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Expression of a novel cadherin (EP-cadherin) in unfertilized eggs and early *Xenopus* embryos

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Summary

Two distinct cadherin cDNA clones of *Xenopus laevis* were isolated from a stage 17 embryo cDNA library. Analysis of the complete deduced amino acid sequences indicated that one of these molecules is closely homologous to chicken and mouse N-cadherin, while the other displays comparable homology to both E- and P-cadherins and was thus denoted EP-cadherin. This molecule has an apparent relative molecular mass of 125×10^3 (compared to approx. 138×10^3 or approx. 140×10^3 of E-cadherin and N-cadherins, respectively). Northern and Western blot analyses indicated that N-cadherin is first expressed at the neurula stage while EP-cadherin is the only cadherin detected in unfertilized eggs and cleavage stage embryos. Immunolabeling of *Xenopus* eggs with antibodies prepared against a fusion protein, containing a segment of EP-cadherin, indicated that the protein is highly enriched at the periphery of the animal hemisphere. EP-cadherin was also found in A6 epithelial cells derived from *Xenopus* kidneys, and was apparently localized in the intercellular adherens junctions.

Key words: cell adhesion, cadherins, adherens junctions, *Xenopus laevis*.

Introduction

Cadherins are a family of structurally and functionally related molecules that mediate Ca^{2+}-dependent intercellular adhesion (Takeichi, 1988). These molecules were primarily localized in areas of cell–cell contact, often associated with actin microfilaments adherens type junctions (Volk and Geiger, 1984; Boller *et al.* 1985; Hirano *et al.* 1987). Based on their spatial relation to the cytoskeleton, it was suggested that cadherins play a role in the generation of intercellular and intracellular forces, which are of fundamental importance in cellular dynamics and embryonic morphogenesis (Geiger *et al.* 1984; Edelman, 1985). This notion was also supported by the spatial and temporal correlation between the expression of different cadherins during development and specific morphogenetic events (Hatta *et al.* 1987; Duband *et al.* 1988). Furthermore, transfection of cadherin-specific cDNA into non-expressing cells affected cellular morphology, leading to an apparent epithelialization of the cells (Matsuzaki *et al.* 1990). Thus the study of cadherin expression and function is of major importance for the understanding of the mechanisms underlying cellular interactions in development.

In view of the vast information available on the cellular and molecular aspects of *Xenopus* development, this system appears to be most suitable for exploring the particular involvement and contribution of cadherins to embryonic morphogenesis. The presence of a Ca^{2+}-dependent intercellular adhesion system in *Xenopus* embryonic cells was reported by Nomura *et al.* (1986). Since then, additional information concerning specific adhesion molecules was obtained. This includes the identification of a molecule, antigenically related to E-cadherin, in a cultured epithelial cell line and in gastrulating embryos (Nomura *et al.* 1988; Choi and Gumbiner, 1989), as well as the cloning and sequencing of *Xenopus* N-cadherin (Detrick *et al.* 1990). It was further shown that the latter is first expressed at the neurula stage. In addition, a cadherin-like molecule, distinct from both N-cadherin and E-cadherin, was reported to be present in late stage oocytes (Choi *et al.* 1990).

Here we report on the cloning, sequencing and analysis of expression of *Xenopus* N-cadherin as well as a new cadherin molecule denoted EP-cadherin, which displays a comparable homology to both E- and P-cadherins. We show that both the mRNA and protein products of this gene are present in the unfertilized egg. Furthermore, immunolabeling with antibodies raised against a bacterial fusion protein containing EP-cadherin sequences indicated that the protein is particularly enriched at the periphery of the animal hemisphere.

Materials and methods

Animals, eggs and embryos

Mature frogs (both wild type and albino) were purchased from *Xenopus* 1 Ltd (MI, USA). Females were induced to lay eggs by injections of hCG according to Newport and Kirschner (1982). Eggs were collected directly to 1×MMR (0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 0.1 mM EDTA, 5 mM Hepes, pH 7.8). Embryos were obtained by *in vitro* fertilization and maintained in 0.1×MMR. Eggs and early embryos were dejellied in 2% cysteine in 1×MMR or 0.1×MMR neutralized to pH 7.8 with NaOH. Embryos were staged according to the Normal Table of *Xenopus laevis* (Nieuwkoop and Faber, 1967).

Cloning and sequencing of Xenopus *cadherins*

The cadherin cDNA clones were isolated from a stage 17 λgt10 cDNA library kindly provided by D. Melton, Harvard University, Cambridge, MA (Kintner and Melton, 1987). The library was screened under low-stringency conditions with a chicken N-cadherin cDNA probe (Hatta *et al.* 1988). The isolated clones were subcloned into either pGEM (Promega, USA) or Bluescript (Stratagene, USA) plasmids. Restriction enzymes were purchased mainly from New England Biolabs (USA). Southern blot analysis was carried out according to Maniatis *et al.* (1982), using high-stringency conditions. The nucleotide sequences of the isolated clones were derived from single-strand templates using the dideoxy chain termination method of Sanger *et al.* (1977) as modified for the Sequenase kit (U.S. Biochemicals, USA).

Northern blot analysis

Total RNA was extracted using the LiCl–urea procedure (LeMeur *et al.* 1981). RNA was electrophoresed in agarose–formaldehyde gels, as described by Maniatis *et al.* (1982). 25 μg of total RNA were loaded on each lane. The RNA was blotted onto a Hybond-N membrane (Amersham, UK), stained with methylene blue and subjected to hybridization using high-stringency conditions. DNA probes (^{32}P-labeled) were prepared using a random-priming DNA-labeling kit (Boehringer, FRG).

