Differential expression of αN-catenin and N-cadherin during early development of chicken embryos

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ABSTRACT **α-Catenins are a group of proteins associated with cadherin cell-cell adhesion molecules, and play indispensable roles in the function of the cadherins. αN-catenin, a subtype, was identified as a protein associated with N-cadherin. In this study, we investigated the expression pattern of αN-catenin in early chicken embryos, and compared it with that of N-cadherin. αN-catenin was first detected in the closed somites and neural tube, and, at later stages, in many other tissues including the central nervous system (CNS), skeletal muscles, various regions of the overlying ectoderm, and some endodermal layers. In the CNS and skeletal muscles, both αN-catenin and N-cadherin were strongly expressed, and their distribution patterns were similar. However, in some parts of the ectoderm and endoderm, only αN-catenin was expressed. On the other hand, various mesenchymal tissues and peripheral nerves strongly expressed N-cadherin, but their αN-catenin expression was, in general, weak. Thus, the expression of these two proteins did not always correlate with each other. These results suggest that cells use different combinations of a cadherin and an α-catenin in a tissue-specific manner.**

KEY WORDS: *αN-catenin, N-cadherin, chicken embryo, cell adhesion molecule*

Introduction

Cadherin cell-cell adhesion molecules are associated with cytoplasmic proteins collectively called catenins. These include α-catenin (Herrenknecht *et al.*, 1991; Nagafuchi *et al.*, 1991; Hirano *et al.*, 1992), ß-catenin (McCrea *et al.*, 1991) and plakoglobin (Knudsen and Wheelock, 1992). Among them, α-catenin has been best studied in terms of function. Without this class of catenins, cadherins cannot function as adhesion molecules (Hirano *et al.*, 1992; Shimoyama *et al.*, 1992). α-Catenin is divided into two subtypes, αE-catenin and αN-catenin, which have 82% identity to each other. αE-Catenin has been identified as an E-cadherin-binding protein in the mouse (Herrenknecht *et al.*, 1991; Nagafuchi *et al.*, 1991), and this molecule is expressed in a wide variety of tissues, although it is diminished in most regions of the central nervous system (CNS) during development (Nagafuchi and Tsukita, 1994). αN-catenin has been identified as a protein associated with chicken N-cadherin (Hirano *et al.*, 1992). Our previous observations indicated that this protein is expressed in the nervous system as well as in some other tissues (Hirano *et al.*, 1992).

Although each of the α-catenins was identified as a protein associated with a particular cadherin, they can bind to both E-cadherin and N-cadherin at least under certain experimental conditions, as revealed by α-catenin cDNA transfection of L cells expressing these cadherins (Hirano *et al.*, 1992). We also demonstrated, using α-catenin-deficient PC9 cells, that αN-catenin could support the function of E-cadherin (Hirano *et al.*, 1992). Furthermore, our preliminary results indicated that αN-catenin did not always colocalize with N-cadherin. For example, in the heart, αE-catenin but not αN-catenin is expressed together with N-cadherin (Nagafuchi *et al.*, 1991; Hirano *et al.*, 1992). It remains to be investigated what kind of biological functions underlie the differential combination of various cadherins and α-catenins *in vivo*.

For a better understanding of the above problem and also of the role of αN-catenin itself in morphogenesis, we performed detailed analyses of the tissue distribution of this molecule in early chicken embryos, and compared the findings with those on N-cadherin distribution. Our results show that these two molecules are differentially expressed in many kinds of tissues derived from different germ layers.

Results

We immunostained for αN-catenin and N-cadherin in 2- to 8-day chicken embryos. Our previous immunoblotting experiments using 10-day embryos showed that αN-catenin was expressed mainly in the brain (Hirano *et al.*, 1992). In the following analyses, however, we found local expressions of this molecule in other tissues in early

