″). In a larva with affected morphology, almost all of the notochord cells exhibited AP activity (Fig. 4A‴). In some experimental embryos, ectopic AP activity was evident not only in notochord cells but also in endodermal strand cells and nerve cord cells (Fig. 4B). These findings indicate that mis- and/or overexpression of *β-catenin* induces ectopic expression of AP in cells of the notochord, endodermal strand and nerve cord.

The quantity of *Ci-AP* mRNA in *Ci-β-catenin*-electroporated embryos was compared with that in normal embryos. As shown in Fig. 4C, in spite of expansion of the domain with AP expression, the amount of *Ci-AP* mRNA in experimental embryos was about the same as that in normal embryos. It is likely that endogenous *Ci-AP* is strongly expressed so that changes in the amount of the mRNA due to *Ci-β-catenin*-electroporation did not affect the detection level of the mRNA.

Effects of overexpression of *Cs-β-catenin*

In the experiment described above, *Ci-β-catenin* was misexpressed in cells of specific lineages with the aid of the *Ci-fkh* promoter. In that experiment, however, *Ci-β-catenin* was not misexpressed in cells of muscle or epidermis. Promoters that induce misexpression of a given gene in every blastomere of the ascidian embryo have not yet been devised. Effects of ectopic *β-catenin* expression on specification of muscle and epidermis cells were therefore examined by microinjection of synthetic mRNA, using *C. savignyi* eggs because *C. intestinalis* eggs are too fragile for RNA injection. The eggs were injected with *β-catenin* cDNA lacking the N-terminal region (without the phosphorylation sites for GSK3β, the protein is thought to act as the constitutive active form; Fig. 1B).

Eggs injected with 60 pg of synthetic *Cs-β-catenin* mRNA developed into larvae with truncated tails (Fig. 5A′), while control eggs injected with 60 pg of *lacZ* or *GFP* mRNA developed into normal larvae (Fig. 5A; data not shown). Each experiment was repeated at least three times with different batches of eggs. In the *Cs-β-catenin*-overexpressing embryos, AP activity was detected in almost all regions of the injected embryos except the caudal region (Fig. 5B′).

To examine cells with additional AP activity, we took advantage of cleavage-arrested embryos. When early ascidian embryos are immersed in sea water containing cytochalasin B, cytokinesis but not nuclear division of blastomeres is blocked. Blastomeres of these cleavage-arrested embryos express differentiation markers depending on their lineage (Whittaker, 1973). Embryos injected with *Cs-β-catenin* mRNA were arrested at the 110-cell stage, because the developmental fate of almost all of the blastomeres is restricted to one tissue by this stage (Fig. 5C). AP activity in the cleavage-arrested embryos was examined when untreated control embryos hatched. In uninjected embryos, AP activity was detected in 10 vegetal cells, as expected based on the endoderm lineage (Fig. 5D,E). In embryos injected with *Cs-β-catenin* mRNA, AP activity was evident in almost all of the blastomeres except the primary lineage muscle cells (Fig. 5D′,E′). Three series of experiments with different batches of eggs gave the same results.

To examine the amount of *Cs-AP* mRNA, RT-PCR analysis was performed. To carry out quantitative RT-PCR analysis, RNA isolated from 20 late-tailbud embryos was reverse transcribed, one-twentieth of the reverse-transcribed product was used as the template for the following PCR, and the optimal number of PCR cycles was determined empirically (data not shown). Although the domain with Cs-AP expression was expanded in experimental embryos, the RT-PCR assay indicated that the amount of *Cs-AP* mRNA was not increased compared to that in the controls (Fig. 6A), as was also true in the case of *C. intestinalis*.

As shown in Fig. 5D′,E′, primary lineage muscle cells in *Cs-β-catenin*-overexpressing embryos did not express AP. Differentiation of muscle cells was confirmed by histochemical detection of AChE (Fig. 5F,F′). In addition, the muscle actin

gene *Cs-MA1* was expressed in experimental embryos (Fig. 5G,G′) at the same level as in uninjected control embryos (Fig. 6A).

