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Catenins in *Xenopus* embryogenesis and their relation to the cadherin-mediated cell-cell adhesion system

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SUMMARY

In the course of an analysis of cell-cell adhesion in the *Xenopus* embryo, antibodies directed against α- and β-catenin were applied to investigate their relation to the cadherins occurring early in this system. The results demonstrate that α- and β-catenin are provided maternally and increase in amount throughout embryogenesis. Immunoprecipitations indicate that both of the catenins are complexed to U-cadherin in the early phase of embryogenesis and to E-cadherin, when it appears during gastrulation. An excess of α-catenin occurs in free form in the early embryo, whereas all of the β-catenin seems to be complexed to cadherin. Synthesis of the two components throughout early embryogenesis and their binding to newly synthesized cadherins were demonstrated by metabolic labelling.

The spatial distribution of α-catenin was analysed by immunohistology. During cleavage α-catenin is deposited evenly along the plasma membranes within the embryo, while the cell peripheries at the surface of the embryo remain devoid of α-catenin. At later stages, the pattern of α-catenin distribution becomes more complex. Quantitative differences in the intensity of staining along the plasma membranes in the different regions of the embryo can be distinguished. Particularly the appearance of E-cadherin in the gastrula ectoderm is accompanied by conspicous depositions of α-catenin along the respective plasma membranes in this layer. All cells in the later embryo, apart from the neural crest cells, carry α-catenin on their plasma membranes indicating the universal character of cadherin-mediated cell-cell adhesion in the *Xenopus* embryo.

Key words: catenin, cadherin, cell adhesion, embryogenesis, *Xenopus*

INTRODUCTION

The recent identification of different cadherins on the plasma membranes of the early *Xenopus* embryo explains the well-known calcium dependency of interblastomere adhesion. U-cadherin participates in the mutual adhesion of all embryonic cells from the first cleavage division until beyond the gastrula stages (Angres et al., 1991). EP-cadherin, a further cadherin detected in the early embryo (Ginsberg et al., 1991; Levi et al., 1991), resembles U-cadherin in its distribution but is not identical to U-cadherin as recent immunological evidence indicates (Müller et al., personal communication). EP-cadherin is identical to CLP (Choi et al., 1990) and may be an isoform of XB-cadherin (Herzberg et al., 1991). Whether EP-cadherin contributes to cell adhesion in the early embryo and whether it has functions different from those of U-cadherin is not known.

The cadherin-dependent cell adhesion system in the embryo becomes more complex when new members of the cadherin family emerge during gastrulation and neurulation. E-cadherin appears in the gastrula ectoderm and N-cadherin becomes expressed at restricted locations including the neural plate (Angres et al., 1991; Choi and Gumbiner, 1989; Detrick et al., 1990).

Prior to the emergence of this spatially patterned mosaic of cadherins, the embryonic cells exhibit distinct activities that imply a great plasticity and flexibility of the early cell-cell adhesion system. During the first cleavage cycles adjacent blastomeres lose contact in a defined area to allow the blastocoel anlage to form (Kalt, 1971a, 1971b). The maintenance of the blastocoel cavity might require continuous restriction of cell-cell adhesion at specific membrane domains. During gastrulation cells are able to change their relative positions when they intercalate during epiboly and convergent extension. Cell sheets slide past each other in highly organized and complex patterns during involution and germ layer formation (Keller and Winklbauer, 1992). The notochord segregates from the dorsal mesoderm. During these activities, the respective regions of the embryo do not recognizably alter U-cadherin expression on their cell membranes, i.e. movements of cells along adjacent cells and deadhesion of cell populations are achieved in spite of the continuous presence of U-cadherin on the membranes

involved. In fact, U-cadherin-mediated cell-cell adhesion of prospective head mesoderm cells is required for their persistent and directional movement over the blastocoel roof (Winklbauer et al., 1991).

These observations imply that the mere presence of U-cadherin on cell membranes is not an indication of their adhesive properties, but that the efficiency of cadherin-mediated cell-cell adhesion is locally regulated in a dynamic fashion.

Studies on systems other than *Xenopus* embryonic cells support this view. It has been shown that cadherins require intracellular anchoring to actin to be fully active in cell-cell adhesion (Boller et al., 1985; Hirano et al., 1987; Nagafuchi and Takeichi, 1988, 1989; Ozawa et al., 1990; Magee and Buxton, 1991; Takeichi, 1991). Moreover, not all cadherin molecules of a given cell line are associated with microfilaments and the ratio of actin-bound to unbound cadherin can change depending on the cell type or the growth rate of cells (Shore and Nelson, 1991). These findings give rise to the attractive assumption that cadherin activity on embryonic cells may be regulated by association to and dissociation from the very dynamic and versatile microfilament system. This would allow for local modulations of cadherin activity in specific embryonic regions and even on membranes of individual cells.

A group of proteins which associate intracellularly with the cadherins has been termed catenins (Ozawa et al., 1989). They seem to provide the link between the cadherins and the microfilament system. Whereas -catenin has not been well characterized as yet, the primary structure of - and -catenin has recently been established. -catenin shares sequence homology with vinculin (Herrenknecht et al., 1991; Nagafuchi et al., 1991), a protein that is involved in the cytoplasmic anchoring of integrins and is localised in zonulae adherentes and in focal contacts. -catenin exhibits strong homology to plakoglobin and the product of the *Drosophila armadillo* gene (McCrea et al., 1991; Peifer et al., 1992; Butz et al., 1992). While -catenin binds tightly to the cytoplasmic domains of cadherins (McCrea and Gumbiner, 1991), -catenin seems to mediate the association of cadherins to actin fibres (Ozawa et al., 1990; Tsukita et al., 1992).

In general, the catenins may associate with different cadherins (Ozawa et al., 1989; Herrenknecht et al., 1991; Wheelock and Knudson, 1991; Ozawa and Kemler, 1992), but evidence has been reported that catenins associate differentially with one cadherin in cells that express more than one member of this protein family (Herrenknecht et al., 1991). Moreover, an isoform of -catenin, -N-catenin, has recently been identified which complexes with N-cadherin in neural cells. Transfection with -N-catenin of cells that express E-cadherin but not -catenin induces enhanced adhesiveness and a change in multicellular organization. (Hirano et al., 1992). These observations emphasize the great potential for the regulation of cell-cell adhesion, which catenins may provide.

Most of the present knowledge about catenins is derived from the analysis of cultured cell lines and little is known about the expression and function of catenins during embryonic development. Unlike cultured cell lines, the cell population in the embryo is not in a steady state, but is shifting continuously as the individual cells follow their autonomous program of development or respond to inductive stimuli. One might therefore envisage that the concentration and activity of the components of the cadherin-mediated cell adhesion system change continuously to adapt to the demands of the different developmental processes. These considerations, together with the knowledge about the importance of the catenins in regulating cell-cell adhesion, have led us to study - and -catenin in the developing *Xenopus* embryo. We report on their accumulation, their complexing to different cadherins and the regional and subcellular distribution of -catenin at the different developmental stages.

MATERIAL AND METHODS

Embryos

Xenopus embryos were obtained, raised in 1/10 MBSH as described by Fey and Hausen (1990) and staged according to Nieuwkoop and Faber (1967).

