

Expression of Cell Adhesion Molecule E-Cadherin in *Xenopus* Embryos Begins at Gastrulation and Predominates in the Ectoderm

Young-Sook Choi and Barry Gumbiner

Department of Pharmacology, University of California, San Francisco, California 94143

Abstract. The expression of the Ca^{2+} -dependent epithelial cell adhesion molecule E-cadherin (also known as uvomorulin and L-CAM) in the early stages of embryonic development of *Xenopus laevis* was examined. E-Cadherin was identified in the *Xenopus* A6 epithelial cell line by antibody cross-reactivity and several biochemical characteristics. Four independent mAbs were generated against purified *Xenopus* E-cadherin. All four mAbs recognized the same polypeptides in A6 cells, adult epithelial tissues, and embryos. These mAbs inhibited the formation of cell contacts between A6 cells and stained the basolateral plasma membranes of A6 cells, hepatocytes, and alveolar epithelial cells.

The time of E-cadherin expression in early *Xenopus* embryos was determined by immunoblotting. Unlike its expression in early mouse embryos, E-cadherin was not present in the eggs or early blastula of *Xenopus laevis*. These findings indicate that a different Ca^{2+} -dependent cell adhesion molecule, perhaps another

member of the cadherin gene family, is responsible for the Ca^{2+} -dependent adhesion between cleavage stage *Xenopus* blastomeres. Detectable accumulation of E-cadherin started just before gastrulation at stage 9½ and increased rapidly up to the end of gastrulation at stage 15. In stage 15 embryos, specific immunofluorescence staining of E-cadherin was discernible only in ectoderm, but not in mesoderm and endoderm. The ectoderm at this stage consists of two cell layers. The outer cell layer of ectoderm was stained intensely, and staining was localized to the basolateral plasma membrane of these cells. Lower levels of staining were observed in the inner cell layer of ectoderm. The coincidence of E-cadherin expression with the process of gastrulation and its restriction to the ectoderm indicate that it may play a role in the morphogenetic movements of gastrulation and resulting segregation of embryonic germ layers.

E-CADHERIN is a cell-cell adhesion molecule that has been implicated in several Ca^{2+} -dependent cell interactions between epithelial cells. It is also known as uvomorulin in the mouse (19, 35), L-CAM in the chick (10), and cell-CAM 120/80 in the human (8). Although E-cadherin (uvomorulin) is localized to the zonula adhaerens junction (3), it seems to function at an early step in intercellular adhesion and to be required for the assembly of the entire epithelial junctional complex (14). E-Cadherin belongs to a family of Ca^{2+} -dependent cell-cell adhesion molecules known as cadherins, which includes N-cadherin in neural tissues (16), P-cadherin in placenta (32), and probably others yet to be identified. These cadherins are highly related at the amino acid sequence level and probably perform similar functions in different tissues (45). They seem to be particularly important for cell interactions that occur during embryological development, since changes in the expression of cadherin type are often associated with morphogenetic events in embryogenesis (17, 32, 46). The cadherins have been proposed to be responsible for the specific adhesion and intercellular recognition among like cells that regulate the formation and segregation of tissues in morphogenesis (45).

To better understand the developmental significance of the expression of E-cadherin (and other cadherins), it will be important to investigate how E-cadherin expression influences or regulates the behavior of cells in epithelia undergoing morphogenesis. Early developing embryos of the amphibian *Xenopus laevis* provide particularly good experimental material for investigating the behavior of epithelial cells and the role of intercellular adhesion during development. In the earliest cleavage stages, a multicellular blastula is formed, which is held together by a Ca^{2+} -dependent adhesion mechanism (28, 30, 31) and is physiologically separated from the bathing medium by a tightly sealed surface epithelium (27, 38). Up until the midblastula transition, cell division is reductive (24) and the cells are built largely from components stored in the egg (2, 42, 50, 51). The cleavage furrows and resulting plasma membranes in these early embryos seem to be assembled from the exocytosis of vesicles stored in the egg (2). Thus, the assembly of cell contacts and junctions is very extensive, synchronous, and rapid during these stages. Amphibian embryos have also been successful experimental models for the study of cell adhesion and cell behavior in morphogenetic events such as gastrulation (12). In classical

embryology, early amphibian embryos were used to demonstrate the importance of selective cell adhesion in morphogenesis (43, 47). More recently *Xenopus* embryos have been used to study the developmental regulation of gene expression and the molecular biology of tissue induction and differentiation (9).

Surprisingly little is known about cell adhesion molecules in *Xenopus laevis* or other amphibians. In this study, we sought to identify *Xenopus* E-cadherin and to examine the time and tissue location of its expression in the early embryo. E-Cadherin is the earliest adhesion molecule expressed in mouse and chicken embryos (17, 46, 49). Therefore, we asked whether it might be stored in vesicles in the *Xenopus* egg, inserted into the plasma membrane during cleavage, and responsible for the Ca^{2+} -dependent adhesion between early blastomeres. Also, to begin to investigate the influence of E-cadherin on cell behavior and its role in the morphogenesis of epithelial sheets, we examined whether changes in the levels or location of its expression were associated with the formation of the primary germ layers (ectoderm, mesoderm, and endoderm) during gastrulation.

Materials and Methods

Cell Culture and Resistance Recovery Assay

The A6 *Xenopus* kidney cell line was obtained from the American Type Culture Collection (Rockville, MD) and subcloned by limiting dilution. The subclone A6.2 was used throughout these experiments. The cells were grown at 28°C in a humidified atmosphere of 1% CO_2 in air. The growth medium was 85% DME H16 containing 0.85 g/liter glucose and 0.74 g/liter NaHCO_3 , supplemented with 5% supplemented calf serum, 10 mM Hepes, pH 7.4, and 2 mM glutamine. For routine passage, cells were dissociated into single cell by treatment with 0.05% trypsin/0.02% EDTA and seeded at a 1:5 ratio into 75-cm² flasks. They became confluent after 4 d, and the growth medium was changed once at 3 d after seeding.

For the resistance recovery assay, cells were plated onto 12-mm filter chambers (Millicell HA; Millipore Co., Bedford, MA) at a density of 2×10^5 cells/filter and grown for 7 d in the growth medium supplemented with 10^{-7} M dexamethasone. Filters that had a transepithelial resistance $>4,000 \Omega \times \text{cm}^2$ were chosen for this assay. The assay was similar to that described (13) with modification. Cells on filters were washed three times in 85% PBS (0.15 M NaCl, 15 mM Na_2HPO_4 , pH 7.5) and incubated in 85% minimal essential medium for suspension culture (SMEM; Gibco Laboratories, Chagrin Falls, OH) supplemented with 1% dialyzed FCS (13) at 28°C for 6.5 min to open intercellular junctions. Filters were then transferred into humidified wells of a 24-well culture plate, and incubated with 100 μl of a 1:50 dilution of mAb ascites fluid made up in recovery medium (85% SMEM supplemented with 1% dialyzed FCS and 1 mM CaCl_2) for 40 min on ice while rocking gently. Filters were then transferred to recovery medium at 28°C, and transepithelial resistance was measured at the indicated times.

