

Alterations induced by E-cadherin and β -catenin antibodies during the development of *Bufo arenarum* (Anura-Bufonidae)

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Summary. E(epithelial)-cadherin is a member of a calcium-dependent family of cell surface glycoproteins involved in cell-cell adhesion and morphogenesis. Catenins are a large family of proteins that connect the cadherins to the cytoskeleton. They are important for cadherin function and for transducing signals involved in specification of cell fate during embryogenesis. The best characterized catenins include α -, β -, γ -, and p120-catenin. Using specific antibodies, we studied the expression and distribution of E-cadherin, and α - and β -catenin in developmental stages of *Bufo arenarum* toad. The three proteins were found co-localized in stages 19 to 41 of development. Surprisingly, E-cadherin was the only of these three proteins found earlier than stage 19. To test whether E-cadherin and β -catenin have a functional role in *Bufo arenarum* embryogenesis, stage 17 whole embryos were incubated with anti-E-cadherin and β -catenin antibodies. Both anti-E-cadherin and anti- β -catenin antibodies induced severe morphological alterations. However, while alterations produced by the anti- β -catenin antibody, showed some variability from the most severe (neural tube and notochord duplication) to a simple delay in development, the alterations with anti-E-cadherin were homogeneous. These observations suggest a critical role for E-cadherin and β -catenin in the early embryonic development of the *Bufo arenarum* toad. Our results are consistent with the developmental role of these proteins in other species. One of the most surprising findings was the blockage with the anti- β -catenin antibodies on later embryo stages, and we hypothesize that the partial axes duplication could be mediated by the notochord induction.

Key words: Catenins, Cadherins, Morphogenesis, Amphibians

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Introduction

Cadherins are calcium-dependent transmembrane glycoproteins that concentrate at sites of cell-cell contacts and associate with the actin-based cytoskeleton through a family of proteins termed catenins (Hirano et al., 1987; Nagafuchi and Takeichi, 1989; Ozawa et al., 1989; Stappert and Kemler, 1993). Catenins are part of a sub-membranous protein network by which cadherins are connected to other integral membrane proteins (McNeill et al., 1990). Catenins regulate the extracellular adhesive properties of cadherins as well as their interaction with the cytoskeleton (Kemler et al., 1990). Catenins also mediate signals for gene expression during development and tissue morphogenesis (Matsuyoshi et al., 1992; Behrens et al., 1993; Hamaguchi et al., 1993; Rubinfeld et al., 1993; Su et al., 1993; Hoschuetzky et al., 1994; Shibamoto et al., 1994). During development, the *Drosophila* segment polarity gene *armadillo* and its vertebrate homologous β -catenin appear essential for both cadherin-mediated cell-cell adhesion and for mediating signals involved in specifying cell fate (Gumbiner and McCrea, 1993; Heasman et al., 1994; Gumbiner, 1995; Haegel et al., 1995; Klymkowsky and Parr, 1995; Peifer, 1995). The roles of β -catenin include signals for axis formation in vertebrates and changes in gene expression in mesodermal and endodermal embryonic tissues (Miller and Moon, 1996).

Furthermore, different members of the cadherin family are distributed in unique spatiotemporal patterns during embryogenesis. Changes in cadherin expression determine the sorting of cells to form differentiated tissues (Takeichi, 1988, 1991). In this paper we mapped the normal expression of E-cadherin, α - and β -catenin in the *Bufo arenarum* toad embryos and investigated the effects of inhibiting E-cadherin and β -catenin during the development of the *Bufo arenarum* toad tissues, by treating whole embryos with specific antibodies.

Materials and methods

Toad embryos

The production of eggs was induced in *Bufo arenarum* adult females by intraperitoneal injection of 2500 IU hCG (Endocorion, Elea, Buenos Aires). Embryos were obtained by artificial fertilization by mixing eggs with minced testicular tissue, and cultured in Holtfreter's solution (Holtfreter, 1931).

Immunohistochemistry

Embryos and larvae were studied at the following developmental stages: 3 (2 cells), 7 (32 cells), 13 (neural plate), 17 (tail bud), 19 (heart beat), 25 (complete operculum), and larval stages 28 and 41 (Gosner, 1960). Whole animals were fixed in Carnoy's solution for 2 hours at 20 °C, dehydrated in ethanol and embedded in paraffin (Cicarelli®, Buenos Aires). Cross and sagittal 5 µm-thick sections were cut in a Reichert Jung Hn 40 microtome and dried onto 1% gelatin-coated glass slides.

Mouse monoclonal antibodies to E-cadherin (clone 36 mouse IgG2a), to β-catenin (clone 1a mouse IgG1) and to α-catenin (clone 5 mouse IgG1) were from Transduction Labs, Lexington, KY, and were used at a 1:50 dilution in phosphate buffer saline (PBS). A rabbit polyclonal IgG to β-catenin was a gift from Dr. P. McCrea (MD Anderson Cancer Center, University of Texas). Normal mouse serum (Sigma, St. Louis, MO) was used as negative control, diluted 1:100 in PBS.