Extraction of proteins from cultured cells, tissues and eggs

Cultured cells and tissues were extracted in 1×Laemmli sample buffer (Laèmmli, 1970). Dejellied eggs were extracted with 1% NP-40 in 150 mM NaCl, 2 mM $CaCl_2$, 10 mM Hepes pH 7.5, supplemented with protease inhibitors (1 mM PMSF, 20 μg ml^{-1} aprotinin). Detergent-insoluble material was removed by centrifugation at 12 000 *g* for 30 min.

SDS–PAGE and immunoblot analysis

Protein samples were electrophoresed through an 8% polyacrylamide slab gels. The polypeptides were electroblotted onto nitrocellulose paper (Schleicher and Scheull, FRG) and immunolabeled using either the R-156 serum or anti-E-cadherin antibodies (kindly provided by B. Gumbiner, UCSF, USA) at an appropriate dilution followed by alkaline phosphate-conjugated (Promega, USA) or ^{125}I-labeled secondary antibodies.

Preparation of fusion proteins and generation of antibodies

The *Bgl*II fragment of clone c4 (see Results) was ligated into the *Bam*HI site of the Path 2 vector (Dieckmann and Tzagoloff, 1985). Bacteria transfected with this plasmid were induced to express high amounts of the fusion protein with IAA (Sigma, USA) and total protein extracts were injected into rabbits. Following four injections at 2 week intervals, blood was collected and examined for the presence of antibodies.

Cell culture and transfections

The A6 kidney cell line (ATCC, USA) was grown in 85% DMEM supplemented with 8.5% fetal calf serum (FCS), at 28°C in a humidified atmosphere of 5% CO_2 in air. Chinese hamster ovary (CHO) cells were grown in DMEM supplemented with 10% FCS, at 37°C in a humidified atmosphere of 7% CO_2 in air. CHO cells were co-transfected with a pECE plasmid (Ellis *et al.* 1986) containing the *Eco*RI fragment of clone 4 and the pSV2-neo plasmid (Southern and Berg, 1982) using the calcium phosphate transfection procedure (Graham and Van Der Eb, 1973). Transfectants were selected using 700 μg ml^{-1} of G-418 (Geneticin, GIBCO, USA) in the medium. Positive clones were identified by immunoblot analysis using the pan-cadherin antibodies R-156 directed against a synthetic peptide corresponding to the 24 C-terminal amino acids of chicken N-cadherin.

Immunofluorescence of A6 cells

Cells were cultured on glass coverslips, permeabilized for 3 min with 0.5% Triton X-100 in 3% formaldehyde and further fixed with 3% formaldehyde for additional 25 min. Staining of cells was carried out by the indirect immunofluorescence technique. Affinity-purified R-156 antibodies were used at 5 μg ml^{-1}. Sera of rabbits injected with the fusion protein (designated R-827, see above) were used at a 1:500 dilution and preimmune serum up to 1:50 dilution. Rhodamine-labeled secondary antibodies were either prepared as described (Brandtzaeg, 1973) or purchased from Jackson labs (USA).

Staining and sectioning of eggs

Albino *Xenopus* eggs were stained according to the method of Dent *et al.* (1989), using either R-827, R-156 or irrelevant rabbit antibodies and goat anti-rabbit antibodies conjugated to peroxidase (Jackson, USA). Following the enzymatic reaction, eggs were dehydrated in alcohol and embedded in JB4 resin (Polysciences, Inc., PA). The embedded eggs were sectioned (2–3 μm) using the LKB Nova microtome (Sweden) and examined in a Zeiss Axiophot microscope.

Results

Cloning of Xenopus *cadherins*

In order to clone *Xenopus laevis* cadherin molecules, we have screened a λgt10 cDNA library of stage 17 embryos (Kintner and Melton, 1987) with a cDNA-encoding chicken N-cadherin (Hatta *et al.* 1988). Using low-stringency conditions, seven independent clones were isolated. Cross-hybridization studies, under high-stringency conditions, indicated that the isolated clones could be subdivided into two groups, one containing clones Nos. 1, 3, 6 and 8 while the other contained clones 2, 4 and 5. The physical maps of the isolated cDNA clones (Fig. 1) also suggested that they represent two distinct cDNA molecules. It was nevertheless noted that clone 3 varies somewhat from clones 1, 6 and 8 in its restriction map. This variation may be attributed to polymorphism, which is common in the pseudotetrap-