Preparation of Samples

Tryptic Digestion of Whole Cells. Confluent A6 cells were washed three times with 85% PBS⁺ (0.15 M NaCl, 15 mM Na_2HPO_4 , pH 7.5, 1 mM CaCl_2 , 0.5 mM MgCl_2) and scraped into either solution A (10 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM CaCl_2) or solution B (10 mM Hepes, pH 7.4, 150 mM NaCl, 1.5 mM EDTA) on ice at the density of 5×10^6 cells/ml. Cells were incubated with 0.2 mg/ml trypsin, shaken gently for 30 min at 37°C, and the digestion was terminated by the addition of 0.3 mg/ml soybean trypsin inhibitor, 1 mM PMSF, and 1 mM iodoacetamide. Cells were removed by ultracentrifugation at 30,000 rpm for 1 h in a rotor (Ti 80; Beckman Instruments Inc., Palo Alto, CA). To concentrate glycoproteins from the supernatant for some experiments, the supernatant was adjusted to 0.5% Triton X-100, mixed with 50 μl of 1:1 slurry of Con A-Sepharose 4B, and incubated rotating for 1 h at 4°C. Sepharose beads were pelleted at 1,000 rpm for 5 min in a microcentrifuge, washed three times with immunoprecipitation buffer (1% Triton X-100, 0.5% Na deoxycholate, 0.2% SDS, 0.15 M NaCl, 20 mM Hepes, pH 7.4), once with high

salt buffer (0.5 M NaCl, 10 mM Tris, pH 7.4), and once with 10 mM Tris buffer, pH 7.4.

Extraction of A6 Cells with Triton X-114. Confluent A6 cells were washed three times with 85% PBS⁺ and extracted on ice with 1% Triton X-114 in solution A supplemented with a protease inhibitor cocktail (1 mM PMSF, 1 mM iodoacetamide, 1 mM benzamidine, 10 $\mu\text{g}/\text{ml}$ of aprotinin, pepstatin, leupeptin, and antipain, respectively). The phase separation was done as described (4). Glycoproteins in the aqueous phase were concentrated as described above.

Extraction of Tissues from Adult *Xenopus laevis*. The liver was removed from *Xenopus laevis*, cut into small pieces with scissors, rinsed twice with ice cold 85% PBS⁺, and then homogenized in 85% PBS⁺ supplemented with protease inhibitors (10:1 volume/tissue) in a waring blender at low speed for 1 min at 4°C (all of the following procedures were undertaken at 4°C). The homogenate was filtered through gauze to remove remaining connective tissue and centrifuged at 7,000 rpm in a rotor (JA 20; Beckman Instruments, Inc.). The pellets were resuspended in immunoprecipitation buffer (5:1 volume/tissue) and homogenized with five strokes in an homogenizer (Dounce; Kontes Glass Co., Vineland, NJ). Extracts were centrifuged at 12,000 rpm for 30 min in a rotor (JA 20; Beckman Instruments, Inc.) and the supernatant was ultracentrifuged at 30,000 rpm for 1 h in a rotor (Ti 70; Beckman Instruments, Inc.) to remove all the insoluble material. Lung and brain were directly homogenized in immunoprecipitation buffer with a Dounce homogenizer. Protein concentrations were measured by the Amido-Schwartz method (40).

Extraction of Eggs and Embryos. Production of eggs, fertilization, and removal of the jelly coat were done as described (28). Dejellied eggs or embryos were extracted with 1% NP-40 in solution A supplemented with protease inhibitors. This procedure avoided disruption of the yolk platelets. The platelets and other insoluble material were removed by centrifugation at 10,000 rpm for 30 min in a microcentrifuge. The supernatant was bound to Con A-Sepharose 4B or mAbs coupled to Sepharose 4B. In some experiments, eggs or embryos were extracted with immunoprecipitation buffer, which tends to solubilize yolk proteins as well.

Immunoblotting and Antibodies

SDS-PAGE and immunoblotting were performed as described (13, 14). Prestained molecular mass markers (Sigma Chemical Co., St. Louis, MO) were used for the molecular mass estimation of proteins. In one experiment, proteins were electrophoretically transferred to nitrocellulose paper from a fixed and Coomassie blue-stained gel as described (37).

The microaffinity purification of antibodies on proteins bound to nitrocellulose was done as described (5). The extracellular tryptic fragment of canine E-cadherin, purified as described (14), was run on preparative SDS-PAGE, and transferred to nitrocellulose paper. The protein band was located by staining with Ponceau-S (Eastman Kodak Co., Rochester, NY) and cut out for antibody purification. Alkaline phosphatase-conjugated goat anti-rabbit IgG and anti-mouse IgG (Bio-Rad Laboratories, Richmond, CA) were used as second antibodies to detect polyclonal and monoclonal antibodies, respectively. To increase the sensitivity for immunoblotting of egg and embryo extracts, a two-antibody detection method was used. The mouse primary antibodies were detected by first incubating with rabbit anti-mouse IgG, and then alkaline phosphatase-conjugated goat anti-rabbit IgG. p-Nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (Bio-Rad Laboratories) were used as color developing reagents.

Preparation of Antigen and Generation of mAbs

Large amounts of the extracellular tryptic fragment of E-cadherin (E-cad100) were prepared from trypsin digests of A6 cells by scaling up the procedure described above. The supernatant was dialyzed with column buffer (10 mM Hepes, pH 7.4, 2 mM CaCl_2 , 50 mM NaCl), loaded on to a preequilibrated DE-52 column (1.6 \times 5.0 cm), and eluted with a linear gradient of NaCl (0.05–0.5 M). The fractions eluting between 0.1 and 0.2 M NaCl were pooled, bound to Con A-Sepharose 4B, and washed as described above. E-cad100 was further purified by preparative SDS-PAGE (8% acrylamide/0.21% bisacrylamide). The gel was stained lightly, destained, and the E-cad100 band was cut out and washed with distilled water for 2 h with frequent changes. For immunization, gel slices containing $\sim 5 \mu\text{g}$ of E-cad100 were finely minced between two plastic plates of a petri dish, suspended in 0.3–0.5 ml PBS, and emulsified with the same volume of complete Freund's adjuvant (Calbiochem-Behring Corp., La Jolla, CA) for the first injection and incomplete Freund's adjuvant for the second injection. BALB/c mice were injected first subcutaneously and then boosted intraperitoneally after 2 wk. At the third week, the serum titer was checked by immunoblotting.

A mouse whose titer was 1:1,000 was chosen and finally boosted intraperitoneally with $\sim 20 \mu\text{g}$ of E-cad100 in PBS. The fusion and culture of hybridomas was done as described (6).

The hybridoma supernatants were screened by immunoblotting trypsin digests and the aqueous phase of Triton X-114 extracts of A6 cells using a Miniblotter (Immunitics, Cambridge, MA.). Immunoglobulin subtyping of the mAbs was done using a kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). mAbs 8C2 and 5D3 were subclass IgG1, while mAbs 19A2 and 31D2 were IgG2b. Ascites fluids were produced as described (6).

Cell Labeling and Immunoprecipitation

For overnight labeling, 90% confluent A6 cells in 75-cm² flasks were incubated in 0.3 mCi [³⁵S]methionine (ICN Biomedical Inc., Irvine, CA) in 5 ml of methionine-free growth medium supplemented with 5% supplemented calf serum, 10 mM Hepes, and 2 mM glutamine.