Sections from *Bufo arenarum* embryos and tadpoles were deparaffinized in xylene, re-hydrated and permeabilized with 0.1% Triton X-100 (Sigma, St. Louis, MO) in PBS for 15 minutes. The sections were incubated in normal horse serum for 30 minutes followed by overnight incubations with the primary antibodies at 4 °C in a humidified chamber. The sections were rinsed in PBS and incubated with species-specific biotinylated secondary antibodies diluted 1:400 in PBS containing 1.0% BSA and 2.0% normal horse serum. After rinsing in PBS sections were incubated with avidin-biotin complex and 3,3'-diaminobenzidine chromogenic substrate (ABC kit Vectastain Elite®, Vector Burlingame, CA) and slides were mounted with Canada balsam (Biopack®, Buenos Aires). In control sections the primary antibodies were replaced with mouse non-immune serum (Sigma, St. Louis, MO). Sections were examined and photographed in a BX50 Olympus microscope.

Bioassay

Prior to evaluating the effect of antibodies on embryonic morphology, a comprehensive morphological and histological study was performed in a group of stage 17 untreated embryos (Gosner, 1960). Intact embryos were photographed with a Nikon stereomicroscope, and then fixed for 2 hours at room temperature in 3%

glutaraldehyde 3% paraformaldehyde and 1% picric acid in 0.1M cacodylate buffer at pH 7.4, and postfixed in 1% osmium tetroxide. After dehydration in acetone, the embryos were embedded in Durcupan® resin (Fluka, Buchs, Switzerland), and 0.5 µm-thick serial cross-sections were cut in a Reichert Ultracut S ultramicrotome and stained with toluidine blue.

Thirty stage 17 whole embryos were incubated with anti-E-cadherin, anti-β-catenin antibodies or control IgG. Embryonic viability was controlled periodically. All experiments were performed in triplicate. Embryos were incubated for 24 hours at 20 °C in 50 µl of Holtfreter's solution containing 1:2 anti-E-cadherin or anti-β-catenin monoclonal antibodies, or an equivalent concentration of mouse non-immune serum as control (Sigma Chemical Co., St. Louis, MO). After treatment, the embryos were photographed and processed for histological evaluation as described above.

Results

Expression of E-cadherin in *Bufo arenarum* development

The first detection of immunopositive signals with E-cadherin antibody was observed at stage 17 (Fig. 1a). E-cadherin was expressed in the two ectodermal layers of the embryos. It was found localised in the basolateral membrane of cells from the outer ectodermal layer, and, surprisingly, in both the basolateral and apical membranes of inner ectodermal layer cells (Fig. 1a). This expression pattern remained constant through stage 19 (not shown). At stage 25 there was a dramatic increase in expression in the epithelial cells of gills and pharynx (Fig. 1e,f). At this stage E-cadherin was also found weakly expressed in the epithelium of the utricle and saccule of the otic vesicles (Fig. 1e). Expression of E-cadherin increased even further from stages 25 to 41. At stage 28, we observed high membranous expression of E-cadherin in gill and pharyngeal epithelia and in the pericardium. The levels of E-cadherin were comparatively lower in otic vesicles, rhombencephalon, VIII cranial nerve (auditive) and in the choroid plexus (not shown). At stage 41, the levels of E-cadherin expression increased uniformly in pharynx, otic vesicles, auditive nerve and skin (Fig. 1g,h). Additionally, at this stage we detected weakly E-cadherin expression in the myocardium (not shown).

Expression of α-catenin in *Bufo arenarum* development

Alpha-catenin was first detected at stage 25, expressed co-localised with E-cadherin (Fig. 1i,j), but also in E-cadherin-negative tissues. At this stage, α-catenin was also detected in the rhombencephalon (Fig. 1i), the auditive nerve, notochord and pericardium (not shown). Similarly to E-cadherin, α-catenin expression increased dramatically from stages 25 to 41 (Fig. 1i-l). At stage 41, low levels of α-catenin expression were observed in the myocardium, co-localised with E-