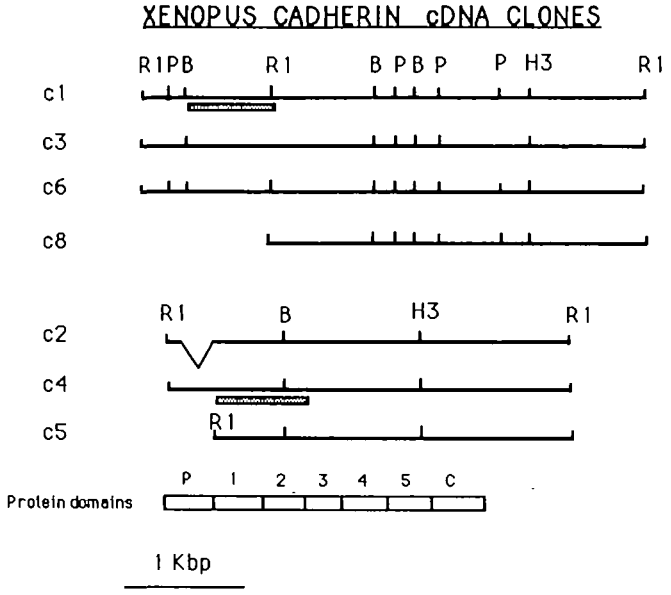

Fig. 1. Restriction maps of the various cDNAs encoding *Xenopus* N-cadherin (C1, C3, C6, C8) and EP-cadherin (C2, C4, C5). The restriction sites marked include: *Eco*RI: (RI;) *Bam*HI: (B); *Hin*dIII: (H3); *Pst*I (P). The boxes under C1 and C4 represent the fragments that were used as probes. A scheme outlining the various cadherin protein domains (including presequences (P), ectodomains 1–5 and the cytoplasmic (C) domain) is shown at the bottom.

loid clawed frog (Kobel and Du Pasquier, 1986). Some variation was also found in the 5′ region of the other group of clones, manifested by the presence of 209 bp in clone 4, which were absent from clone No. 2. Notably, the sequences missing from the latter were flanked by G(233)TG and A(439)GA. These sequences show similarity to the splicing consensus (Mount, 1982), though the presence of splicing variants was not directly established here.

Nucleotide and amino acid sequences

The nucleotide and the deduced amino acid sequences of clone 1 are shown in Fig. 2. The amino acid sequence shows a high degree of homology to chicken N-cadherin, and we thus refer to this clone as the *Xenopus* N-cadherin. Our sequence differs only slightly from the one recently published by Detrick *et al.* (1990). The differences found between the two are marked in Fig. 2.

The nucleotide and the deduced amino acid sequences of clone 4 are shown in Fig. 3. Comparison of the protein sequence to that of known cadherins revealed considerable degree of homology to both E- and P-cadherin is shown in Fig. 4. As can be seen, the 5′ region of clone 4 contains two in-frame ATG sequences. Based of the homology to other cadherins and comparison to the Kozak consensus sequence (Kozak, 1987), it is difficult to select one of the two as the definitive initiation site. We have, thus, chosen the first one as the start of translation. Since this cDNA clone is comparably homologous to the two cadherin molecules and in view of its distinction from E-cadherin (see below), we have designated it EP-cadherin.

Sequencing of the 3′ ends of the clones encoding both N- and EP-cadherins disclosed poly (A) stretches as well as consensus poly-adenylation signals. The length of the 3′ non-coding region in the two clones was about 1 kb, which is similar to the homologous region in other cadherins.

Cadherin expression during early embryogenesis

In order to study the involvement of the newly cloned cadherins in embryonic morphogenesis, we have followed the pattern of expression of EP-cadherin and N-cadherin in early *Xenopus* embryos. The expression of both cadherins was first studied using Northern blot analysis. Total RNA was extracted from embryos at a variety of developmental stages including: unfertilized eggs, blastula at mid-blastula transition (MBT), neurula and tail bud.

As shown in Fig. 5, the EP-cadherin transcript was detected already in the unfertilized egg, indicating that it was a maternal transcript. The levels of EP-cadherin decreased in later stages. The EP-cadherin transcript was about 3.5 kb, in accordance with the size of the cDNA clone. The N-cadherin transcript was first detectable at the neurula stage and persisted in the tail bud. The transcript was about 4.2 kb, again indicating that the cDNA clone is essentially a full-length clone.

The expression and immunolocalization of cadherins

Having found the EP-cadherin transcript in the unfertilized egg, we proceeded by checking whether a cadherin protein was also detectable at that early stage. A protein extract of unfertilized eggs was run on a SDS–PAGE and subjected to immunoblot analysis, using the pan-cadherin rabbit serum (R-156), prepared against a synthetic peptide corresponding to the 24 carboxy-terminal amino acids of chicken N-cadherin (Geiger *et al.* 1990). These antibodies recognize all the cadherins thus far identified. The pan-cadherin antibodies reacted with a 125×10^3 M_r polypeptide in *Xenopus* egg extract (Fig. 6B). Furthermore, the EP-cadherin cDNA was ligated into the pECE eukaryotic expression vector (Ellis *et al.* 1986) and transfected into CHO cells, together with the pSV2-neo vector (Southern and Berg, 1982). Positive clones were identified by Western blotting with R-156 antibodies, disclosing a protein band comigrating with the one found in the eggs (Fig. 6A). This band was not present in non-transfected CHO cells.