For pulse-chase experiments, A6 cells were grown to confluence in 60-mm culture dishes, washed three times with 85% PBS⁺, and twice with methionine- and serum-free growth medium. The cells were then incubated in the methionine-free medium for 10 min at room temperature and pulse labeled with 0.1 mCi [³⁵S]methionine in 1.5 ml of methionine-free medium for 15 min at 28°C. To chase, cells were washed once with the growth medium supplemented with 5% supplemented calf serum, and incubated in 2 ml of the same medium at 28°C. Cells were extracted with ice cold immunoprecipitation buffer at the indicated times, and insoluble material was removed by centrifugation at 10,000 rpm for 30 min in a microcentrifuge.

For immunoprecipitation, mAbs were purified on a protein A-Sepharose column and then coupled to CNBr-activated Sepharose 4B as described (14). All procedures were the same as the Con A-Sepharose precipitation except that antibodies coupled to Sepharose 4B were used. Polypeptides were analyzed by 8% SDS-PAGE. For fluorography, the gel was fixed for 1 h in 10% acetic acid/50% methanol, treated with 1 M sodium salicylate for 30 min at room temperature, vacuum dried, and exposed to X-OMAT-AR (Eastman Kodak Co.) at -70°C .

Immunofluorescence Staining of A6 Cells and Immunocytochemistry of Tissues and Embryos

Indirect immunofluorescence of A6 cells grown on cover glass was performed as described (14). Texas red-conjugated rabbit anti-mouse IgG (Accurate Chemical & Scientific Corp., Westbury, NY) was used to detect the primary mouse mAbs.

Immunocytochemistry on tissues and embryos was performed as described (25). Cryostat sections (16 μm) were prepared from unfixed liver, lung, and embryos embedded in O.C.T. compound (Miles Laboratories Inc., Elkhart, IN) and collected on the cover glass coated with 0.2 mg/ml poly-L-lysine. Sections were fixed with 95% ethanol for 15 min at 4°C. 0.5% BSA/PBS was used as a blocking reagent. Antibodies were applied for 1 h. Samples were examined with an epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY), and Tri-X film (Eastman Kodak Co.) was used for photography.

Results

Identification and Purification of E-Cadherin in the A6 *Xenopus* Kidney Cell Line

A polyclonal antiserum generated against canine E-cadherin (13, 14) was used to identify E-cadherin in the A6 *Xenopus* kidney cell line. Titers of cross-reacting antibodies were low, but sufficient to identify the protein. It was not possible to detect E-cadherin in total SDS extracts of A6 cells by immunoblotting. However, after enrichment of the glycoprotein fraction by binding to Con A-Sepharose, E-cadherin could be easily detected by immunoblotting (Fig. 1, A and B).

E-Cadherin can be released from the cell surface as a protease-resistant fragment by trypsin in the presence of Ca²⁺ (13, 20). In the absence of Ca²⁺, it is degraded extensively by trypsin. This Ca²⁺-dependent change in trypsin sensitivity was also used as a criterion for its identification in *Xenopus laevis*. Whole A6 cells were digested with trypsin in the

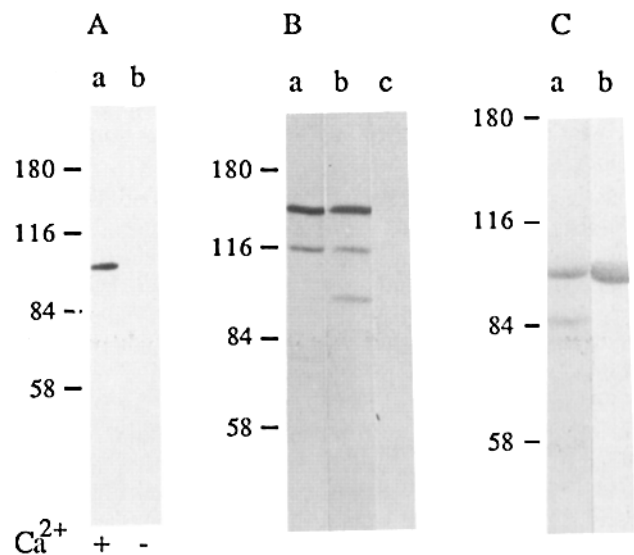


Figure 1. Identification and purification of *Xenopus* E-cadherin. (A) Identification of the 100-kD trypsin-resistant ectoplasmic domain of *Xenopus* E-cadherin (E-cad100). 5×10^6 A6 cells were digested with trypsin in the presence (lane a) or absence (lane b) of Ca²⁺. Con A-bound fractions were electrophoresed and immunoblotted with a polyclonal antiserum against canine E-cadherin. (B) Identification of the 140-kD intact E-cadherin. Con A-bound fractions of the aqueous phase of Triton X-114 extracts of A6 cells were immunoblotted with the polyclonal antiserum against canine E-cadherin (lane a), with the same antibodies after microaffinity purification on canine E-cadherin (lane b), and with preimmune serum (lane c). (C) Purification of E-cad100. (Lane a) Coomassie blue-stained gel of one preparation of E-cad100 obtained from 2×10^8 A6 cells. (Lane b) Immunoblotting of the same gel with polyclonal antiserum to canine E-cadherin. Numbers are molecular masses in kD.

presence or absence of Ca²⁺, and glycoproteins release from the cell surface were concentrated with Con A-Sepharose 4B. Polyclonal antibodies against canine E-cadherin recognized a 100-kD polypeptide in the supernatants of cells trypsinized in the presence of Ca²⁺ (Fig. 1 A, lane a), but not in the absence of Ca²⁺ (Fig. 1 A, lane b).

To identify the intact E-cadherin protein, Triton X-114 extracts of A6 cells were phase partitioned and the glycoproteins in the aqueous phase were concentrated by binding to Con A-Sepharose 4B. Similar to mouse and canine E-cadherin (13, 35), *Xenopus* E-cadherin was preferentially distributed in the aqueous phase of Triton X-114 extracts (not shown), even though it is known to be an integral membrane protein (18, 39). Two polypeptides (140 kD, 116 kD) were strongly recognized by the polyclonal antiserum to canine E-cadherin, and two other polypeptides (100 kD, 80 kD) were stained rather weakly (Fig. 1 B, lane a). None of these polypeptides were recognized by preimmune serum (Fig. 1 B, lane c). After microaffinity purification on highly purified, trypsin-resistant ectoplasmic domain of canine E-cadherin by the Olmstead procedure (5), anti-canine E-cadherin antibodies recognized three polypeptides (140 kD, 116 kD, 100 kD) (Fig. 1 B, lane b). Therefore, these three polypeptides contain E-cadherin epitopes. Also, these three polypeptides exhibited the Ca²⁺-dependent resistance to trypsin di-

gestion (not shown). The 116- and 100-kD polypeptides are probably degradation products of the 140-kD polypeptide because extraction of cells with boiling SDS buffer greatly reduced the levels of these bands (Fig. 2 A). Like E-cadherin in other species (8), *Xenopus* E-cadherin bound to the lectin Con A, but did not bind to wheat germ agglutinin (not shown). These criteria, therefore, identify the 140-kD polypeptide as *Xenopus* E-cadherin and the 100-kD polypeptide as its trypsin-resistant ectoplasmic domain (E-cad100). The molecular masses of both polypeptides are ~20 kD higher than that in other species (1, 8, 10, 13, 34, 35). This molecule may be the same as the 140-kD Ca²⁺-dependent cell adhesion glycoprotein that has been previously identified in A6 cells (31).