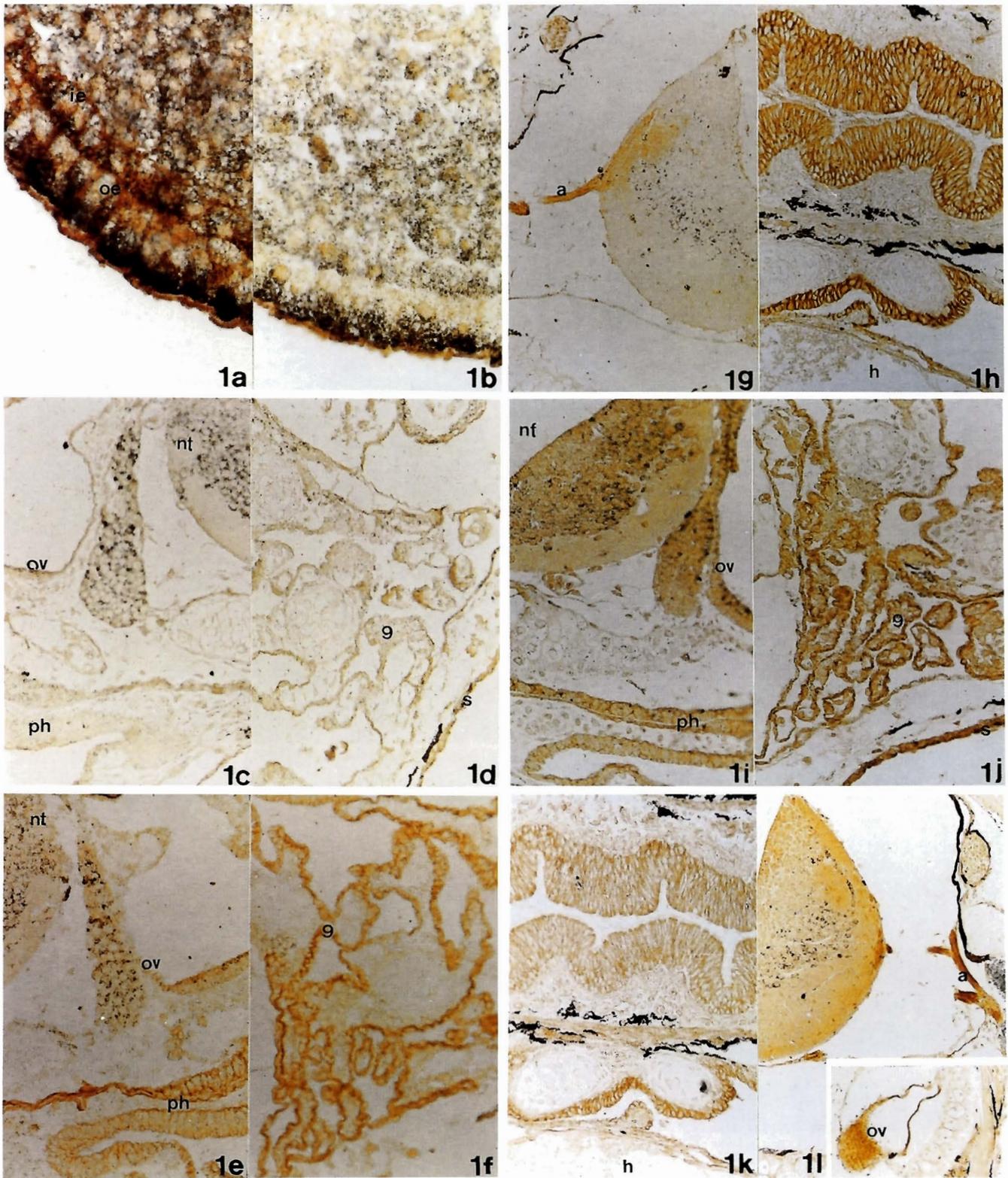


Fig. 1. Cross sections of *Bufo arenarum* embryos. **a.** Stage 17, incubated with mouse anti-E-cadherin. This molecule is expressed in the basolateral membrane of outer ectoderm, and apical and basolateral membrane of inner ectoderm. x 133. **b.** Stage 17, incubated with mouse non-immune serum. x 133. **c.** Stage 25, incubated with mouse non-immune serum. x 66. **d.** Stage 25, incubated with mouse non-immune serum. x 66. **e.** Stage 25 showing a clear expression of E-cadherin in pharynx, and a pale expression in the otic vesicles (auditive epithelium laying the utricle and saccule). x 66. **f.** Stage 25 showing a strong expression of E-cadherin in gill epithelium. x 66. **g.** Stage 41 showing a clear expression of E-cadherin in auditory nerve at rhombencephalon level. x 66. **h.** High expression of E-cadherin in pharynx at stage 41. x 66. **i.** Expression of α -catenin, at stage 25 in pharynx, otic vesicles, rhombencephalon and the auditory nerve. x 66. **j.** Expression of α -catenin, at stage 25 in gills and skin. x 66. **k.** Expression of α -catenin at stage 41 in pharynx. x 66. **l.** Strong expression of α -catenin in otic vesicles (insert), rhombencephalon and the auditory nerve. x 66. **oe:** outer ectoderm; **ie:** inner ectoderm; **nt:** neural tube; **ov:** otic vesicle; **ph:** pharynx; **g:** gill; **s:** skin; **h:** heart.