In order to identify and localize the cadherin molecule, we have raised antibodies against a trpE fusion protein containing amino acids 149–366 of EP-cadherin. The antibodies obtained, denoted R-827, intensely stained cultured epithelial cells of *Xenopus* origin. The antigen recognized by the R-827 serum was localized along areas of cell contacts displaying patterns closely related to those obtained with the pan-cadherin, R-156 antibody (Fig. 7). It was, however, noted that the staining with the latter antibody was somewhat more extensive showing specific labeling along the peripheral

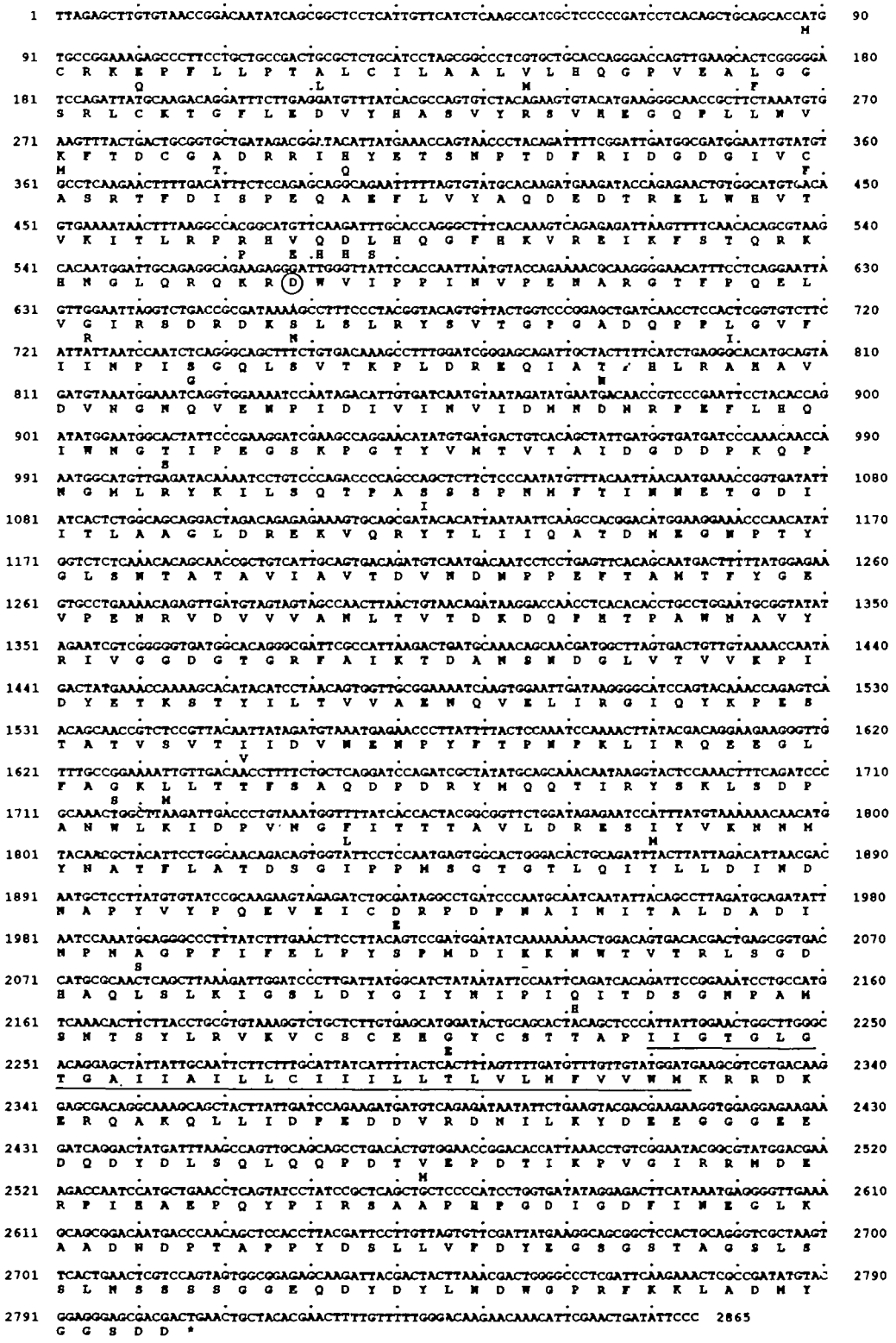

Fig. 2. Nucleotide and deduced amino acid sequences of *Xenopus* N-cadherin (clone No. 1). The N-terminal amino acid of the mature protein is encircled and the transmembrane domain underlined. Amino acid variations from the previously published sequence of *Xenopus* N-cadherin (Detrick *et al.* 1990) are indicated below the sequence. Notice that the dash under lysine (650) marks an in frame deletion.

lamellipodia of the cells (Fig. 7B). It was further found by Western blotting that A6 cells contained three major immunoreactive bands when assayed with R-156 antibodies, one of which comigrated with the major egg molecule and with the EP-cadherin present in transfected CHO cells (Fig. 6C). Unfortunately the R-827