Preparation of extracts

Embryos of different stages were sonicated at 4°C in lysis buffer (2% NP-40, 1 mM $CaCl_2$, 150 mM NaCl, 10 mM Tris pH 7.4, 2 μM aprotinin, 2 μM pepstatin, 2 μM leupeptin, 2 mM iodoacetamide, 1 mM N-ethylmaleimide and 2 mM phenylmethylsulfonylfluoride). 10 μl of lysis buffer per embryo were used. Yolk was sedimented by centrifugation (1 minute at 14 000 revs/minute). Lipids were removed from the supernatant by extraction with an equal volume of 1,1,2-trichlorotrifluoro-ethane and centrifuged as before. Extracts were used immediately or else stored in liquid nitrogen.

Antibodies

Anti- -catenin antibodies (-M12K) were produced by immunizing rabbits with the synthetic peptide HVNPVQALSEFK, a sequence that is localized near the C-terminus of mouse -catenin as described by Herrenknecht et al. (1991). Anti- -catenin antibodies (-P14L) were produced likewise, using the synthetic peptide PGDSNQLAWFDTDL, a sequence that is localized at the C terminus of mouse -catenin (Butz et al., 1992), for immunization. Antibodies were affinity purified from the immunsera by using the corresponding peptides. Monoclonal antibodies directed against U-cadherin (mAb 6D5) and *Xenopus* E-cadherin (mAb 10H3) (Angres et al., 1991) were purified from ascites fluids on a protein A column. Control P3, an inert IgG, is produced by a nonfused mouse myeloma cell line.

SDS-PAGE and immunoblotting

Equivalents of 5 embryos for one lane were heated in sample buffer for 10 minutes to 95°C and subjected to 7% SDS-PAGE (Laemmli, 1970). Proteins were transferred electrophoretically onto a nitrocellulose membrane. Blots were treated for 30 minutes with blocking buffer (0.05% Tween20, 10% low fat milk powder in PBS) and with antibodies as described by Fey and Hausen (1990). Affinity-purified anti- -catenin and anti- -catenin antibodies were diluted to approximately 1 μg/ml and 5 μg/ml respectively in blocking buffer. Anti-U-cadherin and anti-E-cadherin antibodies were used at an 1:5000 dilution of the ascites fluids. Peroxidase-coupled antibodies (Dianova) were diluted 1:3000 for use as secondary antibodies. Blots were developed with the ECL western blot detection system (Amersham).

Labelling procedures

Fertilized eggs were dejellied (2% cysteine, pH 8.0) 30 minutes after fertilization, transferred into 1/10 MBSH containing 5% Ficoll (Pharmacia) and injected with approximately 2 μCi of L-[^{35}S]methionine (cell labelling grade, Amersham) in 20 nl of MBSH. Embryos were incubated up to stage 8. Labelling of gastrulae was performed by injection into the blastocoel of stage 11.5 embryos and incubation up to stage 13. Embryos of each sample were extracted with lysis buffer containing 10 mM L-methionine.

Immunoprecipitations

Immunoprecipitations were carried out as described by Müller et al. (1992). 10 μg of purified mouse antibodies were coupled to 15 μl of a protein G-sepharose suspension (Pharmacia) or, in the case of rabbit antibodies, to 80 μl of a 10% suspension of protein A-sepharose (Pharmacia). Lysates for immunoprecipitation were precleared by centrifugation (1 hour, 25000 revs/minute, TL100 ultracentrifuge, TLA 100.2 rotor) and incubated for 2 hours with the bead-coupled antibodies. Beads were washed once with washing buffer (0.05% NP-40, 0.5 M NaCl, 50 mM Tris pH 8.8, 2 mM $CaCl_2$, 1 mg/ml ovalbumin supplemented with protease inhibitors). Bound proteins were eluted with SDS-PAGE sample buffer at 95°C and processed for immunoblotting. For immunoprecipitation of labelled proteins, the beads were preabsorbed with lysates from non-labelled embryos before antibody coupling. Electrophoresis gels were blotted on nitrocellulose membranes, processed for fluorography by impregnation in amplify TM (Amersham), dried and exposed to Cronex TM X-ray films (Du Pont de Nemours). For controls, the inert P3-IgG was coupled to the beads and the samples were processed identically. In the case of anti- -catenin antibody, specific precipitation of -catenin could be inhibited by adding the peptide antigen at 50 μg/ml to the precipitation reaction.

Sedimentation analysis

Extracts from 20 embryos were layered onto 3.8 ml 5-20% (wt/wt) sucrose gradients prepared in lysis buffer. After centrifugation at 44 000 revs/minute for 16 h at 4°C in a SW65 rotor (Beckmann instruments) the gradients were fractionated from bottom to top. The fractions were analysed by SDS-PAGE and immunoblotting. Protein standards of known S values were centrifuged on replicate gradients (catalase, 11.4 S; aldolase, 7.4 S; BSA, 4.6 S).

Immunohistological procedures

Whole-mount staining of embryos was performed as described elsewhere (Angres et al., 1991). Embryos of different stages were fixed in 20% dimethylsulfoxide in methanol overnight at −20°C (Dent et al., 1989). After several washes in PBS, the specimens were incubated overnight at 4°C in 5 μg/ml anti- -catenin antibody in 20% bovine serum in PBS. Control embryos were treated with P3 IgG. In a further control sample, 100 μg/ml of the peptide M12K was added during anti- -catenin incubation. During the following day the specimens were washed in PBS at room temperature with several changes of the buffer. In the same way, DATF-conjugated goat anti-rabbit IgG (Dianova) and DATF-conjugated donkey anti-goat $F(ab)_2$ were applied overnight as secondary and third antibodies. After the last wash, embryos were fixed with paraformaldehyde in PBS for 1 hour at room temperature and washed twice with PBS for 10 minutes. Specimens were placed into 2% agar, dehydrated in two changes of 3,2 dimethoxypropane of 1 hour each at room temperature and embedded in glycolmethacrylate (Technovit 7100, Kulzer, FRG). Immunofluorescence was observed on 5 μm sections with a Zeiss Axioplan with epifluorescence optics.

RESULTS

Antibodies against mouse α- and β-catenin cross-react with *Xenopus* catenins

Rabbit antibodies against mouse - and -catenin are known to cross-react with the respective catenins of various species (Herrenknecht et al., 1991; Butz et al., 1992). The following results prove that these antibodies can be used to identify - and -catenin in the *Xenopus* embryo unambiguously. On immunoblots of embryo extracts, anti- -catenin recognizes a component of M_r 105×10^3 (Fig. 1A, lanes 5 and 6) and anti- -catenin reacts specifically with a component of M_r 94×10^3 (Fig. 1A, lanes 7 and 8). These components are nearly identical in size with mouse - and -catenin (Ozawa et al., 1989).

Xenopus U-cadherin (M_r 125×10^3) and E-cadherin (M_r 140×10^3) are recognized by monoclonal antibodies described previously (Angres et al., 1991) (Fig. 1A, lanes 1 and 2; Fig. 1A, lanes 3 and 4). When these antibodies were used for immunoprecipitations, the two components that react with the anti-catenin antibodies, coprecipitate from embryo extracts with either U-cadherin (Fig. 1B, lanes 1, 3 and 5) or E-cadherin (Fig. 1C, lanes 1, 3 and 5). This coprecipitation is a typical feature of catenins. Control samples, mock precipitated with the inert IgG P3, showed none of the specific components (Fig. 1B and 1C, lanes 2, 4 and 6).