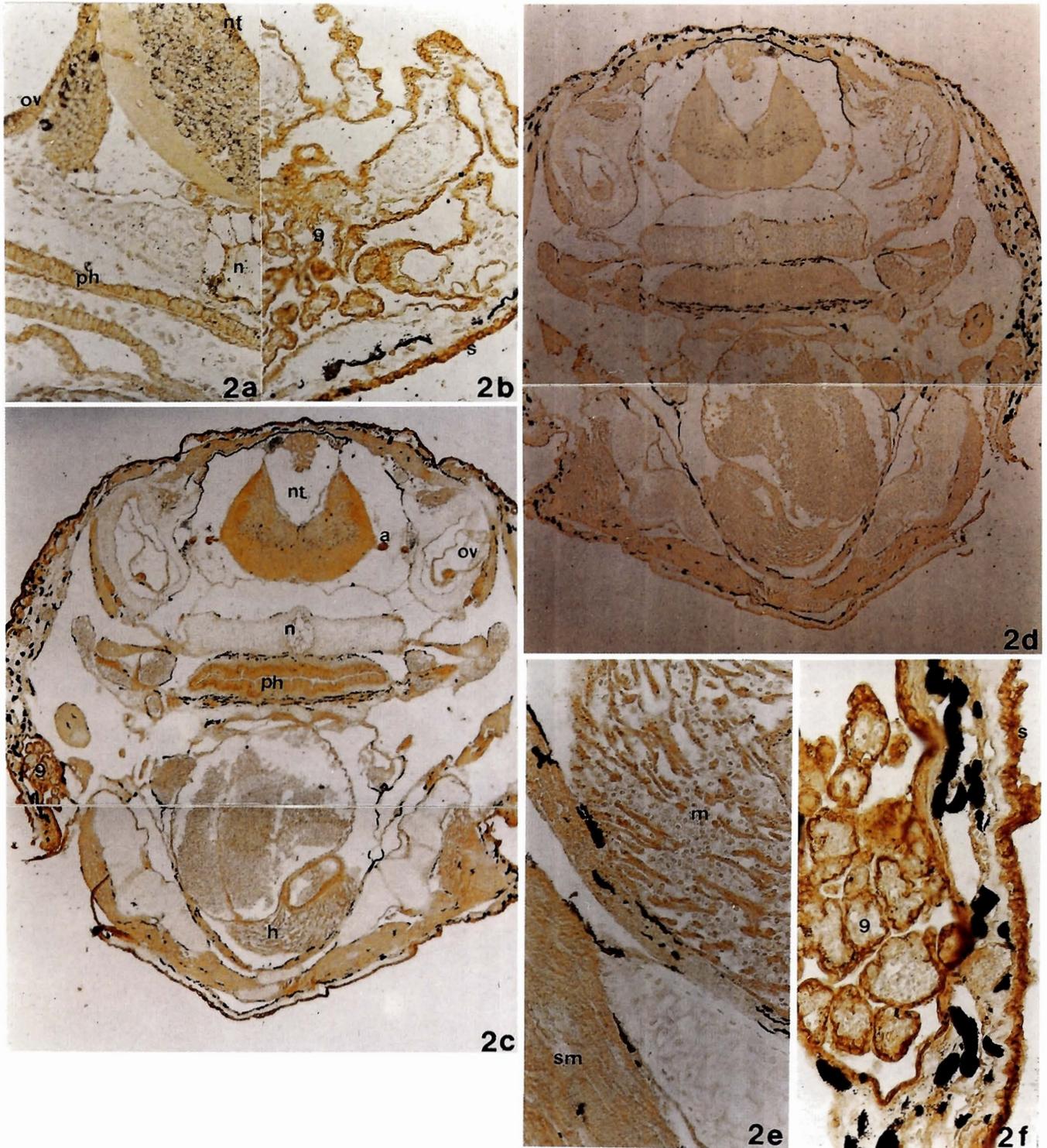


Fig. 2. Cross sections of *Bufo arenarum* embryos. **a.** β -catenin expression at stage 25 in the rhombencephalon, with a higher label in the periventricular cells, in the otic vesicles, auditory nerve, notochord and pharynx. x 66. **b.** Note the high expression of β -catenin in gills and skin. x 66. **c.** Strong β -catenin expression at stage 41 in the rhombencephalon, choroid plexus, notochord, otic vesicles, auditory nerve, heart, skeletal muscle, pharynx, gills and skin. x 13. **d.** Control section incubated with mouse non-immune serum. x 13. **e.** Detail of β -catenin expression in myocardium and skeletal muscle. x 66. **f.** Detail of β -catenin expression in gill epithelium and skin. x 66. nt: neural tube; n: notochord; ph: pharynx; ov: otic vesicle; h: heart; m: myocardium; sm: skeletal muscle; g: gills; s: skin; a: auditory nerve.

cadherin (not shown).

Expression of β -catenin in Bufo arenarum development

A strong β -catenin signal was detected at stage 19 in

both outer and inner ectoderm layers (not shown). At stage 25 β -catenin was detected in the epithelial cells of otic vesicles, notochord, pharynx, gills and auditory nerve (Fig. 2a,b), choroid plexus, pericardium and myocardium (not shown). The expression in the