For the generation of mAbs specific to *Xenopus* E-cadherin, E-cad100 was purified on a DE-52 column, concentrated by binding to Con A-Sepharose, and then purified by SDS-PAGE. In one preparation (Fig. 1 C, lane a), ~3 μg of E-cad100 was obtained from 2 × 10⁸ cells. Immunoblotting of the same gel with the polyclonal antiserum against canine E-cadherin showed that the purified polypeptide is the trypsin-resistant fragment of E-cadherin (Fig. 1 C, lane b).

Characterization of *Xenopus* E-Cadherin with mAbs

Four independent mAbs to *Xenopus* E-cadherin were generated (see Materials and Methods). They were characterized by the immunoblotting of a total protein SDS extract of A6 cells. The 140-kD polypeptide was the major band recognized by mAbs 5D3, 19A2, 8C2, and 31D2 (Fig. 2 A, lane a-d). The lower molecular mass minor bands observed before with polyclonal antiserum were also detected, and probably represent degradation products. Another minor band migrating at 155 kD, which had not been detected with the polyclonal antibodies, was also recognized. In this particular experiment, mAb 31D2 appeared to recognize only the 140-kD polypeptide. However, it detected all of the polypeptides when used at higher concentrations (not shown).

The 155-kD polypeptide is a good candidate for a precursor to *Xenopus* E-cadherin, since an E-cadherin precursor has been observed in biosynthetic labeling of mouse (36), chicken (11), human (Damsky, C., unpublished observation), and canine cells (Chernov-Rogen, T., and B. Gumbiner, unpublished observation). To determine whether the 155-kD

polypeptide is a precursor of the 140-kD mature E-cadherin, a pulse-chase experiment was carried out (Fig. 2 B). The 155-kD polypeptide was heavily labeled with [³⁵S]methionine after the pulse, and was progressively chased into the 140-kD mature form. The half-life of the precursor was ~45 min, and labeling of the precursor was persistent up to 120 min of chase. Processing of the precursor in *Xenopus laevis* was slower than conversion in other species. This slow conversion probably explains the detection of the precursor by immunoblotting. The prominent ~100-kD polypeptide in Fig. 2 B, observed only by immunoprecipitation, is distinct from E-cad100 and is a candidate for a cytoplasmic protein that binds to E-cadherin (McCrea, P., and B. Gumbiner, unpublished observation). A similar molecular mass polypeptide has also been found associated with E-cadherin immunoprecipitated from cells of other species (36, 48) (Gumbiner, B., unpublished observation).

mAbs 8C2 and 31D2 are capable of distinguishing between the Ca²⁺-dependent molecular conformations of E-cad100. It is possible to reversibly induce the (-) Ca²⁺ conformation of E-cad100 after its isolation in the presence of Ca²⁺. [³⁵S]Methionine-labeled E-cad100 was allowed to convert into the (-) Ca²⁺ conformation by removing Ca²⁺ with EDTA for 5 min on ice. mAbs 5D3 and 19A2 were able to immunoprecipitate E-cad100 in both the (+) Ca²⁺ and the (-) Ca²⁺ conformations (Fig. 3, lanes a-d). However, mAbs 8C2 and 31D2 recognized only the (-) Ca²⁺ conformation of E-cad100 (Fig. 3, lanes e-h).

To determine whether mAbs to *Xenopus* E-cadherin could interfere with its adhesion function in cultured cells, a resistance recovery assay for measuring the sealing of tight junctions was used (13). The assay was modified slightly from the original method so it could be used on the amphibian A6 cells. Intercellular junctions were opened by incubation in Ca²⁺-free medium for 6.5 min at 28°C, and cells were treated with various antibodies for 40 min on ice. After returning the monolayers to Ca²⁺-containing medium at 28°C, the recovery of transepithelial electrical resistance was followed over time as a measure of the rate of resealing of tight junctions. All three of the anti-E-cadherin mAbs tested strongly inhibited the recovery of transepithelial resistance (Fig. 4). A mixture of the three mAbs inhibited more strongly than the individual mAbs. Therefore, the protein recognized by

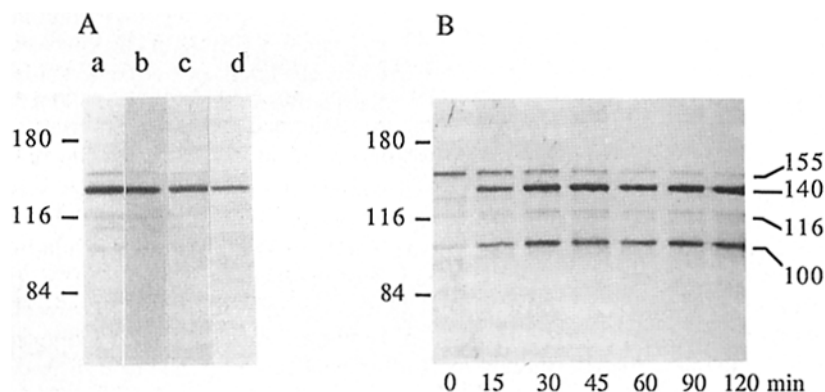


Figure 2. Characterization of *Xenopus* E-cadherin by mAbs. (A) Immunoblotting of SDS extracts of A6 cells (2 × 10⁶ cells/lane) with the hybridoma supernatants of mAbs 5D3 (lane a), 8C2 (lane b), 19A2 (lane c), and 31D2 (lane d). (B) Identification of the 155-kD precursor of the 140-kD mature E-cadherin. A6 cells were pulse labeled with [³⁵S]methionine for 10 min, and then chased for the indicated times. Cell extracts were immunoprecipitated with the mixture of mAbs 5D3, 8C2, and 19A2, and analyzed by SDS-PAGE and fluorography. Numbers on the left side are standard molecular masses in kD. Numbers on the right side are the calculated molecular masses in kD of the precursor (155 kD), mature E-cadherin (140 kD), its degradation product (116 kD), and its associated protein (100 kD).

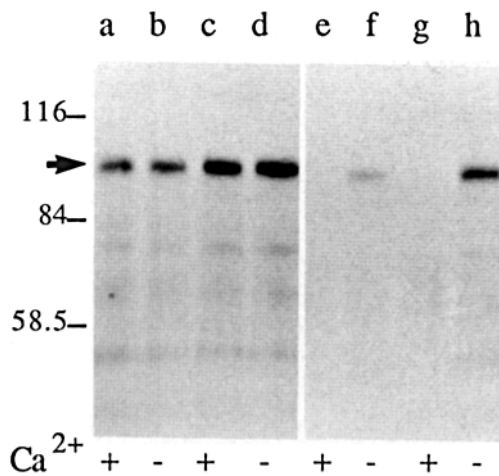


Figure 3. Conformation specificity of mAbs against *Xenopus* E-cadherin. [³⁵S]Methionine-labeled E-cad100 in the absence (-) or presence (+) of Ca²⁺ was immunoprecipitated with mAbs 5D3 (lanes a and b), 19A2 (lanes c and d), 31D2 (lanes e and f), and 8C2 (lanes g and h) and analyzed by SDS-PAGE and fluorography. Arrow, E-cad100. Numbers are molecular masses in kD.

these mAbs functions in the formation of intercellular contacts, as does E-cadherin in Madin-Darby canine kidney cells (14).