α- and β-catenin are present in the embryo throughout early development

Embryos of different stages were analyzed for - and -catenin and for U- and E-cadherin by use of the immunoblotting technique. As shown in Fig. 2, both - and -catenin are present in embryos of all stages. Although a detailed quantification was not carried out, it is apparent from the signal intensity of the bands that the proportion of the amounts of the two components changes with progressing development. The level of -catenin supplied by the egg rises only moderately up to stage 11, whereas -catenin accumulates significantly during this interval. The rate of U-cadherin production resembles that of -catenin.

These changes in their relative amounts indicate that the three components are not confined to a typical cadherin-catenin complex throughout development. In particular the excess of -catenin in the early stages suggests its presence in a form not linked to cadherin. The following experiments were aimed at this question.

α- and β-catenin are complexed with U-cadherin and E-cadherin during development

Detergent extracts were prepared from embryos of different stages and immunoprecipitates with anti-U-cadherin and anti-E-cadherin were analyzed for the presence of the cadherins and catenins.

Between stage 1 and 10.5 both - and -catenin coprecipitate with U-cadherin (Fig. 3A). While the total amount of the precipitated complex increases with advancing development, the relative proportions of the three components within the complex appear to remain constant.

A similar result was obtained when the E-cadherin-

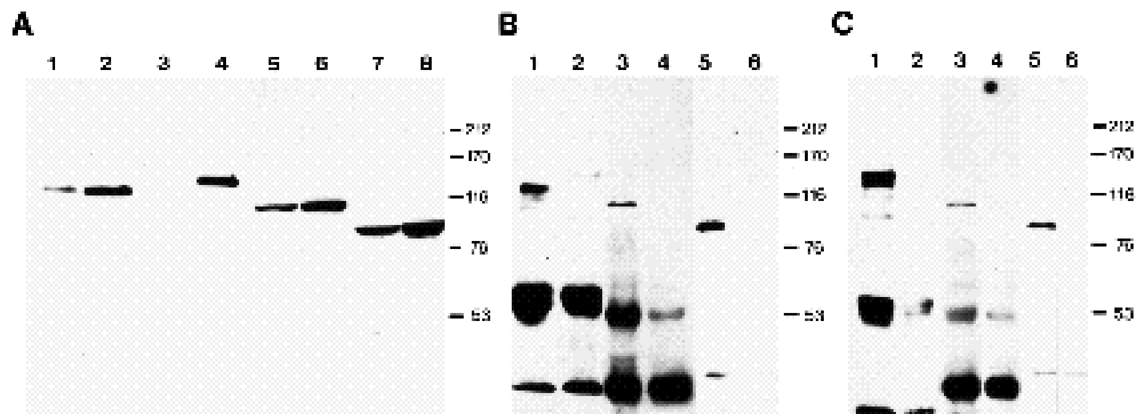

Fig. 1. Cross-reaction of antibodies directed against mouse -catenin and -catenin with *Xenopus* catenins. (A) Extracts of five embryos each of stage 10 (lanes 1, 3, 5 and 7) and stage 17 (lanes 2, 4, 6 and 8) were electrophoresed. Blots were developed with anti-U-cadherin (lanes 1 and 2), anti-E-cadherin (lanes 3 and 4), anti- -catenin (lanes 5 and 6) and anti- -catenin (lanes 7 and 8). (B) Components precipitated by anti-U-cadherin from extracts of 50 stage 10 embryos were analysed by immunoblotting using anti-U-cadherin (lane 1), anti- -catenin (lane 3) and anti- -catenin (lane 5). (C) Components precipitated by anti-E-cadherin from extracts of 50 stage 17 embryos were analysed by immunoblotting using anti-E-cadherin (lane 1), anti- -catenin (lane 3) and anti- -catenin (lane 5). For controls in B and C (lanes 2, 4, and 6), mock precipitates using an inert IgG were used for electrophoresis. The strong signals in the lower part of B and C result from IgGs which were released into the samples from the precipitation beads and which bind the second antibodies. The position (bars) and size (M_r) of molecular weight markers are indicated.

catenin complex was precipitated from extracts of stage 12.5-22 embryos (Fig. 3B). Neither cadherin nor catenin are found in the precipitates, when anti-E-cadherin was used for immunoprecipitation at the pregastrula stages (not shown).

These results indicate that the gross composition of the cadherin-catenin complexes remains constant as development advances.

A major portion of α- and β-catenin in the early gastrula is not complexed to U-cadherin

To test whether all of the catenins of the early gastrula embryo are complexed to U-cadherin, depletion experiments were performed. U-cadherin is efficiently removed from stage 10 embryo extracts by two sequential immunoprecipitations with anti-U-cadherin antibody (Fig. 4, compare lanes A, B^s and C^s), the bulk of the cadherin remaining in the precipitates (Fig. 4, B^p and C^p). However, the levels of - and -catenin remain virtually unaffected by the depletion of the extract with anti-U-cadherin (Fig. 4, compare lane A with B^s and C^s). It can be concluded from this observation that the major portion of the two catenins in the extract is not complexed to U-cadherin. Figure 4 also reveals that the relative proportion of - and -catenin in the precipitated complex differs from that contained in the depleted extract. (Fig. 4, compare lanes C^s and B^p). It is therefore unlikely that all the catenin that has remained in the extract is assembled in a typical cadherin-catenin complex, which would then have to contain a cadherin other than U-cadherin.

To further assess whether the catenins are free or complexed, sedimentation velocity experiments were performed. Extracts from stage 10 embryos were fractionated by sucrose gradient centrifugation and the individual samples were assayed by immunoblotting for U-cadherin, - and -catenin.

Fig. 5 shows that U-cadherin and -catenin can indeed be separated by gradient centrifugation. U-cadherin peaks at around fraction 10-11 and -catenin at around fraction 12 with a slight asymmetry towards the bottom of the gradient. The estimated sedimentation constant of the putative U-cadherin-catenin complex agrees with that described for other cadherin-catenin complexes from mouse cells (Ozawa and Kemler, 1992). In contrast, the large excess of -catenin sediments at a slower rate and seems to occur in a free form.

Interestingly, -catenin shows a distribution in the gradient which is congruent with that of U-cadherin, indicating that most of this protein is bound in a complex which

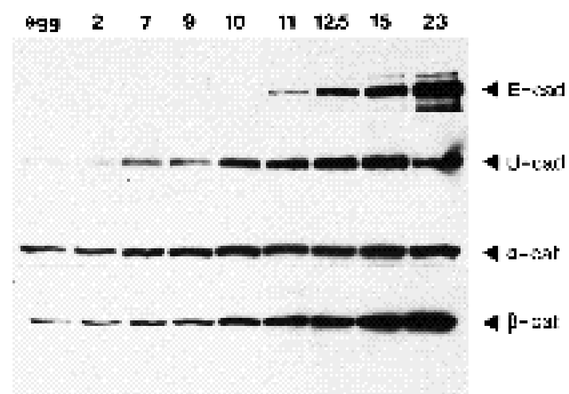

Fig. 2. Western blot analysis of -catenin, -catenin, E-cadherin and U-cadherin during early embryogenesis. Extracts of embryos of different stages (five embryos per lane) were analyzed by immunoblotting with mAb 10H3 for E-cadherin, mAb 6D5 for U-cadherin, -M12K for -catenin and -P14L for -catenin. The developmental stages are indicated.