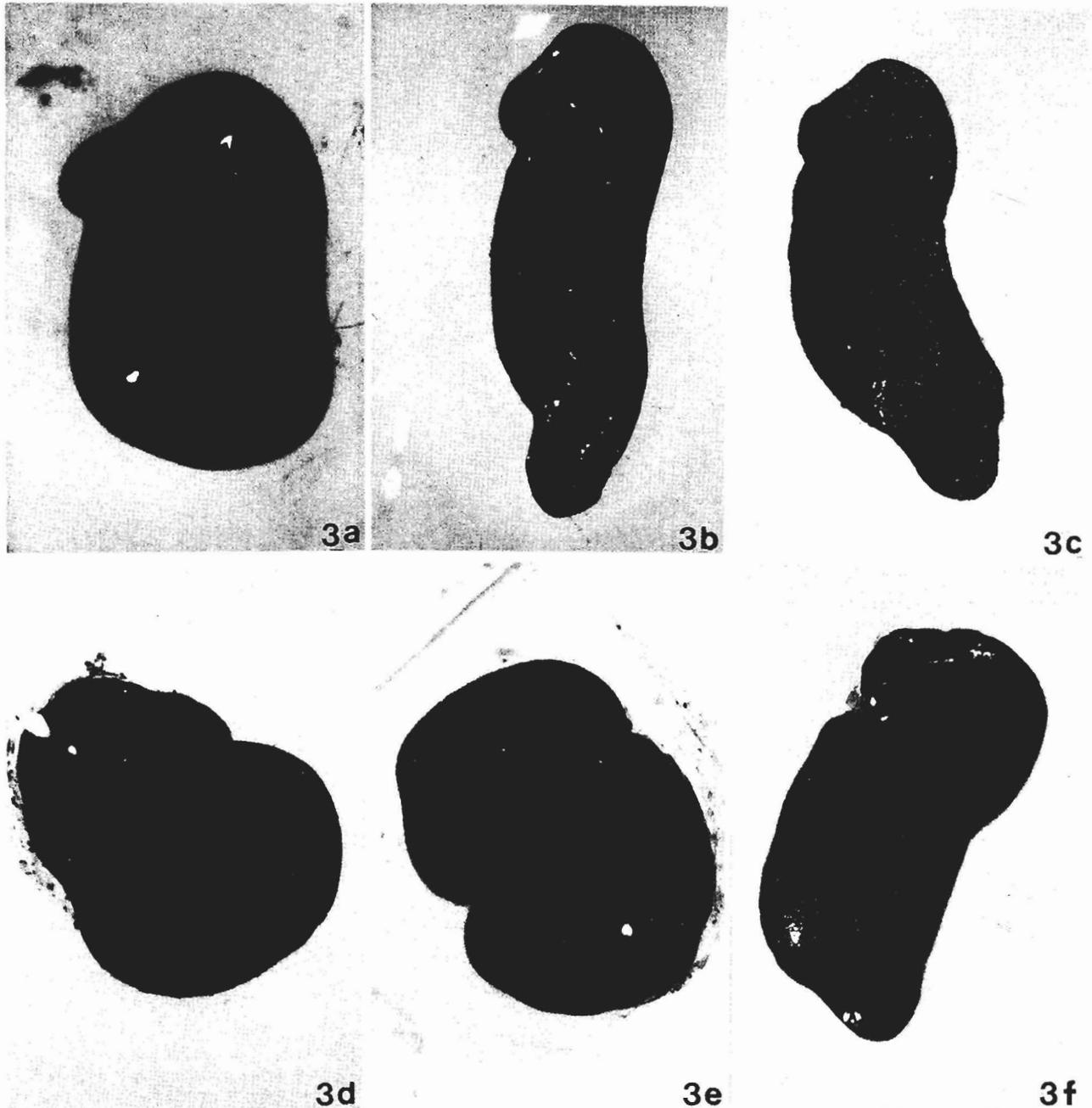


Fig. 3. Control and antibody-treated *Bufo arenarum* embryos. **a.** Stage 17 embryo at the beginning of the incubation period. X 15. **b.** Stage 19 embryo after 24 h of incubation in Holtfreter's solution (x 12.5). **c:** stage 19 embryo after 24 h of incubation in mouse non-immune serum. x 12.5. **d.** Embryo after 24 h of incubation in anti-E-cadherin monoclonal antibody, dilution 1:2. Note the deep alteration of its external morphology, showing a rounded-shaped body and lacking of the typical cephalic and tail-bud differentiation. x 15. **e.** Embryo after 24 h of incubation in anti- β -catenin monoclonal antibody, dilution 1:2. This embryo exhibits changes similar to E-cadherin treatment and represents about 33% of treated embryos. x 15. **f.** Embryo after 24 h of incubation in anti- β -catenin monoclonal antibody, dilution 1:2. Approximately 66% of the treated embryos exhibit an external morphology equivalent to stage 18 of development. x 15

rhombencephalon showed particularly high levels in periventricular cells (Fig. 2a). There was a uniform increase in β -catenin expression at stages 28 (not shown) and 41 (Fig. 2c,e,f). The distribution of β -catenin at this stage included the heart, skeletal muscle, pharynx, rhombencephalon, notochord, otic vesicles, auditory nerve and gills (Fig. 2c,e,f). Surprisingly, β -catenin in the heart was found in the endocardium, but not in the pericardium at stage 41 (not shown).