Expression of E-Cadherin in Other Epithelial Tissues

The E-cad100 for the production of mAbs was purified from the A6 kidney epithelial cell line. To determine whether E-cadherin is expressed in other epithelial tissues, extracts

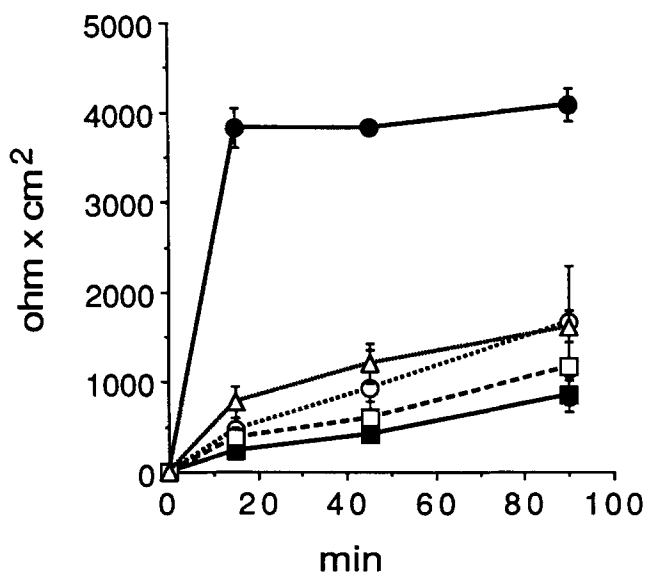


Figure 4. Inhibition of tight junction formation between A6 cells by mAbs to *Xenopus* E-cadherin. Monolayers of A6 cells grown on filters were incubated in Ca²⁺-free medium at 28°C. At 6.5 min, they were then treated with mAbs 5D3 (○), 19A2 (□), 8C2 (△), a mixture of mAbs 5D3, 19A2, and 8C2 (■), or control mAb rrl (●) for 40 min on ice. The recovery of transepithelial resistance was measured at the indicated times after transfer into the recovery medium at 28°C. Values are mean ± SEM; n = 3.

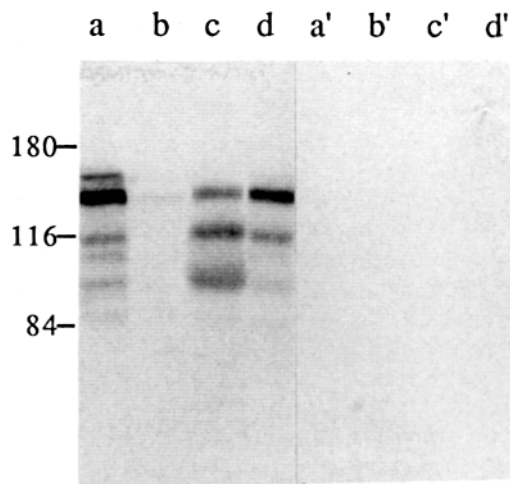


Figure 5. Expression of *Xenopus* E-cadherin in epithelial tissues. Extracts of cultured A6 cells (a), brain (b), liver (c), and lung (d) from *Xenopus laevis*, all containing the same amounts of protein, were concentrated by binding to Con A-Sepharose, and immunoblotted with the mixture of mAbs 5D3, 8C2, and 19A2 (lanes a-d), or with nonimmune antibody for the control (lanes a'-d'). All were then reacted with alkaline phosphatase-conjugated anti-mouse IgG. Numbers are molecular masses in kD.

of lung, liver, and brain of adult *Xenopus laevis* were analyzed by immunoblotting with a mixture of anti-E-cadherin mAbs (Fig. 5). High levels of E-cadherin expression were observed in lung and liver (Fig. 5, lanes c and d), but very little was detected in brain (lane b). The intact 140-kD E-cadherin molecule and its degradation products were detected in both lung and liver. More extensive proteolysis seems to occur during extraction of these tissues than during the extraction of A6 cells. Nonimmune antibody did not react any of these polypeptides (Fig. 5, lanes a'-d').

Immunofluorescence staining of A6 cells and cryostat sections of tissues with anti-E-cadherin mAbs are shown in Fig. 6. In A6 cells, mAb 19A2 stained the basolateral plasma membrane (Fig. 6 A). Hepatocytes were also stained at the basolateral plasma membrane (Fig. 6 C). It was not easy to discern the apical plasma membrane facing small bile canaliculus in these thick sections. Epithelial cells of the lung were stained with mAbs against E-cad100 (Fig. 6 E). Again, staining was localized at the basolateral plasma membrane. Endothelial cells were not stained.

Expression of E-Cadherin in Embryos of *Xenopus laevis*

To determine whether E-cadherin is stored in eggs, and involved in the Ca²⁺-dependent adhesion of early blastomeres and/or later developmental processes of *Xenopus* embryos, its time of expression was examined. Eggs and embryos at different stages were extracted with an NP-40-containing buffer. The glycoprotein fraction was enriched by binding to Con A-Sepharose 4B and immunoblotted with anti-E-cadherin mAbs (Fig. 7 A). Detectable accumulation of E-cadherin started at 10 h after fertilization (stage 9½ as defined by Nieuwkoop [29]) and increased rapidly up to 24 h (stage 15). The 155-kD precursor, the 140-kD mature protein, and degradation products, which had been observed in A6 cells, were also detected in embryos. The lack of detection of