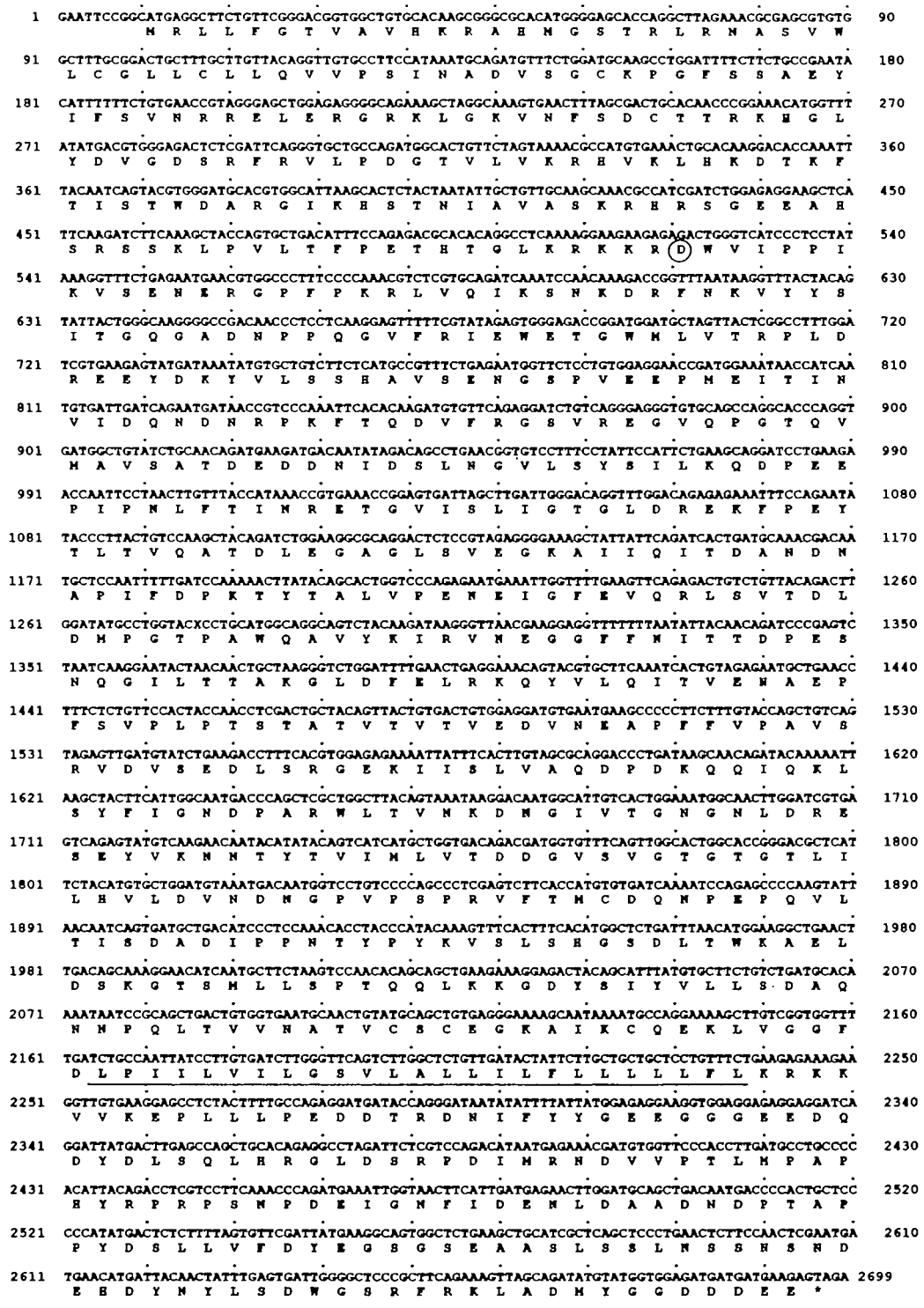

Fig. 3. Nucleotide and deduced amino acid sequences of *Xenopus* EP-cadherin (clone No. 4). The N-terminal amino acid of the mature protein is encircled and the transmembrane domain underlined.

antibodies did not react in either immunoblot or immunoprecipitation assays and thus its exact specificity (especially its capacity to distinguish between E- and EP-cadherin) is not unequivocally defined.

The R-827 antibodies were subsequently used to determine the spatial distribution of the respective cadherin protein in the egg. Fig. 8 shows that the cadherin in the eggs was specifically localized at the periphery of the animal hemisphere. Immunolabling without prior permeabilization did not yield specific labeling, suggesting that the cadherins were not available to the antibodies on the surface of the egg.