The expression pattern of AP in *Cs-β-catenin*-overexpressing and cleavage-arrested embryos suggested that epidermis cells, which are derived from blastomeres of the animal hemisphere, were transformed into endoderm cells. To examine this, the *Cs-β-catenin*-mRNA-injected embryos were subjected to in situ hybridization with a probe of the epidermis-specific *Cs-Epi1* gene. The embryos were arrested at the 32-cell stage. As shown in Fig. 5H, uninjected control embryos exhibited strong *Cs-Epi1* expression in the animal blastomeres. In contrast, no expression of *Cs-Epi1* was detected in *Cs-β-catenin*-mRNA-injected embryos (Fig. 5H′). This was further confirmed by RT-PCR analysis. The quantity of *Cs-Epi1* mRNA was significantly reduced in experimental embryos, while the level of cytoplasmic actin (*Cs-CA1*) mRNA was the same in experimental and control embryos (Fig. 6A).

When nuclear β-catenin accumulation was examined with the antibody, the accumulation was evident not only in the vegetal blastomeres (Fig. 3E′) but also in the animal blastomeres of the manipulated 110-cell embryos (Fig. 3E). These results indicate that the injection of *Cs-β-catenin* mRNA induces nuclear accumulation of β-catenin in the animal and vegetal blastomeres, and thus an ectopic expression of endoderm-specific AP due to transformation of cells with epidermis and mesenchyme fate into cells with endoderm fate. Injection of *Cs-β-catenin* mRNA did not affect the differentiation of primary lineage muscle cells.

Effects of downregulation of Ci-β-catenin by overexpression of Ci-cadherin

β-catenin is a molecule with dual functions: one is as a factor downstream of the Wnt signaling pathway and the other is as a cytoskeletal component that is associated with the cytoplasmic domain of cadherin (Nagafuchi and Takeichi, 1988; Ozawa et al., 1989; Gumbiner, 1993). Taking advantage of this feature, cadherin has been used to disturb the β-catenin function (Heasman et al., 1994; Sanson et al., 1996; Wikramanayake et al., 1998; Logan et al., 1999). To confirm this, we isolated a cDNA clone for *C. intestinalis cadherin* (*Ci-cadherin*), as described above. To deplete Ci-β-catenin, *Ci-cadherin* or *delE-Ci-cadherin*, in which the extracellular domain of the molecule is deleted, was misexpressed with the aid of the *Ci-fkh* promoter (Fig. 1A). Larvae with *Ci-cadherin* misexpression showed normal morphology and the endoderm seemed to be normally developed, although northern hybridization with a *Ci-AP* probe showed that the amount of *Ci-AP* mRNA was slightly reduced in the *Ci-cadherin*-misexpressing embryos (Fig. 6B). This kind of misexpression experiment, however, has several problems, one being mosaic incorporation of the gene, meaning that the expression is not uniform but is restricted to cells of several lineages. Another problem is that the efficiency of expression of the introduced gene is different from embryo to embryo.

To avoid mosaic expression and to express the gene more efficiently, mRNA injection was performed. The injection was performed using *C. savignyi* eggs for the reason mentioned above. As shown in Fig. 1B, the three types of *Ci-cadherin* mRNAs prepared were *Ci-cadherin*, which contains the complete coding region, *delE-Ci-cadherin*, which encodes a mutant molecule lacking the extracellular domain, and *delC-Ci-cadherin*, which encodes only the extracellular domain and the transmembrane domain. When 60 pg of the *Ci-cadherin* mRNA was injected into *C. savignyi* fertilized eggs, blastomeres tightly adhered to one another after the 110-cell stage. Experimental embryos developed at almost the same time as control embryos, although the morphology of the larvae was disturbed (Fig. 7A). No nuclear accumulation of β-catenin was observed even in the vegetal blastomeres of the manipulated 110-cell embryo (Fig. 3F,F′). In these larvae, AP activity was not detected or was considerably reduced (Fig. 7D). The injection of *delE-Ci-cadherin* mRNA affected the embryogenesis more severely (Fig. 7B,E). In these embryos, adhesion between blastomeres seemed to be reduced. The *delE-Ci-cadherin*-mRNA-injected larvae exhibited almost no AP activity (Fig. 7E). RT-PCR analysis showed that the amount of *Cs-AP* mRNA was also reduced in the *delE-Ci-cadherin* mRNA overexpressing embryos (Fig. 6A). On the other hand, embryos injected with *delC-Ci-cadherin* mRNA developed normally (Fig. 7C), and all of the experimental larvae exhibited normal AP activity (data not shown). These results provide additional evidence for the essential role of β-catenin in the endoderm specification of ascidian embryos.