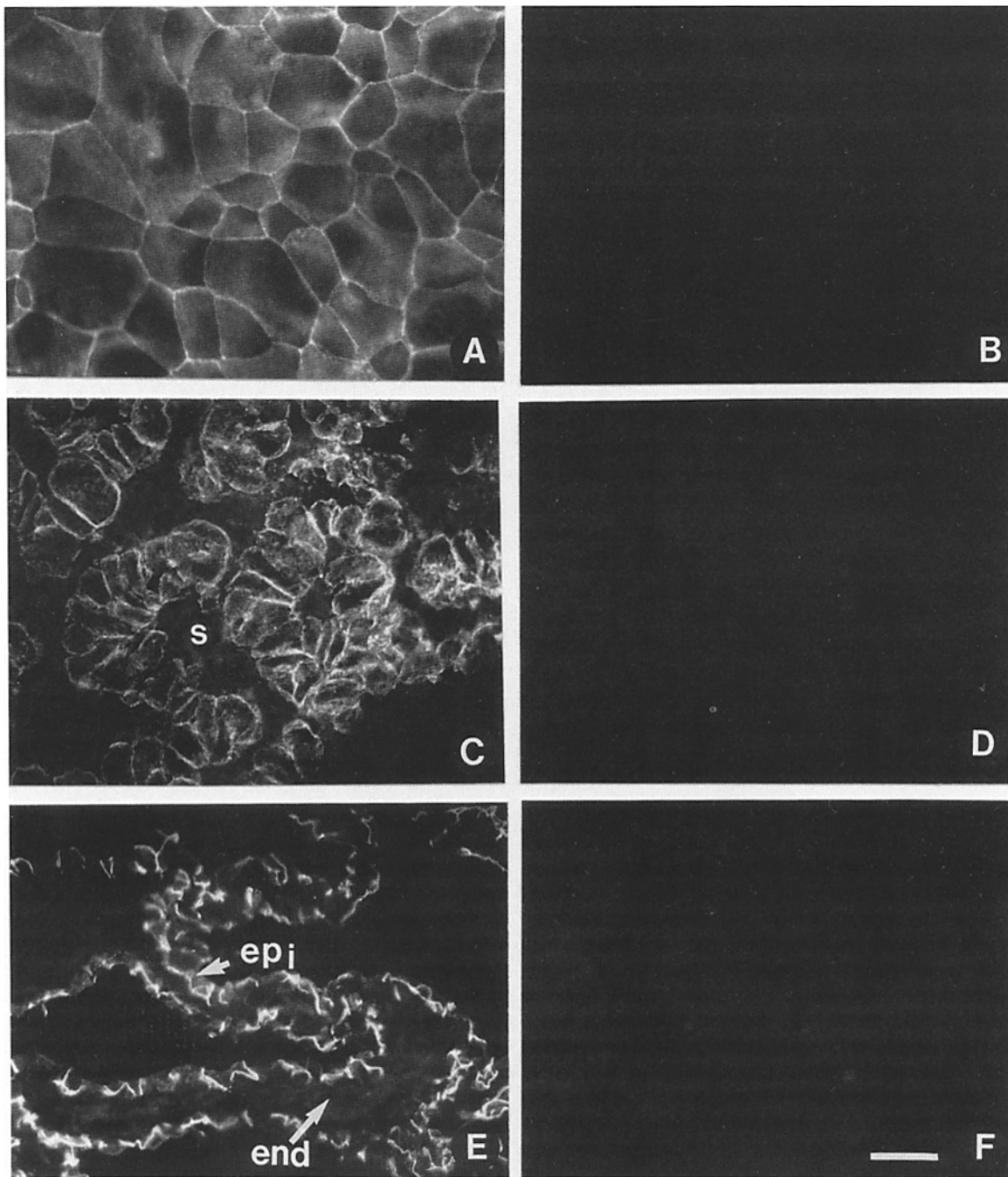


Figure 6. Immunocytochemistry of E-cadherin in A6 and epithelial cells. A6 cells fixed with methanol and acetone were stained with mAb 19A2 (A) or with nonimmune antibody as the control (B). Cryostat sections (16 μm) of liver (C and D) and lung (E and F) fixed with 95% ethanol were stained with the mixture of mAbs 5D3, 8C2, 19A2, and 31D2 (C and E) or with nonimmune antibody as the control (D and F). S, sinusoid; epi, alveolar epithelial cells; end, endothelial cells. Bar, 40 μm .

E-cadherin before stage 9½ was probably not due to difficulties with extraction by NP-40. The extractability of E-cadherin by NP-40 from embryos after stage 9½ was comparable to extraction with the harsher conditions using a mixture of SDS, deoxycholate, and Triton X-100 (not shown). Nor could the lack of detection be attributable to using the Con A enrichment step before SDS-PAGE. Enrichment by immunoprecipitating with a combination of the mAbs and subsequent immunoblotting gave the same result (not shown).

To determine whether E-cadherin might be stored in eggs at a lower concentration, 2,000 eggs (20 times more eggs than that used in Fig. 7 A) were extracted with the NP-40-containing buffer. Extracts were immunoprecipitated either with a mixture of mAbs 8C2, 5D3, and 19A2 or with individual mAbs (Fig. 7 B, lanes d–g) or the mixture (Fig. 7 B, lanes a–c). No specific E-cadherin polypeptide was detected, even when the blot was overdeveloped. Therefore, by

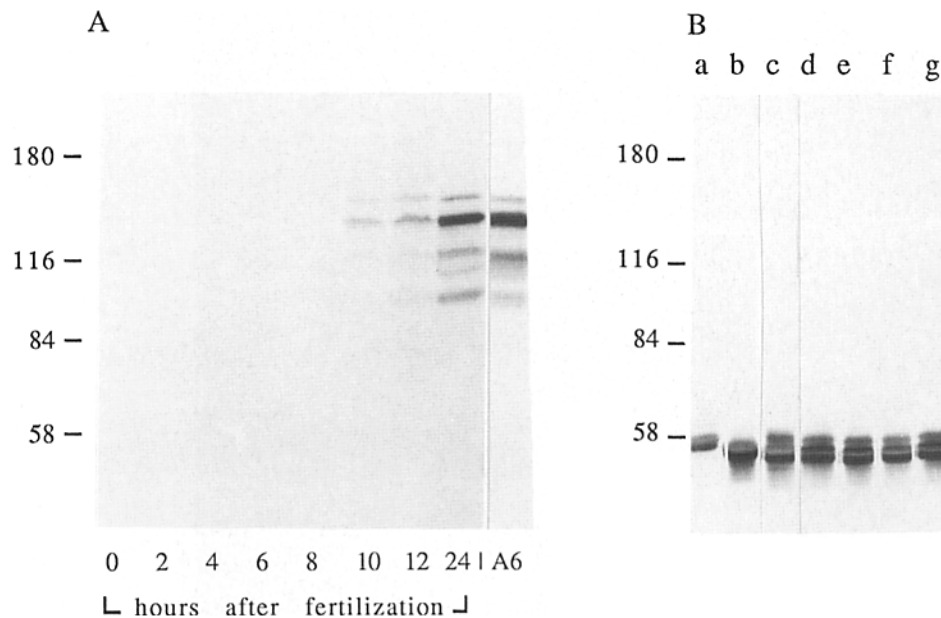


Figure 7. Expression of E-cadherin in *Xenopus* embryos. (A) E-Cadherin expression at various times after fertilization. 150–200 eggs or embryos/sample were extracted with NP-40 at the indicated times after fertilization. The glycoprotein fractions were enriched by binding to Con A-Sepharose and immunoblotted with the mixture of mAbs 5D3, 8C2, 19A2, and 31D2. Lane A6 shows an extract of A6 cells for comparison. (B) Attempt to detect E-cadherin in *Xenopus* eggs. 2,000 eggs/sample were extracted with immunoprecipitation buffer, immunoprecipitated with mAbs 5D3 (lane a), 8C2 (lane b), and 19A2 (lane c), and immunoblotted with the mixture of mAbs 5D3, 8C2, 19A2, and 31D2. The same samples were immunoprecipitated with the mixture of mAbs (lanes d–g) and immunoblotted with individual mAb 5D3 (lane d), 8C2 (lane e), 19A2 (lane f), and 31D2 (lane g). The ~55-kD bands are heavy chains of mouse IgGs used for immunoprecipitation. Numbers are molecular masses in kD.

the criteria of binding of these four different mAbs, E-cadherin did not seem to be present in eggs of *Xenopus laevis*.