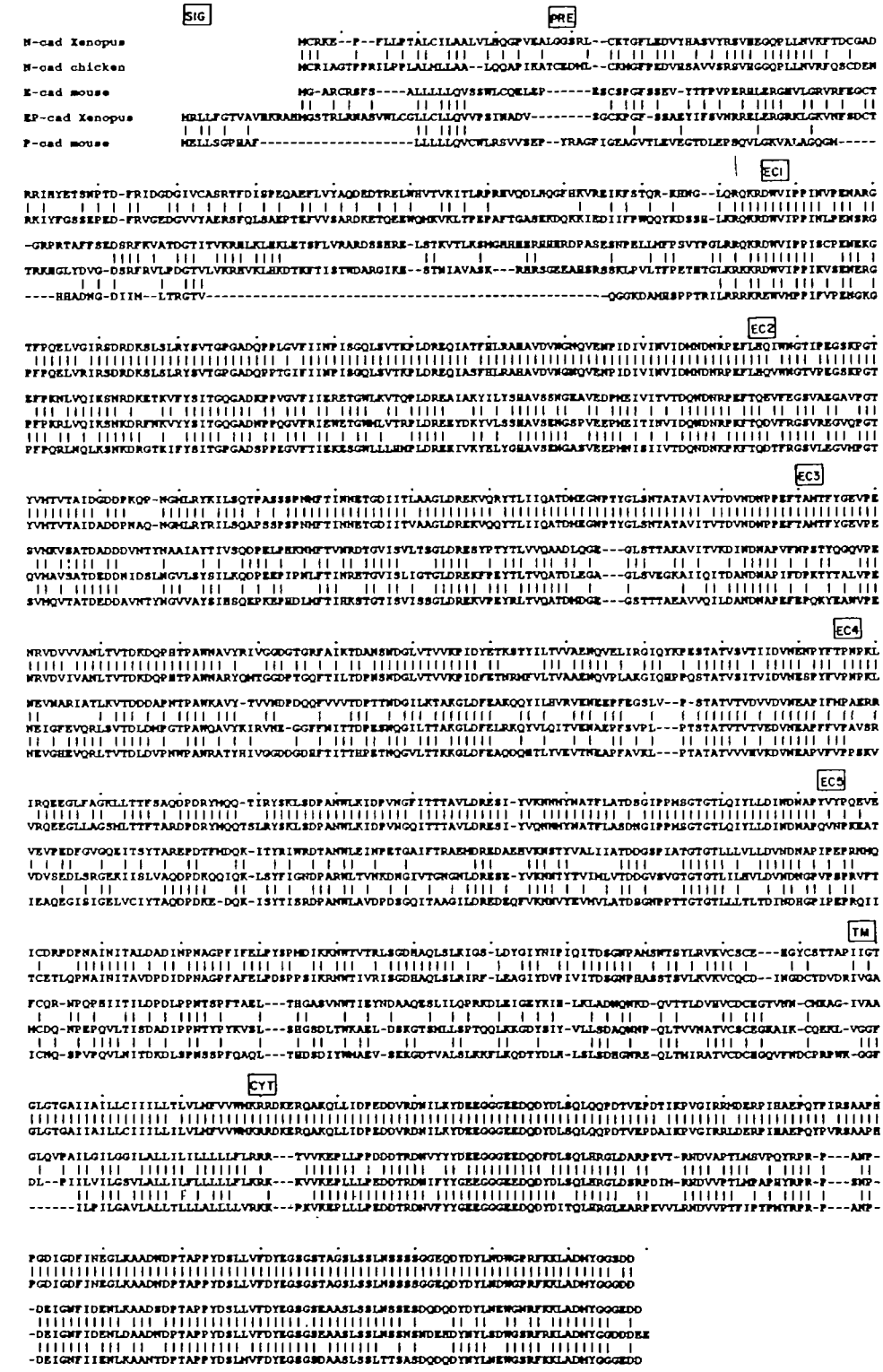

Fig. 4. Comparison of the predicted amino acid sequences of *Xenopus* N-cadherin to chicken N-cadherin (Hatta *et al.* 1988) and of *Xenopus* EP-cadherin to both mouse E- and P-cadherins (Nagafuchi *et al.* 1987 and Nose *et al.* 1987, respectively). Gaps were inserted such that all five molecules will be grossly aligned. The approximate borders of the various cadherin domains (signal peptide (sig), presequences (pre), ectodomains 1–5 (EC1–EC5), the transmembrane (TM) and cytoplasmic domain (cyt)), are marked.

Expression of cadherins in the adult frog

In order to check whether the cadherins expressed during embryogenesis are also found in adult tissues, we have studied the tissue distribution of N-cadherin and EP-cadherin at the RNA level. Total RNA was extracted from heart, lung, liver, skin, intestine and

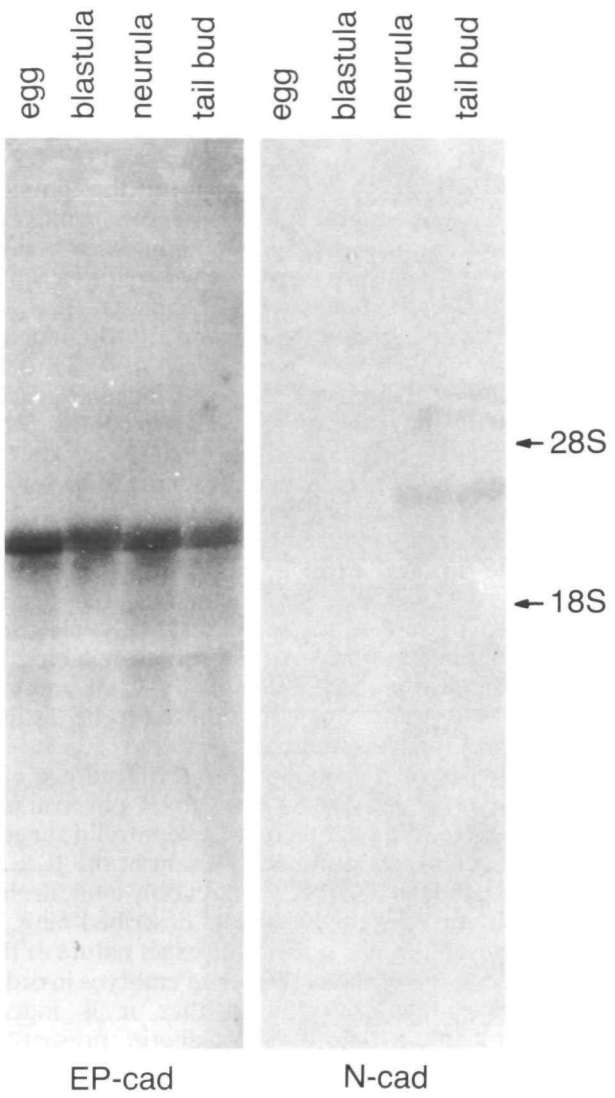

Fig. 5. Northern blot analysis of RNA from early embryos reacted with either an EP-cadherin (EP-cad) or a N-cadherin (N-cad) probe. 25 μg of total RNA from unfertilized eggs, blastula at MBT, neurula and tail bud embryos were run on an agarose–formaldehyde gel and transferred onto a Hybond-N membrane. All samples showed the same intensity following methylene blue staining of the blot. The position of 28S and 18S ribosomal RNAs is indicated.

testis and subjected to Northern blot analysis at high stringency. As shown in Fig. 9, EP-cadherin displayed a rather restricted distribution, being expressed at significant levels only in skin and lung. Thus, its tissue distribution is generally similar (though not identical) to that of E-cadherin, as observed by immunohistochemistry (Choi and Gumbiner, 1989 and see Discussion).