We examined whether the failure of endoderm differentiation is associated with effects on the differentiation of muscle cells and epidermis cells. Muscle cell differentiation was evident in the experimental larvae, as shown by histochemical staining of AChE (Fig. 7G). However, RT-PCR suggested that the amount of muscle actin mRNA was slightly reduced (Fig. 6A). Therefore, in situ hybridization of *Cs-MA1* was carried out in *Ci-cadherin*-overexpressing gastrulae. Control embryos arrested at the 110-cell stage expressed *Cs-MA1* in the presumptive muscle cells, as reported before (Fig. 6I; Chiba et al., 1998). Experimental embryos also expressed *CsMA1* (Fig. 7I′).

Differentiation of epidermis cells was examined by in situ hybridization and RT-PCR with a *Cs-Epi1* probe. Experimental embryos injected with *Ci-cadherin* mRNA were arrested at the 32-cell stage with cytochalasin B, because the injection of *Ci-cadherin* mRNA disrupted the morphogenesis during gastrulation and this made it difficult to identify the blastomeres. Hybridization signals were observed throughout the experimental embryos (Fig. 7J′), while in the control embryos the signal was restricted only to several animal cells (Fig. 7J). RT-PCR analysis also showed that the quantity of *Cs-Epi1* mRNA was increased (Fig. 6A), suggesting that the injection of *Ci-cadherin* mRNA causes disturbance of endoderm differentiation, which leads to differentiation of excess epidermis cells.

Relationship between endoderm and notochord specification

Differentiation of ascidian notochord cells requires inductive signal(s) emanating from endoderm cells (Nakatani and Nishida, 1994). In the present study, the differentiation of endoderm cells was experimentally induced or inhibited, providing an opportunity to examine whether these alterations of endoderm differentiation affected notochord specification.

As mentioned above, mis- and/or overexpression of *Ci-β-catenin* in the notochord precursor cells induced ectopic expression of *AP* in notochord cells. *Ci-Bra* is a gene key for