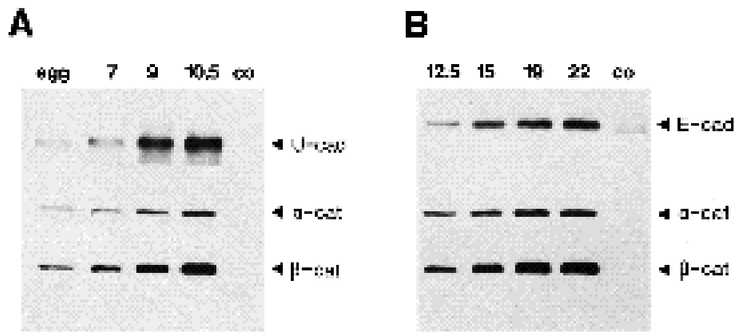

Fig. 3. Coprecipitation of - and -catenin with U-cadherin and E-cadherin from extracts of embryos of different stages. (A) Immunoprecipitates obtained with anti-U-cadherin from extracts of 50 embryos at different developmental stages were analysed by immunoblotting for U-cadherin, and -catenin and -catenin as before (see Fig. 2). (B) Immunoprecipitates obtained with anti-E-cadherin from extracts of 50 embryos of different developmental stages were analysed by immunoblotting for E-cadherin and -catenin and -catenin as before (see Fig. 2). The developmental stages are indicated. co, controls.

is similar in size to cadherin-catenin complexes. Since the depletion experiment described above indicates that the major fraction of -catenin is not complexed with U-cadherin (Fig. 4), the bulk of the -catenin in the extract might conceivably be bound to a different cadherin.

Metabolic labelling of the U-cadherin-catenin complex

In a search for further components with which U-cadherin and the free -catenin might be associated, immunoprecipitates from metabolically labelled material were analyzed. Fertilized eggs and stage 11.5 gastrulae were injected with [^{35}S]methionine and the embryos were cultured up to stages 8 and 13 respectively. Immunoprecipitates from the labelled extracts were subjected to gel electrophoresis, blotting and autoradiography (Fig. 6).

The anti-U-cadherin antibody specifically precipitates four labelled components (Fig. 6A,B, lanes 3) that are not present in the mock precipitated controls (Fig. 6A,B, lanes 1). By staining the blots with the different antibodies after fluorography, these components were identified as indicated in Fig. 6. U-cadherin and -catenin are heavily labelled in stage 8 and stage 13 extracts, whereas -catenin shows only moderate labelling. The fourth protein coprecipitating with U-cadherin has a molecular mass slightly smaller than -catenin and might represent the cadherin-associated -catenin. Anti- -catenin precipitates only -catenin in significant amounts (Fig. 6A,B, lanes 2) and a trace of -catenin. No further coprecipitating components were detected. This lack of additional components in the precipitate is not due to a conceivable specificity of the antibody for non-complexed -catenin. From extracts of labelled *Xenopus* tissue culture cells, cadherins readily coprecipitate with this antibody (Herrenknecht et al., 1991).

We interpret the results from the labelling experiment as follows: an excess of labelled -catenin is present in free uncomplexed form in embryonic cells and is precipitated as such with anti- -catenin from the extracts. All components of the complex precipitating with anti-U-cadherin - the catenins and U-cadherin - are synthesized during embryogenesis and no further labelled proteins remain attached to the complex after extraction. For complex formation in vivo -catenin from a pool of free maternal material intermingles with newly synthesized labelled -catenin. This results in a weak labelling of -catenin in the complex (see Fig. 6 lane 3). This maternal influence on the labelling of the -catenin pool can at least be detected up to stage 13.

Anti- -catenin antibody did not give satisfying results in immunoprecipitations.

Immunolocalization of α-catenin in embryos

Embryos of stages 1-27 were fixed, whole-mount stained with the anti-catenin antibodies, embedded in plastic and sectioned. Immunohistology with the anti- -catenin antibody yielded only unsatisfactory results. The following description is therefore restricted to the distribution pattern of -catenin.

Egg and cleavage stages (Fig. 7)

The cytoplasm of the fertilized egg exhibits a finely punctated pattern of -catenin staining predominantly in the animal hemisphere (Fig. 7A). This intracellular distribution of -catenin is maintained during early cleavage (Fig. 7B,E) and disappears later. At the same time -catenin becomes associated with all newly formed cleavage membranes inside the embryo (Fig. 7B, arrowheads Fig. 7E). Though the staining along the membranes appears smooth and even in cross sections, a finely punctated distribution becomes apparent when a cell border is sectioned tangentially (Fig. 7C, arrowheads). Staining is absent or greatly diminished along membranes not lined by an adjacent membrane. This is apparent at clefts, which are sometimes present between the early blastomeres (Fig. 7D, arrowheads) and on the

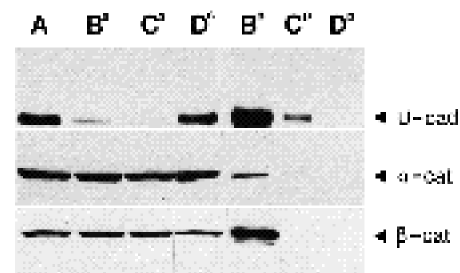

Fig. 4. Catenins in extracts depleted of U-cadherin by sequential immunoprecipitation. Extracts of 50 stage 10 embryos were subjected to two consecutive immunoprecipitations with anti-U-cadherin. Aliquots of the original extract (A) and the supernatants of the first and the second immunoprecipitation (B^s and C^s respectively) and the corresponding precipitates (B^p and C^p) were analyzed for U-cadherin, -catenin and -catenin by immunoblotting as before (see Fig. 2). D^s and D^p are supernatant and precipitate of the control samples in which inert P3-IgG was applied for precipitation.

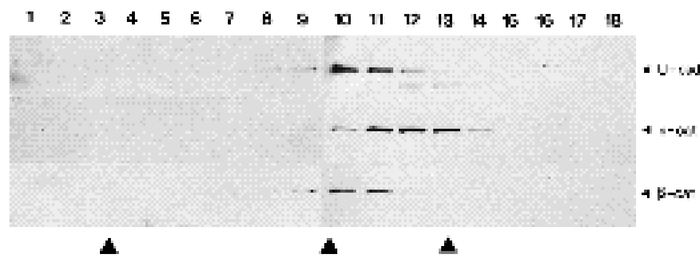

Fig. 5. Separation of -catenin from U-cadherin and -catenin by sucrose gradient centrifugation. An extract of 20 stage 10 embryos was fractionated on a sucrose gradient (see Material and Methods) and the fractions (fraction numbers from bottom to top) were analyzed for U-cadherin, -catenin and -catenin by immunoblotting as before (see Fig. 2). The arrowheads indicate the peak distribution of protein standards of known S values (from left to right: 11.4 S, 7.4 S, 4.6 S).

membrane domains bordering the blastocoel (Fig. 7E, arrowheads).

The egg is free of -catenin along the plasma membrane (Fig. 7A, arrowheads). This property of the egg periphery is maintained during cleavage when it forms the apical domains of the blastomeres at the surface of the embryo (Fig. 7E).