Induction of morphological alterations in *Bufo arenarum* by antibodies against E-cadherin and β -catenin

Control embryos incubated with Holtfreter's solution alone or with mouse non-immune serum for 24 hours developed normally from stage 17 to 19. Minor morphological variations between individuals have been attributed to normal variables within the species or to the small volumes of media used in the assay (Fig. 3a-c). In contrast, embryos incubated with anti-E-cadherin or anti- β -catenin antibodies showed notable developmental alterations. All anti-E-cadherin-treated embryos showed similar and dramatic changes in external morphology (Fig. 3d), including rounded shaped body and lack of the typical cephalic or tail differentiation. Anti- β -catenin-treated embryos exhibited less homogeneous alteration patterns. Some exhibited changes similar to E-cadherin treatment (Fig. 3e) and others showed developmental features of delayed embryos (equivalent to stage 18 embryos) (Fig. 3f).

Histological analysis

The histological analysis of *B. arenarum* embryos at stages 17 –before the beginning of the experiment– (Fig. 4a) and 19 (Fig. 4b,c) incubated in Holtfreter's solution alone or with mouse non-immune serum respectively (Fig. 4b,c) showed normal tissue structures and parameters. Treatment with anti-E-cadherin antibody did not induce a significant developmental delay, the histology of the structures were the same as such a developmental stage should contain, but with severe alterations, including the shortened and folded neural tube and notochord, deformed and ectopic pharynx and

segmented somitogenic mesoderm, folded spinal chord, and globular tail (Fig. 4d), and in some embryos we were able to distinguish malformed heart and eye vesicles (not shown). A very surprising observation was the absence of heart tube and gills. The epidermis showed a normal double layer of cells, a ciliated epithelial layer and a sensory one (Fig. 4d).

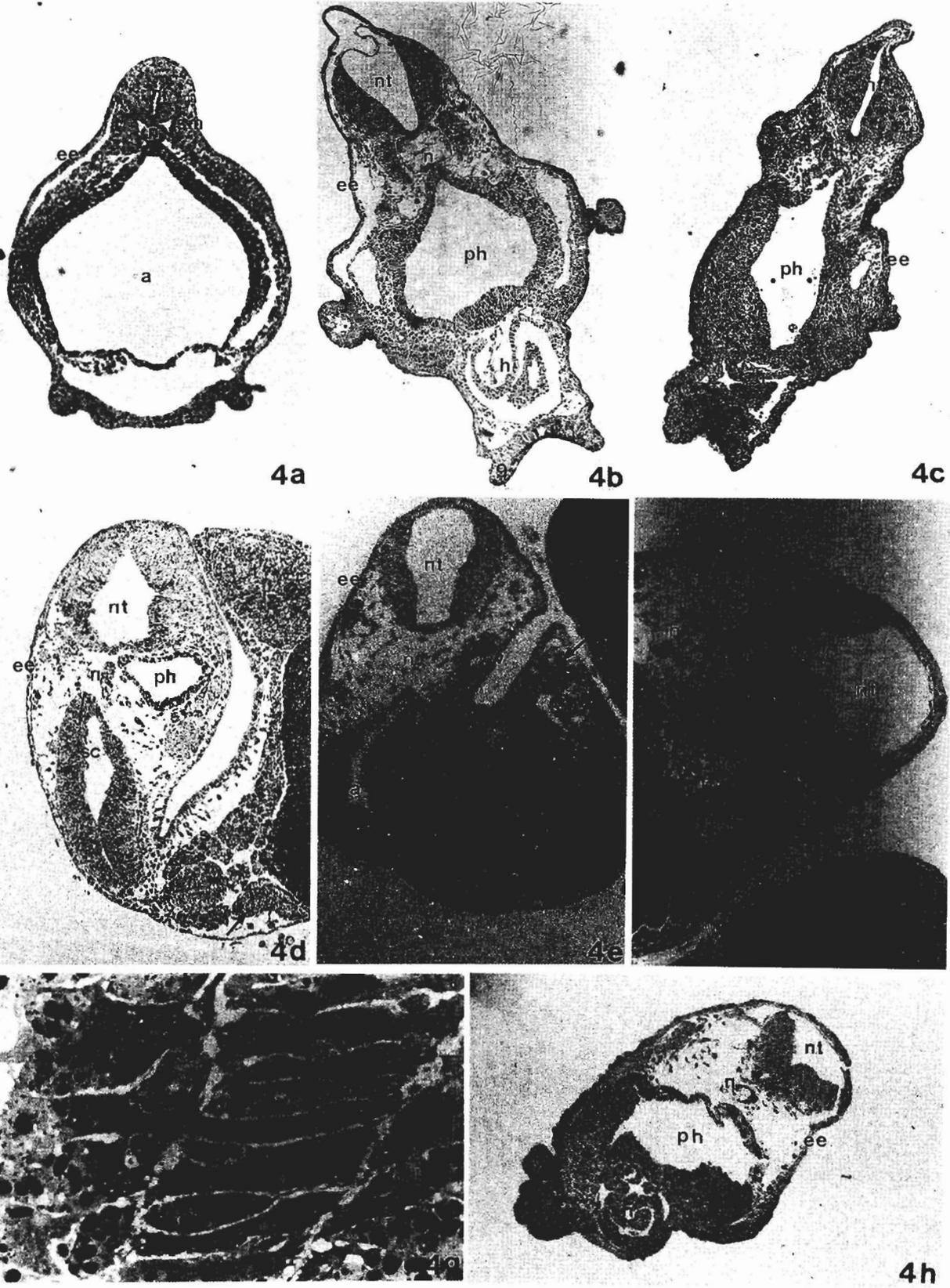
Stage 17 embryos incubated with anti- β -catenin antibodies did not exhibit homogenous defects. Anti- β -catenin treatment induced incomplete axis duplication. Neural tube and notochord duplication was observed in 33% of the embryos studied. These structures were separated by skeletal muscle tissue at the cardiac beat-gill circulation stage. (Fig. 4e-g). The remaining 66% reached developmental stage 18 (Fig. 4h) and showed less severe alterations in neural morphology, although heart tissues were severely malformed and were constituted by an amorphous mass of hypertrophied cardiac cells (Fig. 4h).