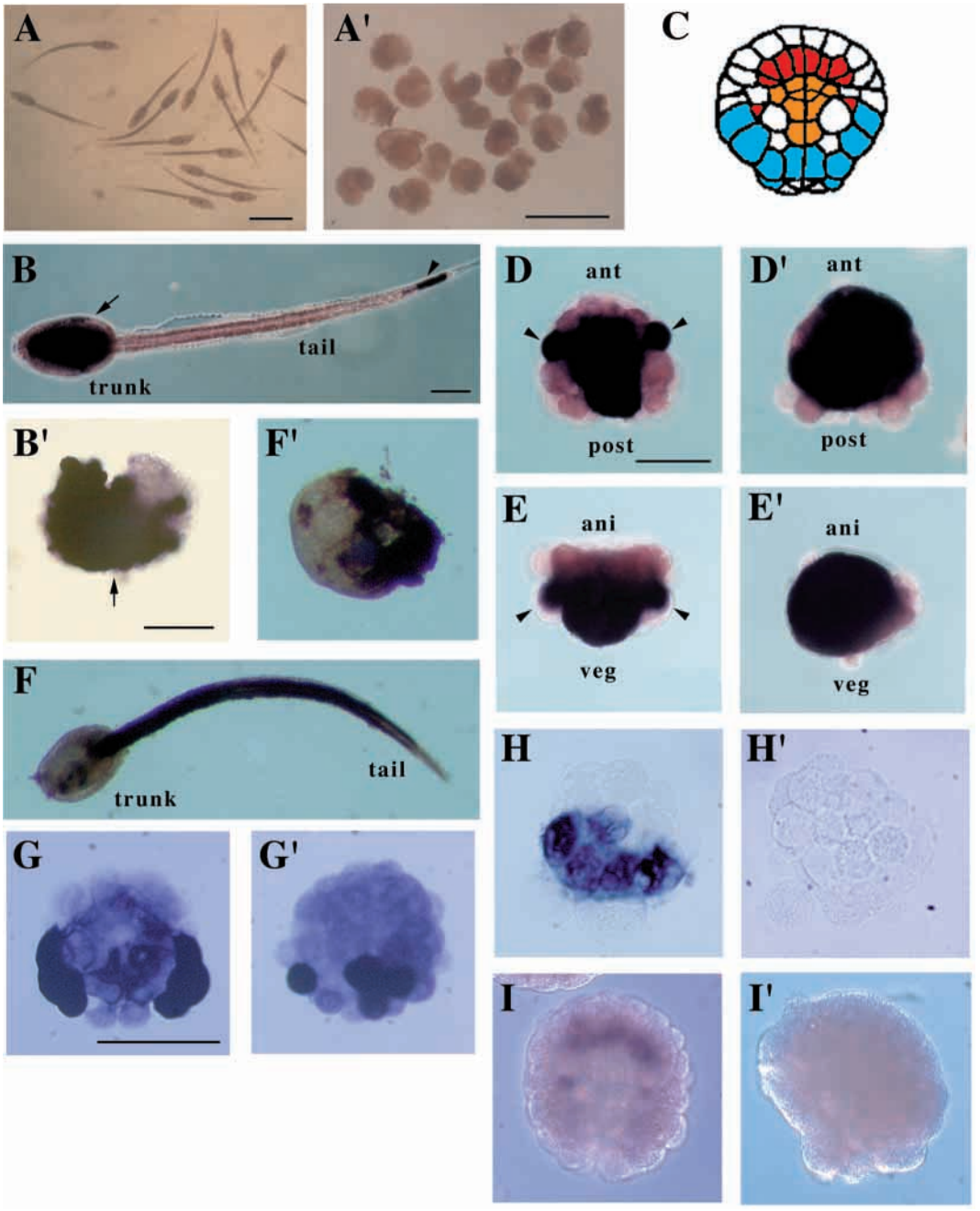

Fig. 5. Effects of *Cs-β-catenin* overexpression on *C. savignyi* embryonic cell specification. (A,A′) Larvae developed from eggs injected with *lacZ* mRNA (A) or *Cs-β-catenin* mRNA (A′). (B,B′) Histochemical staining of AP in larvae developed from eggs injected with *lacZ* mRNA (B) or *Cs-β-catenin* mRNA (B′). (B) In a control larva, endoderm cells (arrow) and cells at the tip of the tail (arrowhead) are stained. (B′) In the *Cs-β-catenin*-overexpressing larva, the region with AP expression is expanded (arrow). (C) Drawing of fates of blastomeres in the 110-cell embryo. Presumptive endoderm cells are shown in yellow, primary lineage muscle cells in blue and notochord cells in red. (D,D′,E,E′) Histochemical staining of AP in embryos arrested at the 110-cell stage. (D,E) In a *lacZ*-mRNA-injected embryo, AP expression is detected only in cells of endoderm lineage. In the embryo, pigment cells that are formed in the brain of normal larvae are also differentiated (arrowheads). (D′,E′) In a *Cs-β-catenin*-mRNA-injected embryo, all of the cells except primary lineage muscle cells express AP. (D,D′) vegetal pole (veg) views; (E,E′) anterior (ant) pole views. Post, posterior; ani, animal pole. (F,F′) Histochemical staining of AChE in experimental larvae. (F) In a *lacZ*-mRNA-injected larva, muscle cells are exclusively stained. (F′) In a *Cs-β-catenin* overexpressing larva, activity of AChE is detected in muscle cells. (G,G′) Expression of muscle actin gene *Cs-MA1* in a cleavage-arrested 110-cell stage embryo with *lacZ*-mRNA injection (G) and in a *Cs-β-catenin*-overexpressing embryo (G′) revealed by in situ hybridization of whole-mount specimens. (H,H′) Expression of an epidermis-specific gene, *Cs-Epi1*, in cleavage-arrested 32-cell stage embryos revealed by in situ hybridization of whole-mount specimens. Although a significant amount of *Cs-Epi1* mRNA was detected in a *lacZ*-mRNA-injected control embryo (H), no *CsEpi1* expression was detected in a *Cs-β-catenin*-overexpressing embryo (H′). (I,I′) Expression of *Cs-Bra* in a 110-cell stage embryo injected with *lacZ*-mRNA (I) and in a *Cs-β-catenin*-overexpressing embryo (I′). Bars, 500 μm (A,A′); 100 μm (B-I′).