The spatial localization of E-cadherin expression was examined by immunofluorescence staining of cryostat sections of embryos with a mixture of mAbs 8C2, 5D3, and 19A2. In stage 15 embryos, specific staining was discernable only in ectoderm, but not in mesoderm and endoderm (Fig. 8 B). The ectoderm at this stage consists of two cell layers. The outer cell layer of ectoderm was stained with mAbs, of which staining was exclusively at the basolateral plasma membrane (Fig. 8 C). At higher magnification, the high level of the basolateral staining of E-cadherin in embryos always coincided with the presence of cortical pigment granules (Fig. 8 D), which is a good marker for the outer cell layer of ectoderm (Keller, R., personal communication). A lower level of staining was observed in the inner cell layer of the ectoderm (Fig. 8, B and C). With nonimmune antibody as a control, the ectoderm was not stained and only background fluorescence was observed (Fig. 8 A). No specific staining was ever observed in any internal cell layers at stage 15. Very low levels of expression of E-cadherin in the mesoderm and endoderm cannot be excluded by these experiments. Low levels of staining would not be detectable above the background autofluorescence from yolk platelets. It was not possible to discern specific staining in any cells of the embryo at stage 9½ and 10½ (not shown). E-Cadherin expression levels at this stage were probably still too low to be detected by this method. Again, autofluorescence from yolk platelets increased in the background and probably interfered with the detection of low levels of staining.

A preliminary experiment was carried out to test the functional role of E-cadherin in gastrulation. A highly concentrated mixture of three purified mAbs (5D3, 8C2, and 19A2) was microinjected into the blastocoel cavity of embryos at stage 8. No obvious effect on subsequent development was observed by simple morphological examination with a dissecting stereomicroscope. Future experiments involving tissue explants and examination of more subtle changes may reveal a perturbation of some component of gastrulation.

Discussion

We wished to undertake the study of E-cadherin (also known as uvomorulin and L-CAM), the Ca²⁺-dependent epithelial adhesion molecule, in early developing embryos of *Xenopus laevis*. This protein had been identified previously in several other species including mouse (19, 35), chick (10), human (8), and dog (1, 13). To investigate the function of E-cadherin in developing *Xenopus* embryos, it was first crucial to unambiguously identify the same protein in this species. The identification of E-cadherin in *Xenopus laevis* in this study was established by several criteria. First, antibodies raised to canine E-cadherin recognized the homologous protein in the *Xenopus* A6 epithelial cell line. This protein exhibited the Ca²⁺-dependent resistance to trypsin digestion that is characteristic for the cadherins in all species (13, 20). It was first synthesized as a higher molecular mass precursor as in other species (11, 36), (Damsky, C., unpublished observation; Chernov-Rogen, T., and B. Gumbiner, unpublished observation). Like E-cadherin in all species, this protein in *Xenopus laevis* was expressed at high levels in epithelial tissues,

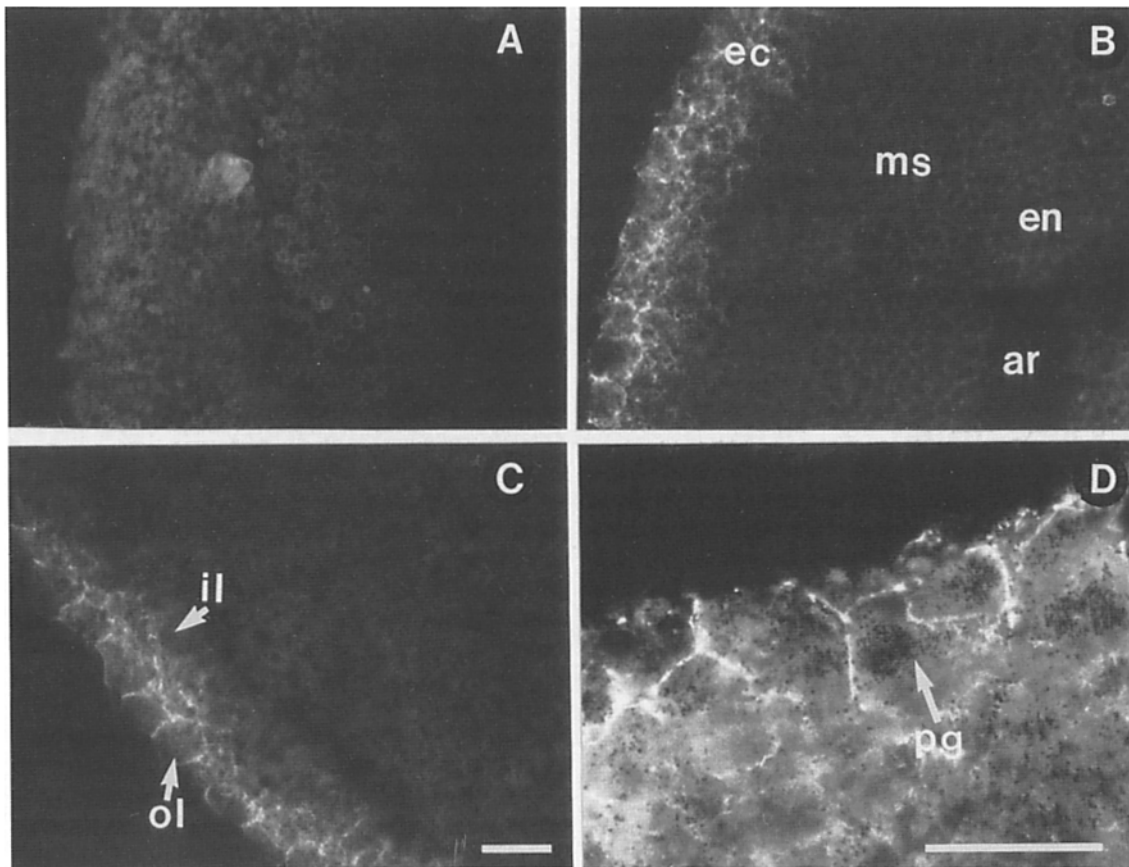


Figure 8. Localization of E-cadherin in the ectoderm of *Xenopus* embryos. Indirect immunofluorescence staining of transverse cryostat sections (16 μm) of stage 15 embryos. (A) Background staining control by nonimmune antibody. (B–D) Staining with a mixture of mAbs 5D3, 8C2, 19A2, and 31D2. *ec*, ectoderm; *ms*, mesoderm; *en*, endoderm; *ar*, archenteron; *ol*, outer layer of ectoderm; *il*, inner layer of ectoderm; and *pg*, cortical pigment granule. A–C are the same magnification; D is a higher magnification of ectodermal staining. Bars, 40 μm .

but not in brain and endothelia. As expected for an adhesion molecule, *Xenopus* E-cadherin was localized to the basolateral plasma membrane domain in different cell types. Finally, the functional criterion for being an adhesion molecule was satisfied by the ability of anti-E-cadherin mAbs to inhibit the formation of cell junctions between cultured epithelial cells. Thus, we believe that the mAbs generated in this study can be considered to be specific probes for E-cadherin in *Xenopus laevis*.