N-cadherin was highly expressed in the heart and a considerable level of expression was also observed in the testis, in line with the reported distribution of the homologous molecule, in the chicken (Hatta *et al.* 1987; Duband *et al.* 1988). It is noteworthy that *Xenopus*

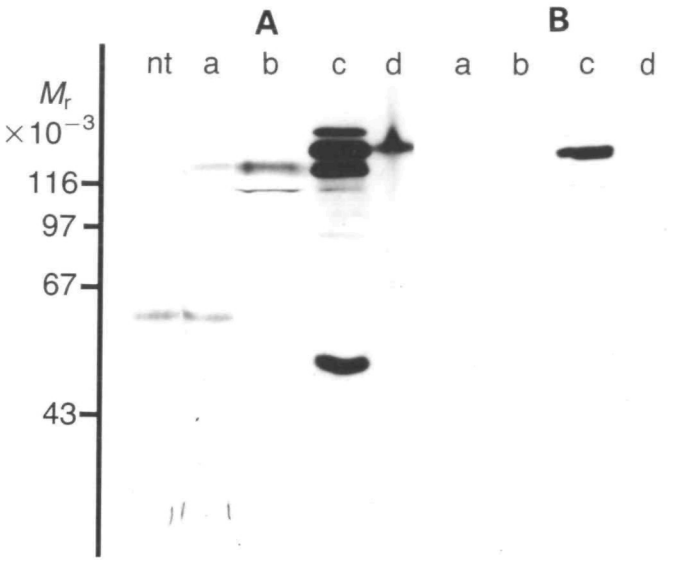

Fig. 6. Immunoblot analysis of protein extracts from non-transfected CHO cells (nt), CHO cells transfected with EP-cadherin (a), eggs (b), A6 cells (c) and heart tissue (d) reacted with either the pan-cadherin R-156 antibodies (A) or anti-E-cadherin antibodies (B).

N-cadherin was previously reported to be also expressed in the brain of developing embryos (Detrick *et al.* 1990).

Immunoblot analysis of adult tissues using the R-156 antibody revealed a multitude of immunoreactive polypeptides. These include the three definitive cadherins (N-cadherin, E-cadherin which migrates slightly faster on these gels and EP-cadherin which has a lower apparent molecular weight) as well as additional bands. Further characterization will be needed to determine whether these are additional unidentified cadherins or rather are precursor forms or breakdown products.

Discussion

We have used, in this study, a molecular genetic approach for the identification and characterization of novel cadherins from *Xenopus laevis*. We have cloned two distinct cDNA species showing variable homologies to known cadherins. The *Xenopus* N-cadherin clone isolated here is highly homologous to all the N-cadherins so far studied. Particularly relevant is its comparison to the molecule recently described by Detrick *et al.* (1990). While the two were nearly identical at the deduced amino acid level, we have detected significant differences in the 5′ non-coding sequences of the two clones and some scattered substitutions along the coding region. It is interesting to note that some of these variations lead to non-conservative sequence changes as may be appreciated from Fig. 2. It might prove most interesting to compare the functional properties of the products of the two clones. As far as the genetic basis for these variations is concerned, it seems most likely that they stem from