differentiation of notochord cells in the ascidian embryo (Corbo et al., 1997a; Takahashi et al., 1999). We therefore examined whether the upregulation of *Ci-β-catenin* affects the *Ci-Bra* expression by northern blot and RT-PCR analyses. As shown in Fig. 4D, northern blot analysis demonstrated that the band intensity of *Ci-Bra* mRNA in *Ci-β-catenin*-overexpressing embryos was considerably weaker than that in control embryos, suggesting the downregulation of *Ci-Bra* by *Ci-β-catenin* overexpression in the notochord precursor cells. This result was confirmed by RT-PCR (Fig. 4D). Both assays therefore showed that *Ci-Bra* was downregulated in the *Ci-β-catenin*-overexpressing embryos. We also examined the effects of *Cs-β-catenin* overexpression of *Cs-Bra* expression. As shown in Fig. 5I′, *Cs-β-catenin* overexpression suppressed *Cs-Bra* expression.

In *Ci-cadherin*-overexpressing embryos, differentiation of

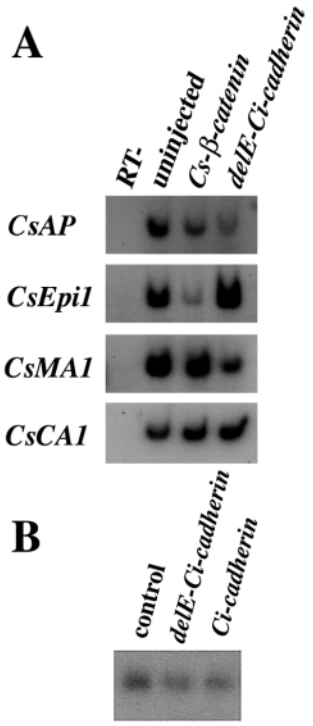

Fig. 6. (A) Quantitative RT-PCR analysis. PCR reactions were performed using cDNA that was reverse-transcribed from an amount of RNA corresponding to that extracted from one embryo. A negative control without reverse transcriptase is shown in the left lane (RT−). (B) Northern blotting with *Ci-AP* probe. RNAs were extracted from *C. intestinalis* embryos into which DNA constructs encoding *delE-Ci-cadherin* and *Ci-cadherin* were electroporated. Lanes were loaded with 1.5 μg of poly(A)$^+$ RNA.

endoderm cells was inhibited. To examine the effect of this inhibition of endoderm differentiation on notochord specification, experimental 110-cell embryos were subjected to in situ hybridization with *Cs-Bra* antisense probe. For this experiment, *Ci-cadherin*-mRNA-injected embryos were used, because the injection of *delE-Ci-cadherin* mRNA severely disturbed the cell adhesion and made the embryos difficult to use for subsequent analysis. In control uninjected embryos, *Cs-Bra* expression was evident in cells of the notochord lineage (Fig. 7H). On the other hand, no or very few cells expressed *Cs-Bra* in *Ci-cadherin*-mRNA-injected embryos (Fig. 7H′). Therefore, without endoderm differentiation, specification of notochord cells does not take place in the ascidian embryo.

DISCUSSION

An essential role for β-catenin in the endoderm specification of ascidian embryos

β-catenin is known to have dual functions: one is as a transcription factor and the other as a cytoskeletal component that interacts with the cytoplasmic domain of cadherin (reviewed by Willert and Nusse, 1998). While it is unlikely that β-catenin functions as a cytoskeletal component in ascidian