Late blastula and early gastrula (Fig. 8)

All cells at these stages display -catenin along their membranes. From the midblastula stage onwards, the staining along the membranes of the animal region becomes more pronounced compared to those of the vegetal region. The intensity of the staining is retained when the animal material is shifted vegetally by epibolic movement (Fig. 8A). With the appearance of E-cadherin at stage 11.5, -catenin staining along the cell borders of the ectoderm becomes significantly stronger. At late gastrula, the ectoderm and the blastopore lip region have become the dominantly stained regions of the embryo (Fig. 8D). The apical domains of the blastomeres at the surface of the embryo remain free of -catenin (Fig. 8A-C) and this feature is retained when the prospective endoderm cells involute to form the archenteron roof and floor (Fig. 8D, arrowheads). Accumulations of -catenin at the apical rim of the lateral membrane domains at the embryonic surface indicate junctional complexes (Fig. 8B, arrowhead). This staining is also a conspicuous feature of the apically constricted bottle cells of the blastopore groove (Fig. 8C, arrowhead).

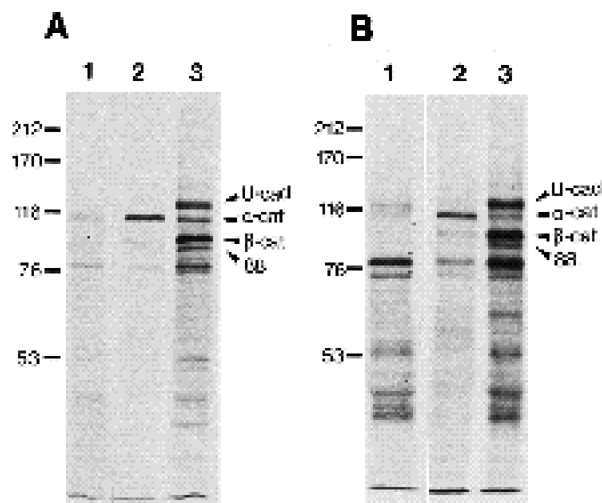

Fig. 6. Analysis of metabolically labelled proteins that coprecipitate with U-cadherin and -catenin. 30 fertilized eggs were injected with [^{35}S]methionine and cultured until stage 8 and 30 stage 11.5 gastrulae were injected with [^{35}S]methionine into the blastocoel and cultured until stage 13. Aliquots of extracts were immunoprecipitated with anti- -catenin and with anti-U-cadherin respectively. Electrophoretic patterns of radioactive proteins were visualized by fluorography. After fluorography, U-cadherin, -catenin and -catenin were identified by staining the blots with the corresponding antibodies. Their position is indicated by arrowheads. The unidentified coprecipitating protein at M_r 88×10^3 might be -catenin. In the control samples, an inert IgG was applied in the precipitation. (A) Extracts of labelled stage 8 embryos. (B) Extracts of labelled stage 13 embryos. Lanes 1: controls; lanes 2: precipitates obtained with anti- -catenin; lanes 3: precipitates obtained with anti-U-cadherin.

Neurula stages (Fig. 9)

The staining of the ectodermal cells including the neural plate is most prominent (Fig. 9A), but -catenin is also present along the membranes of all mesodermal and endodermal cells throughout the embryo (Fig. 9B,C). Within the mesoderm, the dorsal axial structures exhibit the strongest staining. The absence of -catenin from the apical domains at the embryonic surface and the archenteron roof is conspicuous (Fig. 9C). When bottle cells form during invagination of the neural tube, -catenin accumulates in the apical constrictions and remains in this position upon closure of the neural tube (Fig. 9D,E). When the neural tube, notochord and somites have segregated from the surrounding tissues, -catenin begins to disappear from the outer borders of these organanlagen (arrows in Fig. 9D). Removal of -catenin from these sites follows, in time and spatially, the deposition of basal laminae at these locations (Fey and Hausen, 1990).

Tailbud stages (Fig. 10)

In general, the intensity of the staining along the cell peripheries has increased markedly, but it varies locally and becomes differentiated. Most conspicuously, migrating neural crest cells, which now populate large parts of the head region, are completely devoid of membrane-bound -catenin (Fig. 10F). As before, the apical domains of the outer epidermal cells are free of -catenin (Fig. 10A and H). Newly formed epithelia lining secondary cavities like the neural tube (Fig. 10F) and the ear vesicle (Fig. 10G) likewise exhibit no staining on their apical cellular domains and have -catenin concentrated at the apical junctional complexes. When such epithelia bend during morphogenetic events and bottle cells are formed, -catenin accumulates at the apical constrictions of these cells (Fig. 10A, pronephros and neural tube). Cell membranes adjacent to basal lamina that has now formed along the sensorial layer

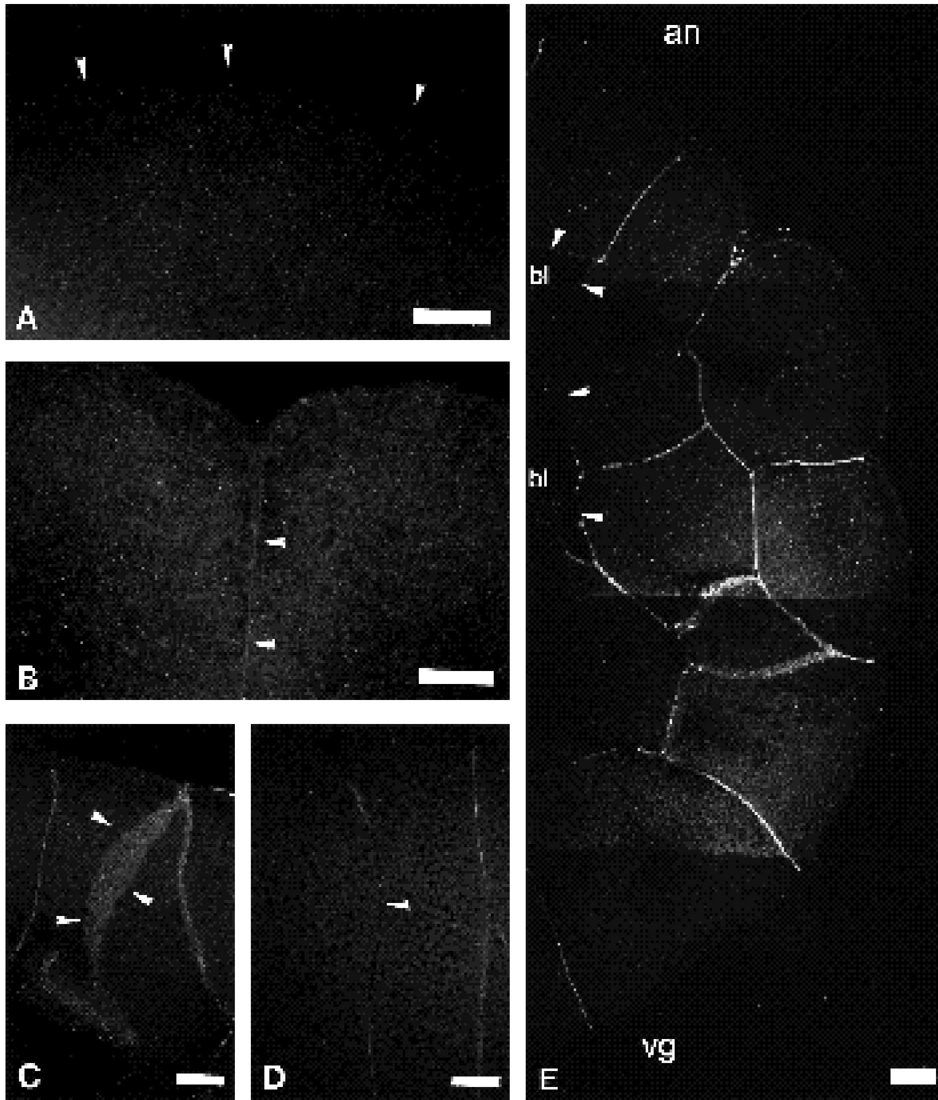

Fig. 7. Distribution of -catenin in early cleavage stage embryos. Embryos were immunostained with -M12K antibody, embedded in glycolmethacrylate and sectioned. (A) Animal pole region of a fertilized egg. Arrowheads indicate the plasma membrane. (B) Animal pole region of an eight cell stage. The internal plasma membrane is indicated by arrowheads. (C) Blastomeres near the animal pole of a stage 6 embryo. The lateral membrane was sectioned tangentially (arrowheads). (D) Cleft (arrowheads) between vegetal blastomeres of a stage 6 embryo. (E) Transversal section of a stage 6 embryo. Arrowheads indicate the cell membranes adjacent to the blastocoel. No staining was obtained in controls. an, animal pole region; bl, blastocoel; vg, vegetal pole region; Bars: 50 μm.