Discussion

The establishment of the basic body plan of amphibian embryos is determined by signals originating from cell-cell interactions (Asashima et al., 1991; Dohrmann et al., 1993; Regabliati et al., 1993). In this paper we studied the distribution of E-cadherin, α -catenin and β -catenin and the effects of inhibiting β -catenin and E-cadherin molecules with monoclonal antibodies during the embryogenesis of *Bufo arenarum*.

In *Xenopus*, E-cadherin is expressed from gastrulation (Choi and Gumbiner, 1989; Angres et al., 1991; Levi et al., 1991). β -Catenin is involved in *Xenopus* axis formation (McCrea et al., 1993) and was found in all *Xenopus* embryo cells, particularly in areas undergoing dynamic morphogenetic changes, such as the marginal zone of blastulae and gastrulae. However, there is no correlation between the levels of β -catenin expression and cell-cell adhesion strength, and β -catenin is found in plasma membranes without cell-cell contacts (Fagotto and Gumbiner, 1994). Those findings suggest that β -catenin may have functions dissociated from its cadherin-linked cell-cell adhesion role during amphibian embryogenesis.

Fig. 4. Cross-sections of control and antibody-treated *Bufo arenarum* embryos. **a.** Embryo stage 17. Note the presence of epidermal ectoderm (ee), neural tube (nt), notochord (n), somitogenic mesoderm (sm) and archenteron (a). $\times 13$. **b.** Stage 19 embryo after 24 h of incubation in Holtfreter's solution. Note the normal development of the embryo. nt: neural tube; n: notochord; ph: pharynx; h: heart; g: mucous gland; s: somites. $\times 20$. **c.** Stage 19 embryo after 24 h of incubation in mouse non-immune serum. We distinguish them as normal structures: neural tube (nt), notochord (n), pharynx (ph), heart (h), mucous gland (g), somites (s). $\times 20$. **d.** Embryo after 24 h of incubation with the anti-E-cadherin monoclonal antibody, dilution 1:2. Observe the folded neural tube (nt) and notochord (n), deformed and ectopic pharynx (ph), somites (s), unsegmented somitogenic mesoderm (arrow), folded spinal chord with central canal (sc), endoderm (e), double-layered epidermal ectoderm (ee) and very well developed tail (t). $\times 23$. **e.** Embryo after 24 h of incubation, with the anti- β -catenin monoclonal antibody, dilution 1:2. Neural tube and notochord duplication (arrow and arrowhead) is observed in approximately 33% of the embryos studied. nt: neural tubes; n: notochord; ev: ear vesicle; a: archenteron; s: somites; e: endoderm; ee: double-layered epidermal ectoderm. $\times 23$. **f.** Detail of an embryo after 24 h of incubation, with the anti- β -catenin monoclonal antibody, dilution 1:2. Note the duplication of neural tube (nt) and notochord (n), both separated by well developed skeletal muscle fibers (sm). $\times 33$. **g.** Detail of skeletal muscle fibers. $\times 333$. **h.** Embryo after 24 h of incubation with the anti- β -catenin monoclonal antibody, dilution 1:2. Approximately 66% of the treated embryos reach developmental stage 18 and show less severe alterations in neural tube morphology, although heart tissues are severely malformed and are constituted by an amorphous mass of hypertrophied cardiac cells. nt: neural tube; n: notochord; ph: pharynx; e: endoderm; ee: double-layered epidermal ectoderm; h: heart; g: mucous gland. $\times 13$



In contrast to the findings in *Xenopus*, in this study we could not detect E-cadherin, α -catenin or β -catenin during early development (stage 3 to tail bud) in *Bufo arenarum* embryos. The first expression of E-cadherin in *Bufo arenarum* was found at stage 17 in the ectodermal layers. High levels of E-cadherin were detected all around the inner ectodermal cells, at the interface with the mesoderm, and in the basolateral membrane of the outer ectoderm. Only at stage 19 was E-cadherin found co-localised with β -catenin along the basolateral membranes. From stage 25, the E-cadherin and β -catenin basal staining in the inner ectoderm disappeared, but their levels remained high at the interface between the inner and the outer layers. As previous findings in *Xenopus*, the loss of epidermal basal staining could be correlated with the separation from adjacent tissues by a thick extracellular matrix. Co-localization of E-cadherin with both α -catenin and β -catenin suggests the formation of a complete cadherin/catenin complex from stage 25, which remains highly expressed in epithelial cells from epidermis, gills and pharynx.