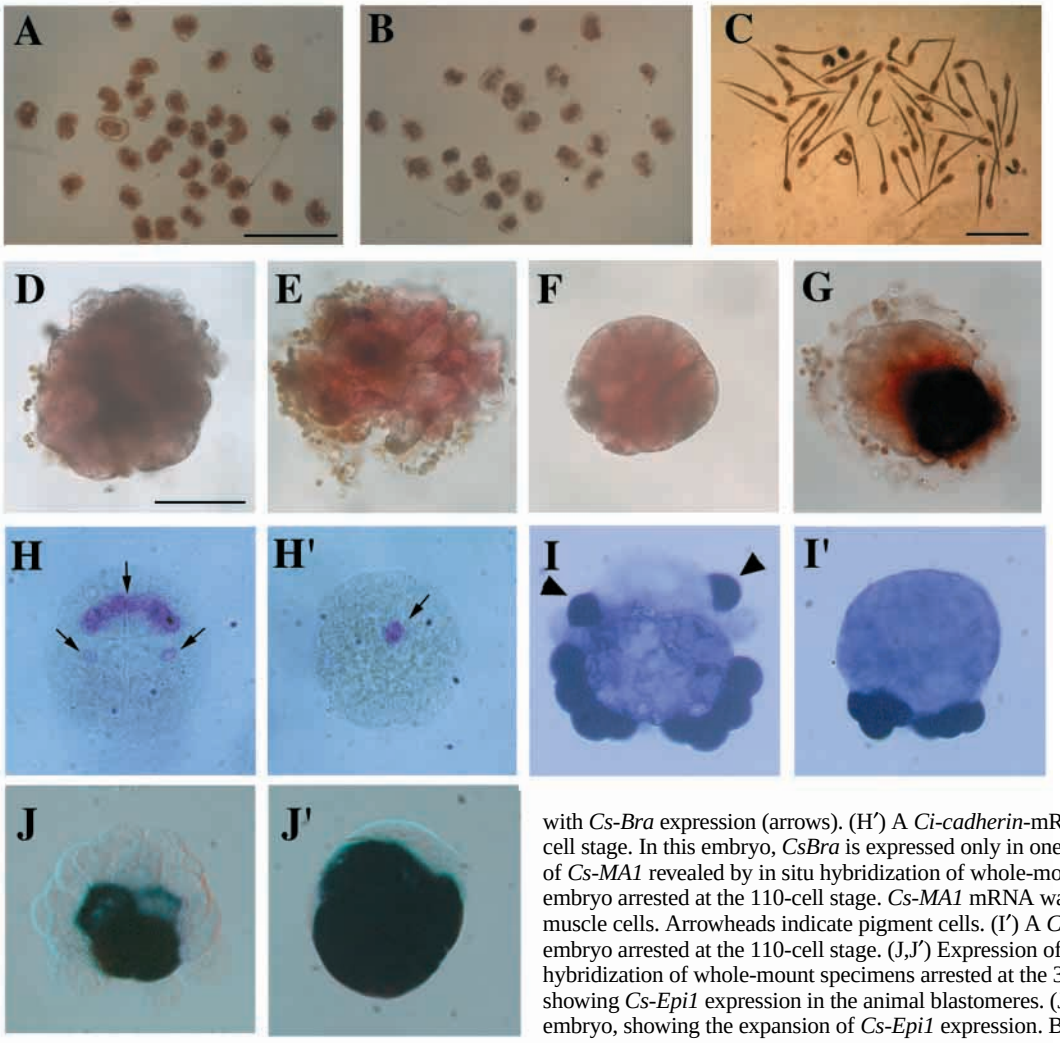

Fig. 7. Effects of *Ci-cadherin* overexpression on *C. savignyi* embryonic cell specification. (A) Larvae injected with *Ci-cadherin* mRNA. Their development was disturbed. (B) Larvae injected with *delE-Ci-cadherin* mRNA. Their development was also disturbed. (C) Larvae injected with *delC-Ci-cadherin* mRNA showing normal morphology. (D, E) Histochemical staining of AP in an embryo injected with *Ci-cadherin* mRNA (D) or *delE-Ci-cadherin* mRNA (E). No AP expression is detected in these embryos. (F) Histochemical staining of AP in an embryo injected with *Ci-cadherin* mRNA and arrested at the 110-cell stage. No AP expression is detected. (G) Histochemical staining of acetylcholinesterase in an embryo injected with *Ci-cadherin* mRNA, showing differentiation of muscle cells. (H,H′) Expression of *Cs-Bra* revealed by in situ hybridization of whole-mount specimens. (H) A control embryo at the 110-cell stage showing 10 notochord cells with *Cs-Bra* expression (arrows). (H′) A *Ci-cadherin*-mRNA-injected embryo at the 110-cell stage. In this embryo, *CsBra* is expressed only in one presumptive cell. (I,I′) Expression of *Cs-MA1* revealed by in situ hybridization of whole-mount specimens. (I) A control embryo arrested at the 110-cell stage. *Cs-MA1* mRNA was detected in the primary lineage muscle cells. Arrowheads indicate pigment cells. (I′) A *Ci-cadherin*-mRNA-injected embryo arrested at the 110-cell stage. (J,J′) Expression of *Cs-Epi1* revealed by in situ hybridization of whole-mount specimens arrested at the 32-cell stage. (J) A control embryo, showing *Cs-Epi1* expression in the animal blastomeres. (J′) A *Ci-cadherin*-mRNA-injected embryo, showing the expansion of *Cs-Epi1* expression. Bars, 1 mm (A-C); 100 μm (D-J).