of the epidermis (Fey and Hausen, 1990) are devoid of -catenin (Fig. 10H, arrows). In some instances, staining occurs along the membranes in local condensations, which may form a fairly regular pattern (Fig. 10B, in the somite cells and Fig. 10C, along the cell membranes of the columnar cells of the cement gland).

DISCUSSION

Protein biochemical studies

Under the premise that the amounts of the components and their state of complexing in the extracts are representative for the in vivo conditions, one may subsume our results in the following tentative model:

(1) In the early embryo some U-cadherin and -catenin is supplied maternally, but the rate of production of these components during cleavage is so high that virtually all of the material present in the early gastrula is derived from new synthesis. In contrast, the amount of -catenin from the maternal pool suffices to contribute an appreciable fraction of the total until beyond the gastrula stages.

(2) U-cadherin is complexed to -catenin and -catenin and no free U-cadherin is detectable at any stage. Thus, the rise in U-cadherin during development is mirrored in a rise in the cadherin-catenin complex. This complex maintains a constant composition with regard to the relative proportion of the constituents. When E-cadherin appears at midgastrula, it is also complexed to the catenins in the typical proportion.

(3) The major fraction of -catenin is present in free form throughout early development. Maternal and newly synthesized -catenin provide a common pool from which material for the newly forming cadherin-catenin complexes is taken.

(4) The major portion of -catenin in the early gastrula is not complexed to U-cadherin, but it does seem to be associated with a different cadherin.

The presence of a large pool of free -catenin in the egg and the early embryo implies that the synthesis of -catenin and the other components of the complex are not interdependent during oogenesis and embryogenesis. The excess of free -catenin makes it unlikely that binding of cadherins

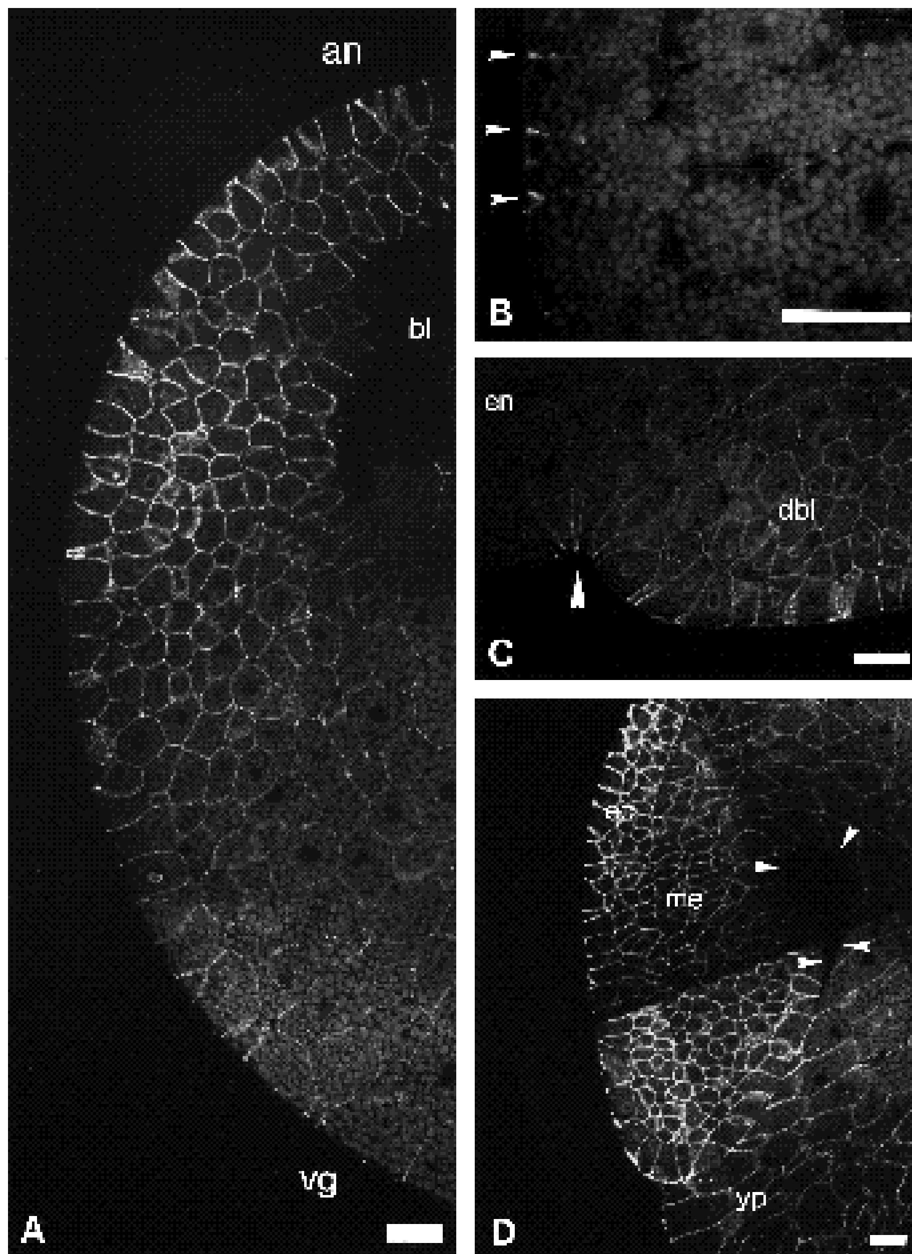

Fig. 8. Distribution of -catenin in blastula and gastrula stages. (A) Ventral portion of a sagittal section through a stage 10 embryo. (B) The marginal region of a stage 9 blastula. Apical concentrations of -catenin along the lateral membranes of the outer ectodermal blastomeres are indicated with arrowheads. (C) Dorsal blastoporal lip region of an early gastrula with apically constricted bottle cells (arrowhead). (D) Lateral blastopore lip region of a stage 12.5 embryo. Arrowheads indicate apical membranes of the cells that form the archenteron roof and floor. The unspecific staining of the yolk platelets is due to autofluorescence. an, animal region; bl, blastocoel; dbl, dorsal blastopore lip region; en, endoderm; ec, ectoderm; me, mesoderm; vg, vegetal pole region; yp, yolk plug. Same histological procedure as in Fig. 7. Bars: 50 μm.

to the cytoskeleton is regulated by the availability of -catenin. It might also explain recent observations by Kintner (1992). When a construct of N-cadherin mRNA lacking the sequence of the extracellular domain is injected into fertilized eggs, the truncated protein synthesized from the construct is expected to compete with the endogenous cadherins for catenin binding thereby inhibiting cell-cell adhesion. This presumed effect was only found in the late gastrula stages, when, as we would interpret, sufficient amounts of the construct protein have formed to inactivate the excess of -catenin.