The cadherins constitute a family of genetically related molecules, and it is likely that additional members have yet to be identified (45). Although the expression of the known cadherins does overlap in some tissues during embryonic development, E-cadherin (or L-CAM) is the only cadherin identified so far that is expressed permanently at high levels in the majority of adult epithelial tissues, such as the lung, kidney, and liver (45, 46). Therefore, we believe it is very likely that the epithelial cadherin molecule we have identified in *Xenopus laevis* is most closely related to E-cadherin in mouse and L-CAM in chicken. It remains possible that the cadherin molecule we have identified in *Xenopus laevis* is not the only epithelial-specific cadherin in this species. If another *Xenopus* cadherin molecule that is also expressed permanently in adult epithelial tissues should be discovered in the future, cloning and sequencing of their two cDNAs will determine

which one is more closely related genetically to mouse E-cadherin or chicken L-CAM.

Although blastomeres in early cleavage stage *Xenopus* embryos form an epithelial-like tightly sealed blastula (27, 38) and are held together by a Ca^{2+} -dependent adhesion mechanism (28, 30, 31), E-cadherin appeared not to be involved in cell–cell interactions in the early *Xenopus* blastula. It began to accumulate to detectable levels only at stage 9½, just before gastrulation. E-Cadherin did not seem to be stored in the egg, unlike many components required for the assembly of cell organelles in cleavage stage *Xenopus* embryos (2, 42, 50, 51). It was undetectable in extracts of a large number of eggs. The use of four independent mAbs to assay E-cadherin makes it very unlikely that E-cadherin went undetected simply because of a masked or missing epitope. These findings suggest that another Ca^{2+} -dependent adhesion molecule, perhaps another member of the cadherin gene family (45), is responsible for the adhesion between cleavage stage *Xenopus* blastomeres and in mediating the assembly of tight epithelial junction between surface blastomeres.

In contrast to our findings, a previous publication on *Xenopus* embryos using antibodies raised to chicken L-CAM concluded that L-CAM or E-cadherin was expressed in most, if not all of the early blastomeres of *Xenopus laevis* (26). However, no biochemical analysis of E-cadherin levels in early

embryos was reported in that study. Also, the antibodies to chicken L-CAM stained diffusely the cytoplasm of both embryonic cells and adult hepatocytes. Diffuse cytoplasmic staining, while not impossible, is inconsistent with the known functions and subcellular distributions of the cadherins. Our finding of a later time of expression is supported both by a biochemical analysis of E-cadherin levels and by localization at the basolateral plasma membrane, a location typical of many other adhesion molecules. The reported staining of early blastomeres with anti-chicken L-CAM may have been due to nonspecific reactivity of the antibodies on *Xenopus* tissues.

This late expression of E-cadherin in *Xenopus laevis* contrasts with its expression in the mouse and chick embryos. E-cadherin is present on the surface of the mouse egg, is already being synthesized in two-cell mouse embryos (49), and functions in compaction at cell stage 8–16 to form the epithelial trophectoderm of the blastocyst (8, 41, 48). E-Cadherin is also expressed in the inner cell mass, which develops into the embryo proper, before gastrulation (7, 49). In chick embryos, L-CAM was found at low levels in all cells of the blastoderm in pregastrulation embryos (46). These differences between *Xenopus*, mouse, and chick suggest that there is no set developmental order for the expression of cell adhesion molecules for all vertebrate species. Perhaps N-cadherin or P-cadherin, which are usually expressed later in the development of mouse and chick (15), mediate the Ca²⁺-dependent adhesion between early blastomeres in *Xenopus*. Alternatively, a novel cadherin or a new kind of Ca²⁺-dependent adhesion molecule could be responsible for intercellular adhesion in cleavage stage *Xenopus* embryos.

Expression of the E-cadherin protein in *Xenopus* embryos was first observed at stage 9½, just before the start of gastrulation. We do not yet know whether its expression is controlled by the onset of embryonic transcription at the mid-blastula transition (24) or by the posttranscriptional activation of stored maternal messenger RNAs as occurs with fibronectin (25) and nuclear lamin synthesis (44). By the end of gastrulation, E-cadherin accumulated to the greatest extent in the outer cell layer of the ectoderm. At this time, the ectoderm consists of two cell layers. A lower level of immunofluorescence staining was noticed in the inner cell layer of the ectoderm. It is not yet possible from our experiments to completely rule out even lower levels of E-cadherin expression by cells of the endoderm or the mesoderm. The background level of fluorescence from embryonic cells may have been great enough to prevent the detection of very low amounts of the protein. In fact, we were unable to detect E-cadherin by immunofluorescence staining in the early gastrula even though small amounts were demonstrated biochemically in immunoblotting experiments. The high level of E-cadherin expression by the ectoderm does suggest, however, that E-cadherin might play a significant role in the morphogenesis of this tissue during gastrulation.

The pattern of E-cadherin expression in the primary germ layers during gastrulation in *Xenopus* embryos differs from mouse and chick embryos. In *Xenopus* embryos, E-cadherin becomes expressed only in the ectoderm during gastrulation. In mouse embryos, E-cadherin is expressed in the endoderm as well as the ectoderm (7). During gastrulation of chick embryos, expression of L-CAM persists in the ectoderm, but disappears from mesodermal and definitive endodermal cells

as they separate from the ectoderm during ingression (46). These differences in the pattern of E-cadherin expression may reflect the different kinds of cell behaviors involved in the formation of the germ layers during gastrulation in various species.

Previous studies of *Xenopus* gastrulation suggest two possible (and perhaps related) roles for E-cadherin in the morphogenesis of the ectoderm. In one series of experiments, Gerhart and Keller have shown that epithelial cells undergo region specific behaviors during gastrulation (12) that could involve different regional forms of intercellular contact. For example, cells in the involuting marginal zone are highly motile with respect to one another and undergo extensive rearrangements despite the fact that they remain in intimate contact and tightly sealed (22, 23). In contrast, the animal cap, which gives rise to ectoderm, undergoes epiboly, a process of isotropic expansion of the epithelium in which the cells flatten and spread. Perhaps E-cadherin expression at the cell surface is associated with epibolic movement and/or incompatible with cellular rearrangements. In this respect, it is interesting that a decrease in E-cadherin expression has been noted in epithelia undergoing extensive morphogenetic movements, such as the invagination of the ectoderm during formation of the neural tube (32, 46).

Another important role for E-cadherin in gastrulation is suggested by the hypothesis that the cadherins are responsible for the differential cell adhesion that leads to the sorting out of cell types in developing tissues (33, 45). In amphibian embryos, differential cell adhesion is believed to play a role in the segregation of the embryonic germ layers (43, 47). Interestingly, recent experiments have shown that animal cap cells isolated from *Xenopus laevis* become able to recognize and migrate preferentially into the ectoderm only when they are derived from embryos after stage 10 (21). Perhaps this onset in the ability of animal cap cells to recognize the ectoderm is mediated by the expression of E-cadherin, which normally commences at this same time.

It should now be possible to test directly whether either of these two ideas about the function of E-cadherin in *Xenopus* gastrulation is correct. The use of specific probes for adhesion molecules in this experimentally accessible developmental system holds exciting prospects for learning about the molecular and cellular processes controlling the morphogenesis of tissues.

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