endoderm specification, it is very likely that it functions as a transcription factor involved in endoderm cell specification. As expected, β-catenin accumulated during cleavages in the nuclei of vegetal blastomeres, suggesting that it plays a role in the specification of endoderm.

Cadherin molecules have been used experimentally to downregulate β-catenin in the cytoplasm and thus to inhibit nuclear localization of β-catenin (Heasman et al., 1994; Wikramanayake et al., 1998; Logan et al., 1999). To examine the function of β-catenin, we prepared three kinds of *Ci-cadherin* mRNAs: *Ci-cadherin* mRNA containing the entire coding sequence, *delE-Ci-cadherin* mRNA lacking the extracellular domain, and *delC-Ci-cadherin* mRNA lacking the cytoplasmic domain. If the failure of endoderm differentiation is due to the function of the cadherin molecule itself, injection of *Ci-cadherin* mRNA and *delE-Ci-cadherin* mRNA should cause opposite effects on endoderm differentiation. Indeed, the injection of *Ci-cadherin* mRNA strengthened the embryonic cell adhesion while *delE-Ci-cadherin* weakened cell adhesions. However, both types of mRNA suppressed the endoderm differentiation. Therefore, it is unlikely that cadherin plays a key role in ascidian endoderm differentiation. The finding that *delC-Ci-cadherin* mRNA does not affect embryogenesis also supports this notion. Therefore, the suppression of endoderm differentiation by injection of *Ci-cadherin* mRNA or *delE-Ci-cadherin* mRNA is likely to be due to the downregulation of cytoplasmic and/or nuclear β-catenin.

Together with the result that overexpression of β-catenin induces the development of an endoderm-specific AP in presumptive notochord cells and epidermis cells, all of these results suggest an essential role for β-catenin in the endoderm specification of ascidian embryos. An important question yet to be answered is whether or not β-catenin itself can be thought of as an 'endoderm determinant'. Because β-catenin mediates a complex signaling pathway and would therefore be regulated by many other unknown factors, it is possible that the factors which regulate the activity of β-catenin may be the endoderm determinants. Elucidating the pathway by which β-catenin activity is regulated may lead to the identification of endoderm determinants, or the entire pathway itself may be the determinant responsible for ascidian endodermal fate.

β-catenin and its conserved function in embryonic cell specification

β-catenin plays a crucial role in the establishment of the dorsoventral axis in embryos of *Xenopus* and zebrafish (Heasman et al., 1994; Funayama et al., 1995; Guger and Gumbiner, 1995; Kelly et al., 1995; Schneider et al., 1996; Larabell et al., 1997) and of the animal-vegetal axis in sea urchin embryos (Wikramanayake et al., 1998; Logan et al., 1999). As shown in the present study, β-catenin accumulated in the nucleus of vegetal blastomeres. Ascidian embryos in which β-catenin was overexpressed appeared to be vegetalized, because AP expression was expanded into the epidermal territory of the animal hemisphere, whereas embryos with cadherin overexpression appeared to be animalized because AP expression disappeared in the endoderm territory of the vegetal hemisphere. Thus, the role of β-catenin in axis determination seems to be conserved among various deuterostomes.