Increasing amounts of -catenin in the early embryo have also been observed recently by DeMarais and Moon (1992). Our observation that neither -catenin nor U-cadherin accumulate in free form indicates that the production of the two components is coupled in some way. It has been demonstrated that -catenin in fact complexes with E-cadherin while the latter is still in its precursor form (Ozawa and Kemler, 1992).

An apparent contradiction in our data on -catenin has recently been solved. Though most of the -catenin in the extracts does not coprecipitate with U-cadherin, it does sediment at the velocity of a cadherin-catenin complex. Application of an antiserum directed against different *Xenopus* cadherins including EP-cadherin yielded immunoprecipitates from late blastula extracts which contain the bulk of the -catenin (data not shown). It is concluded that -catenin in the early embryo is indeed complexed to cadherin, possibly to EP-cadherin. This cadherin seems to prevail at these embryonic stages.

The continuous binding of catenins allows U-cadherin to connect to the cytoskeleton and to be active in cell-cell

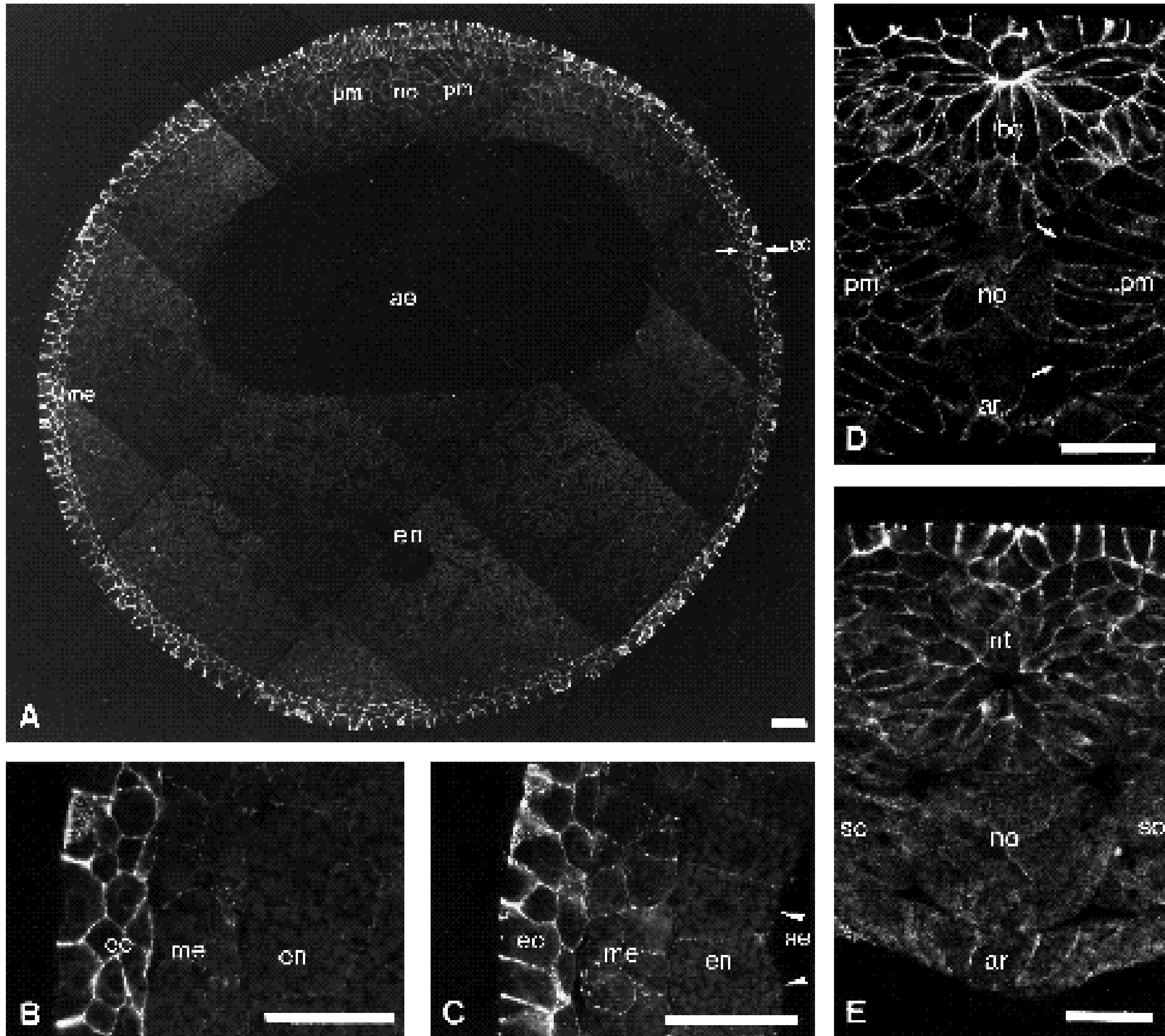

Fig. 9. Distribution of -catenin in neurulae. (A) Transversal section of a stage 14 embryo. (B) Section through the lateral region of a stage 14 embryo depicting the ectodermal, mesodermal and endodermal cell layers. (C) Ventral detail of a sagittal section through a late neurula stage 19 embryo. Arrowheads point to the apical membranes of the endoderm. (D) Embryos at stage 17 and (E) at stage 19 were cut through the central dorsal region transversally. Arrows indicate the regions where cell membranes are devoid of staining. ae, archenteron; ar, archenteron roof; bo, bottle cells; ec, ectoderm; en, endoderm; me, mesoderm; no, notochord; pm, paraxial mesoderm; so, somites. Same histological procedure as in Fig. 7. Bars: 50 μm.

adhesion from early cleavage onwards as we have reported previously (Angres et al., 1991). The question as to how this cell-cell adhesion is regulated locally requires a more detailed analysis.

Immunohistology

The intracellular staining of the egg and early blastomeres

We have recently shown that the bulk of maternal U-cadherin present in the egg derives from new synthesis during oocyte maturation (Müller et al., 1992). The newly synthesized U-cadherin immediately becomes complexed with -catenin and -catenin (Müller, 1992). Interpretation of the stained cytoplasmic granules in the egg and the early blastomeres as cadherin-catenin containing postGolgi structures, which are destined for the newly forming cleavage membranes, is one possibility that has to be confirmed by further investigation.