The high levels of E-cadherin together with α -catenin and β -catenin from stage 25 on suggest the development of stronger adhesion or formation of junctional complexes in the epidermis, gills, pharynx, otic vesicles, and nervous system. In other tissues, however, like the notochord at stages 25 and 28, high levels of β -catenin did not correlate with similar levels of α -catenin, and E-cadherin was absent. These observations suggest β -catenin associations with another cadherin or β -catenin expressed for functions other than cell-cell adhesion (Fagotto and Gumbiner, 1994).

Inhibition of cadherins disrupts monolayered cultures (Damsky et al., 1983; Yoshida-Noro et al., 1984; Behrens et al., 1985; Hatta et al., 1985; Gumbiner and Simons, 1986). Although we do not know how the binding of antibodies to E-cadherin, α - and β -catenin perturbed their functions, our previous report on *B. arenarum* embryos treated with α -catenin antibodies (Casco et al., 1998) and the experiments using E-cadherin, and β -catenin antibodies showed that the morphogenesis of *B. arenarum* embryos was severely affected by the treatments.

All *B. arenarum* embryos incubated in α -catenin antibodies (Casco et al., 1998) showed an external morphology homogeneous and deeply altered. Their appearance exhibited a regression to neural plate stages. Histologically we were able to distinguish three embryo fields: a presumptive neuroectodermal tissue, a vitelogenic tissue (endodermal presumptive cells) and a modified ectodermal layer.

Because many cell types could express multiple cadherins during development, the cell-cell adhesion properties could result from varying combinations of different cell adhesion molecules (Takeichi, 1988). Therefore, the inhibition of one cadherin may not be sufficient to block all cadherin-mediated stages of embryogenesis. However, the generalized effects caused by inhibiting E-cadherin in 17-stage embryos indicate

that E-cadherin is one of the most important cell-cell adhesion proteins for the development of *B. arenarum* embryos. The phenotypical alterations observed in whole embryos treated with anti-E-cadherin antibody included malformations of the eyes and heart and severe alteration of embryonic axis. It was surprising to find that the skin morphology was not affected, considering that E-cadherin is the most abundant cell-cell adhesion molecule of the epidermis. This could indicate that morphology of amphibian skin may be more dependent on integrin-controlled interactions between epidermis and mesenchymal tissues (Sengel, 1976) rather than cadherins. Alternatively, other cadherins could act to compensate for E-cadherin deficiency (Nose and Takeichi, 1986).

Results obtained with anti- β -catenin antibody treatments in stage-17 embryos were more heterogeneous than those observed with anti-E-cadherin. Approximately 33% of the embryos treated with anti- β -catenin showed duplication of the neural tube and notochord both laterally separated by muscular tissue at the cardiac beat-gill circulation stage. These observations are consistent with a role for β -catenin in axis formation, like in *Xenopus*, as demonstrated using antibodies and anti-sense approaches (McCrea et al., 1993; Guger and Gumbiner, 1995; Heasman et al., 2000). Contrary to these studies -carried out on late gastrulae or earlier stages- our findings show that the anti- β -catenin antibodies exert blocking effects on tail bud embryo stages. We postulate that in our model the main target of the blocking effects of the anti- β -catenin antibodies is the notochord. Earlier studies by Takaya (1977) demonstrated that the notochord and the surrounding mesenchyme tissues are a potent inducer of neural tissues not only in the ectoderm of gastrulae, but also in the ventral ectoderm of neurula and early tail-bud embryos. Expression of β -catenin mRNA is regulated by positive and negative signals derived from notochord, neural tube and lateral plate of mesoderm (Schmidt et al., 2000). Our results suggest a role for β -catenin in controlling signals as well as cell-cell adhesion events during the embryonic patterning of *B. arenarum*. Other changes induced by β -catenin inhibition included the presence of hypertrophied cardiac cells. This is surprising, considering that heart cell hypertrophy can be caused by activation of Wnt signals mediated by β -catenin-mediated mechanisms (Toyofuku et al., 2000). However, the role of β -catenin in heart hypertrophy is still unclear, although it seems to depend more on its cell-cell adhesive role than on β -catenin-induced transcription (Toyofuku et al., 2000).

In contrast to anti- β -catenin, anti-E-cadherin did not cause axis duplication. This suggests that although cadherins have a unique spatiotemporal pattern of expression during development, the cadherin-mediated cell-cell adhesion has a role that at least is partially independent of β -catenin during the tissue patterning of stage-17 development of *B. arenarum* embryos.

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