However, if these results are interpreted from another perspective, β-catenin may play a role in the specification of cells situated in the vegetal pole region of invertebrate embryos. These are endoderm cells in ascidian embryos. In sea urchin embryos, the most vegetal cells are micromeres, in which β-catenin first accumulates within their nuclei (Logan et al., 1999). Micromeres are specified autonomously and then they emit signal(s) that induce the endodermal differentiation (reviewed by Davidson, 1990). During *C. elegans* embryogenesis, β-catenin also specifies the endoderm cells, which are situated at the vegetal pole (Thorpe et al., 1997; Rocheleau et al., 1997).

β-catenin and the Wnt pathway involvement in endoderm specification

It has been shown that β-catenin is negatively regulated by GSK3β (reviewed by Cadigan and Nusse, 1997). This negative regulation is thought to be responsible for localizing β-catenin in dorsal blastomeres of *Xenopus* embryos and in vegetal blastomeres of sea urchin embryos. In *Xenopus*, it has been shown that lithium causes embryonic responses that mimic those caused by Wnts (Christian and Moon, 1993; Stambolic et al., 1996). The target of lithium was suggested to be GSK3β (Klein and Melton, 1996). In a previous study, we showed that lithium treatment of *C. savignyi* early embryos causes transformation of notochord cells into endoderm cells, and that microinjection of *Cs-β-catenin* mRNA into A4.1 blastomere causes a similar effect (Yoshida et al., 1998). It was therefore suggested that Wnt signaling cascade molecules, including GSK3β, play a role in the endoderm specification of ascidian embryos. Wnts and its receptor, frizzled, may not, however, be involved in this specification, because endoderm cells are specified autonomously without any cell-cell interaction during early embryogenesis (Nishida, 1992; Shimauchi et al., 1997). A recent study of *Xenopus laevis* has shown that this pathway begins with Dsh (Müller et al., 1999). Future studies should therefore examine the possible roles of Dsh and GSK3β in ascidian endoderm specification.

It is of great interest to identify genes upstream and downstream of β-catenin that are involved in ascidian endoderm specification. For example, *pem* may be involved either directly or indirectly (Yoshida et al., 1998), because the effects of lithium on ascidian embryogenesis are eliminated by *pem* overexpression. We recently isolated several maternally expressed genes (*CsEndo-1*, *CsEndo-2* and *CsEndo-3*) and their mRNAs are segregated with the endoplasm of early *C. savignyi* embryos (Imai et al., 1999). These genes may also be involved in endoderm specification. The earliest known gene that is expressed zygotically in endoderm cells is *fork head/HNF-3β* (Corbo et al., 1997b; Olsen and Jeffery, 1997; Shimauchi et al., 1997). *Cititf1*, a member of the *NK-2* gene family, is also expressed in the endoderm cells of pregastrula *C. intestinalis* embryos, and misexpression of *Cititf1* causes the development of extra endoderm cells (Ristoratore et al., 1999). Future studies will characterize the genetic circuitry involved in the specification of ascidian endoderm cells, and these results will indicate conserved and non-conserved elements of this mechanism when compared to other deuterostome and non-deuterostome embryos.

The relationship between endoderm differentiation and notochord specification in the ascidian embryo

The mechanisms underlying notochord differentiation in

ascidian embryos have been investigated. Endodermal cells secrete signal(s) at the 32-cell stage to induce specification of notochord cells (Nakatani and Nishida, 1994). Candidate signal molecules include bFGF in *H. roretzi* (Nakatani et al., 1996) and Notch in *C. intestinalis* (Corbo et al., 1997a, 1998). The notochord cells then express *Brachyury* at the 64-cell stage, which then triggers the process of notochord formation (Yasuo and Satoh, 1993, 1998; Corbo et al., 1997a; Takahashi et al., 1999). Because ectopic expression of the ascidian *Brachyury* can induce a complete change of presumptive endoderm cells into notochord cells (Yasuo and Satoh, 1998; Takahashi et al., 1999), endoderm cells are competent to differentiate into notochord. As indicated in the present study, presumptive notochord cells are also competent to differentiate into endoderm cells (see also Yoshida et al., 1998). At the 32-cell stage, endoderm cells are in contact not only with presumptive notochord cells but also with neighboring endoderm cells. This suggests that endoderm cells can inhibit their own expression of *Brachyury*, and notochord cells can auto-inhibit the function of β-catenin. Indeed, in embryos overexpressing *β-catenin*, β-catenin inhibits the expression of *Brachyury* in presumptive notochord cells.

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