-catenin along the membranes of the embryonic cells

-catenin is localized along all newly formed membranes of the early embryo. A similar distribution of U-cadherin on the embryonic membranes has previously been described in detail (Angres et al., 1991). Like U-cadherin, -catenin exhibits strong staining in regions of high morphogenetic

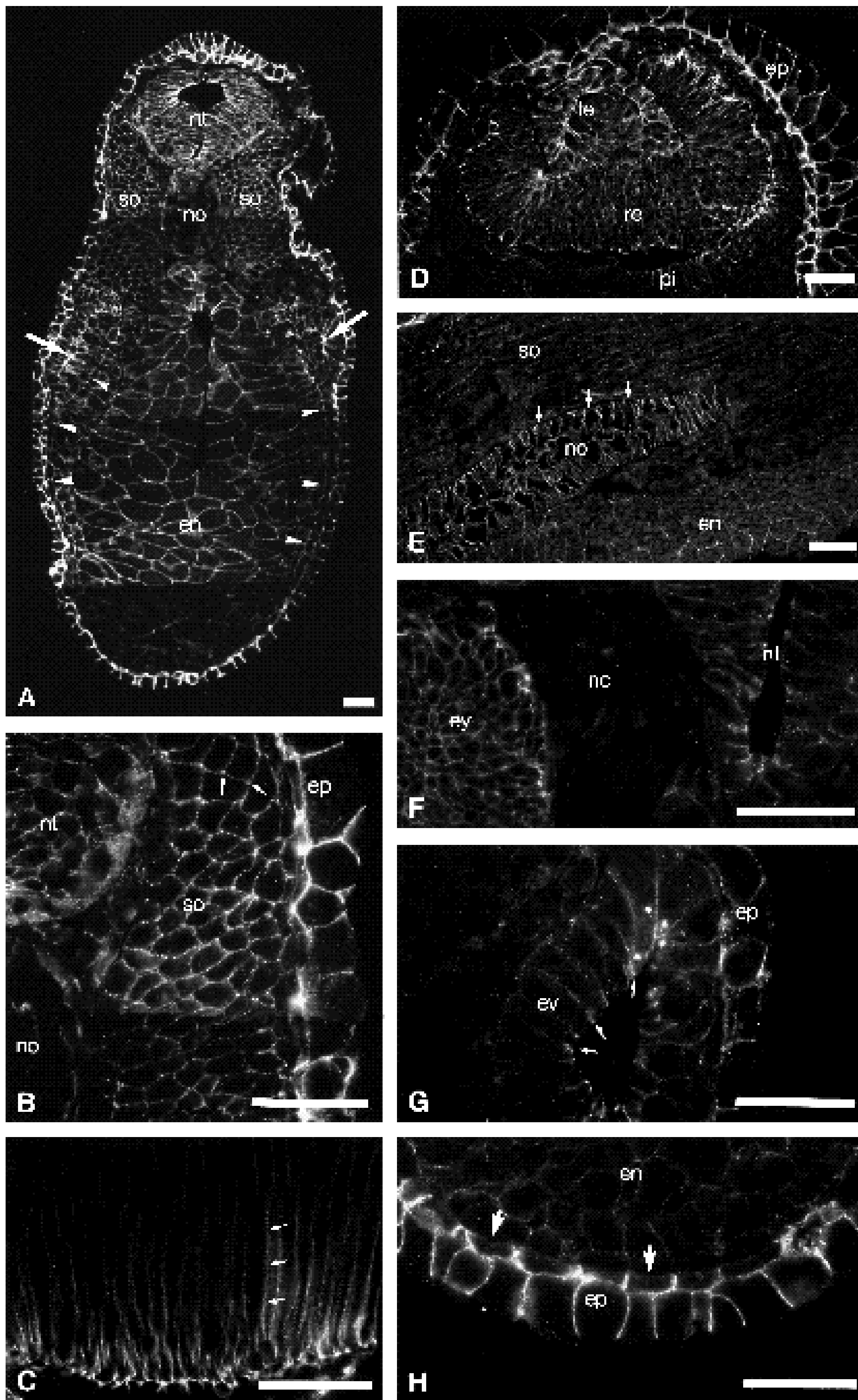

Fig. 10. Distribution of -catenin in the stage 27 tailbud embryo. (A) Transversal section showing staining patterns in different organ anlagen, particulary in the neural tube, pronephros anlage (arrows) and lateral plate mesoderm (arrowheads). (B, C, F, G and H) Details of transversal sections; (D,E) details of parasagittal sections. (B) Somite, arrows indicate the inhomogenuous staining pattern around the somite cells. (C) Cement gland, arrows point at a lateral plasma membrane of a columnar cell. (D) Eye vesicle. (E) Notochord, plasma membranes bordering the basal lamina are indicated by arrows. (F) Neural crest cells are unstained. (G) Ear vesicle, junctional concentrations of -catenin are indicated by arrows. (H) Ventral epidermis, cell membranes of the bilayered epidermis adjacent to the basal lamina exhibit only weak staining (arrows). en, endoderm; ep, epidermis; ev, ear vesicle; ey, eye vesicle; le, lens placode; nc, neural crest cells; no, notochord; nt, neural tube; pi, pigment layer; re, retinal layer; so, somites. Same histological procedure as in Fig. 7. Bars: 50 μm.

activity, e.g. in the blastopore lip region. These observations show that one of our initial questions, whether cadherin-dependent cell-cell adhesion is regulated during morphogenesis by a modulation of the -catenin-mediated binding of cadherins to the cytoskeleton, will remain open until higher resolution methods are used.

After midgastrula, further types of cadherins appear at localized regions of the embryo. Under the premise that the presence of -catenin along the membrane of adjacent cells represents a general marker for cadherin-dependent cell-cell adhesion independent of the cadherin type involved, we may state from our results that all cells in the embryo are joined by cadherins. This finding emphasizes the fundamental role of cadherins in embryonic cell-cell adhesion. Migrating neural crest cells, which are known to lose their N-cadherin when they leave the neurectoderm (Bronner-Fraser et al., 1992; Hatta et al., 1987), appear to be the only exception to this rule. The periphery of these cells is free of -catenin. This observation indicates that neural crest cells not only lack N-cadherin, but that cadherins in general are not functional in these cells during their migratory phase.

A very prominent staining of the ectoderm during gastrulation indicates that cadherin-mediated cell-cell adhesion is particulary strong in this differentiating tissue. During these stages, E-cadherin is added to the U-cadherin in the ectoderm. The staining probably results from -catenin molecules bound by either of the two cadherins. When the neural plate forms from the ectoderm, E-cadherin is removed from this region and is replaced by N-cadherin (Angres et al., 1991; Detrick et al., 1990). As one would expect if the two cadherins were associated with -catenin, this transition is not reflected in a change in -catenin staining.

Absence of -catenin from the cell borders at the embryonic surface

In a previous publication, we reported that U-cadherin is removed from the plasma membrane by endocytosis during oocyte maturation (Müller et al., 1992). As a result, the membranes at the embryonic surface remain free of U-cadherin during development. The present data show that the egg periphery is free of -catenin as well and that cell borders that derive from the egg periphery during cleavage retain this property for prolonged periods. This observation is important since it demonstrates that the lack of cadherins in membrane domains at the outer embryonic surface correlates with changes of submembranal elements.

Subcellular distribution of -catenin

Apart from the absence of -catenin from the peripheral cell cortices at the surface of the embryo, local modulations of -catenin concentrations along membranes of individual cells are evident. Very early during cleavage clearly defined clefts between adjacent blastomeres sometimes occur. The plasma membranes of these regions are free of U-cadherin (Angres et al., 1991) and of -catenin. Similarly, membrane domains facing the blastocoel cavity are not lined by -catenin. It appears, that cadherin-catenin complexes along a membrane require homophilic binding to cadherin on an adjacent membrane to become firmly localized.

During further development, -catenin is deposited along cell membranes in a fashion that exactly mirrors cadherin distribution (Angres et al., 1991). Thus, epithelial adherens junctions are conspicuously marked while membrane domains that are attached to basal laminae become devoid of -catenin. The regular, finely punctuated pattern of -catenin distribution observed between the somite and cement gland cells may reflect the existence of as yet uncharacterized cell junctions.

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