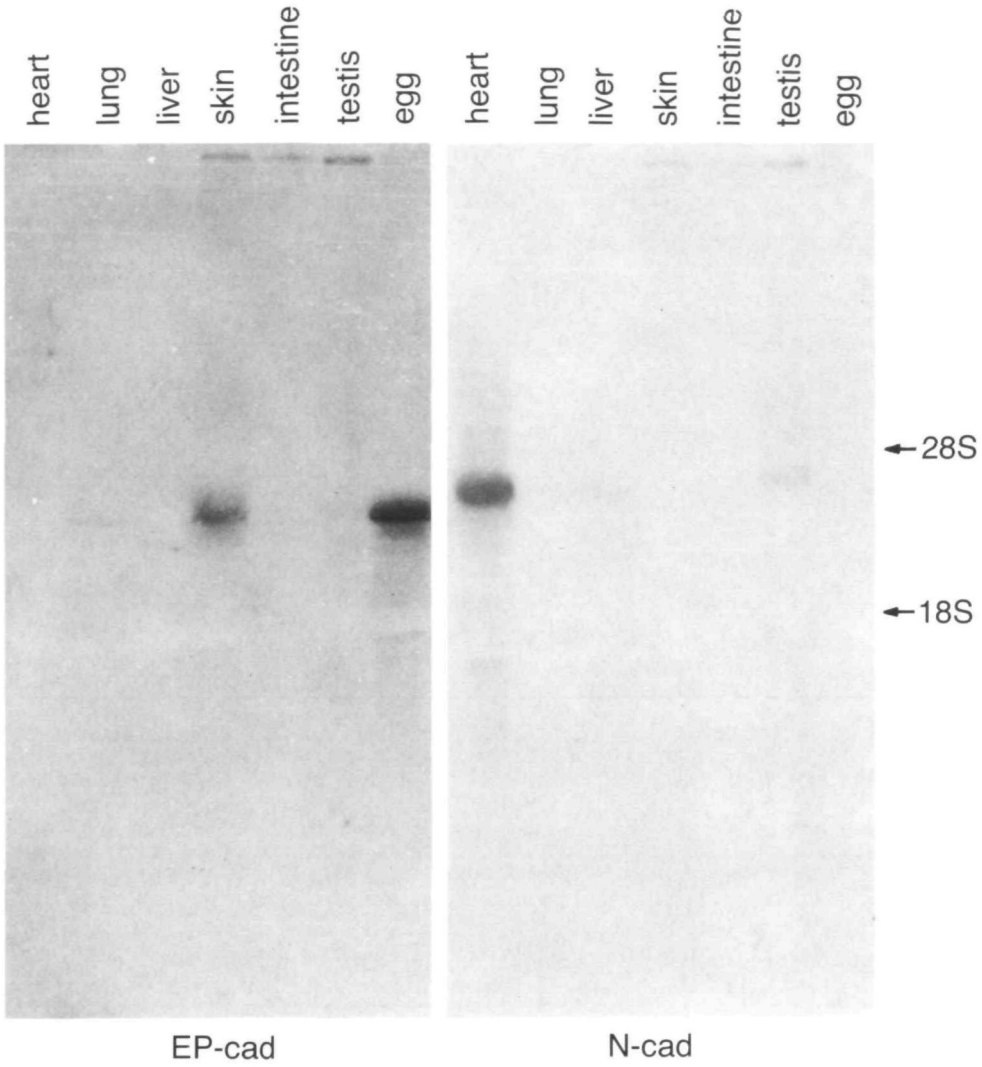

Fig. 9. Northern blot analysis of RNA from adult frog tissues reacted with either an EP-cadherin (EP-cad) or a N-cadherin (N-cad) probe. 25 μg of total RNA from heart, lung, liver, skin, intestine, testis and eggs were run on an agarose–formaldehyde gel and transferred onto a Hybond-N membrane. The methylene blue staining pattern of all samples was comparable. The position of the 28S and 18S ribosomal RNAs is indicated.

animal hemisphere. It is thus anticipated that the animal blastomeres might contain higher levels of the EP-cadherin molecule. This might be related to the fact that the animal blastomeres apparently form tighter intercellular junctions and that most primary epithelia are derived from them (Jones and Woodland, 1986). To substantiate this possibility and determine its physiological significance, it will be necessary to study mRNA distribution in the egg as well as cadherin expression in cleavage-stage embryos both at the protein and mRNA levels.

It is nevertheless noteworthy that the EP-cadherin present at the periphery of the egg is, most likely, not exposed on the egg's surface. This observation is in line with the report by Choi *et al.* (1990) and is based mainly on the observation that positive staining of the egg was obtained only following proper permeabilization. This finding raises the interesting possibility that the EP-cadherin is sequestered into cortical vesicles and may become functional only following fusion of these vesicles with the membrane. This hypothesis is currently under investigation.

Another observation that bears on the specificity of cadherin-mediated interactions is the presence of multiple forms of cadherins in the same tissues and even on the same cells. It has been shown previously that coexpression of two cadherins may occur during epithelial differentiation (for example, N- and E-cadherin in developing kidney (Geiger *et al.* 1989)). It was also demonstrated that, while cadherins may exhibit a preference for homophilic interactions (Nose *et al.* 1988), heterophilic cell junctions may also be formed (Volk *et al.* 1987; Geiger *et al.* 1989). The use of the pan-cadherin serum clearly indicated that coexpression of different cadherins is a rather common phenomenon (Geiger *et al.* 1991 and on Fig. 10 below).

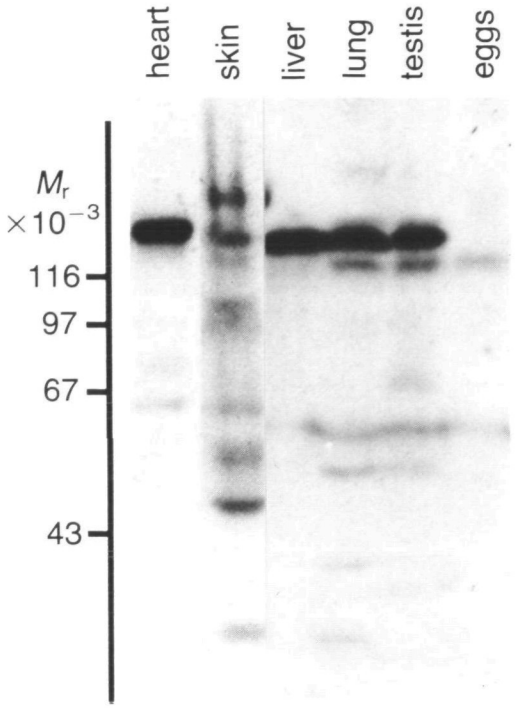

Fig. 10. Immunoblot analysis of protein extracts from heart, skin, liver, lung, testis and eggs reacted with the pan-cadherin, R-156, antibodies.

In agreement with that notion we also show that a cloned cell line such as A6 expresses several (probably 3) distinct cadherins. Does each of these adhesion molecules function independently of the others or do they all act synergistically? Do all the different adhesion molecules participate in junction formation, are they capable of heterophilic interactions and do they similarly trigger the construction of cell junctions and affect cell dynamics and behavior? These issues appear to be among the major challenges of future studies on the molecular basis for cell adhesion.

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