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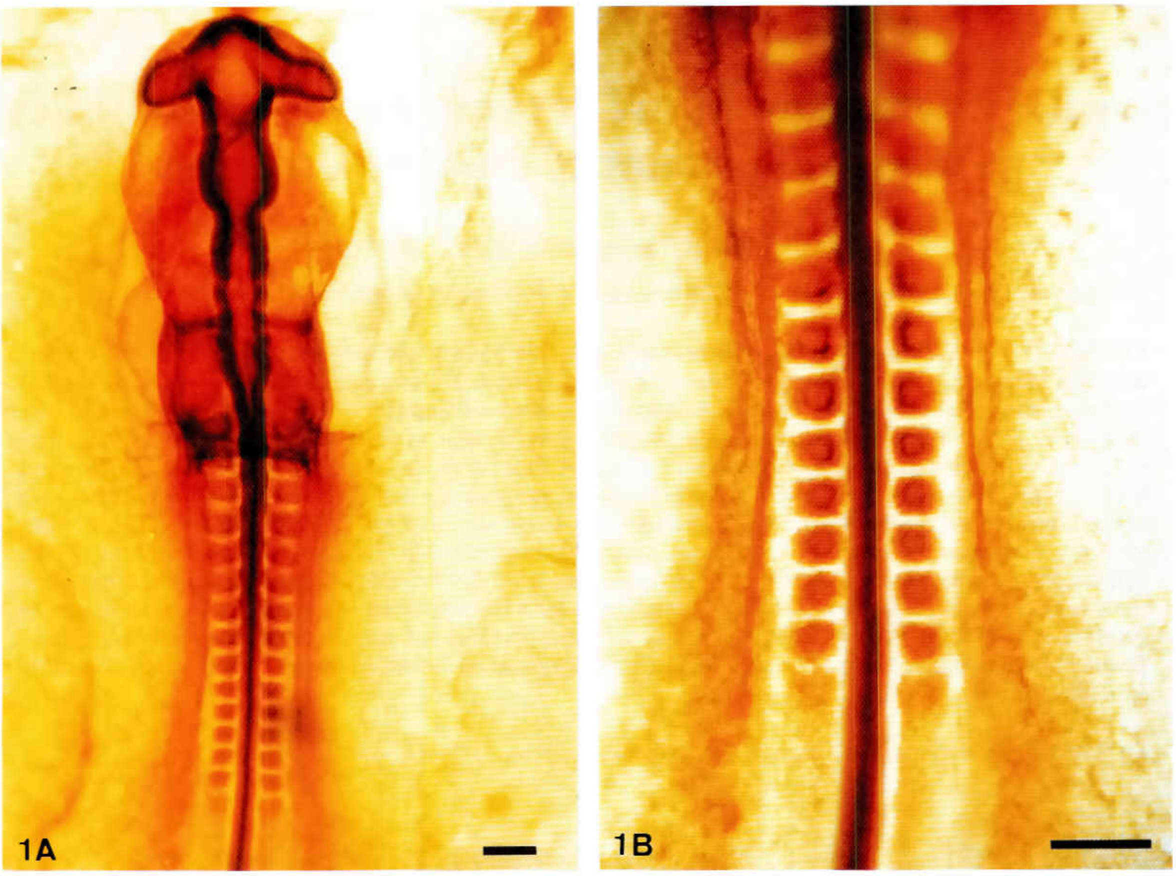

Fig. 1. Whole-mount immunoperoxidase staining for αN-catenin in a 2-day embryo at stage 11. *Focus was adjusted on the neural tube in* **(A)**, *and somites in* **(B)**. *Note that the apical cell-cell contacts in the closed somite epithelium stains for αN-catenin; N-cadherin is known to be concentrated in the same regions (Hatta et al., 1987). Bar, 200 μm.*

embryos. While the expression pattern of αN-catenin was similar to that of N-cadherin in most of these tissues, there were a number of regional differences in distribution between the two molecules.

In 2-day embryos at stage 11, whole-mount staining for αN-catenin showed that this protein was expressed in the neural tube and somites (Fig. 1). During somitogenesis, intense αN-catenin signals first appeared at the apical cell-cell contacts when the somite epithelium had segmented and closed to form vesicles (Fig. 1B). After further differentiation of the somites, αN-catenin was expressed in the myotome, as already reported (Hirano *et al.*, 1992). These expression patterns are similar to those of N-cadherin (Hatta *et al.*, 1987; Duband *et al.*, 1988).

In 4-day embryos at stage 23, many tissues, including the central nervous system (CNS), expressed αN-catenin together with N-cadherin. Other tissues, such as local regions of the overlying ectoderm and the mesenchyme of various tissues, also expressed both αN-catenin and N-cadherin but differentially. For example, αN-catenin was expressed in the ectoderm covering the visceral arches; but, in the same tissues, N-cadherin was expressed only in the visceral furrow regions (Fig. 2A,B). Likewise, the olfactory epithelium as well as the olfactory nerve strongly expressed both αN-catenin and N-cadherin, whereas part of the head ectoderm contiguous to the olfactory epithelium expressed αN-catenin but not N-cadherin (Fig. 2C,D). On the other hand, mesenchymal cells in many tissues expressed N-cadherin, but they, in general, expressed relatively low levels of αN-catenin (Figs. 2 and 5).

In 8-day embryos, we again found correlated or differential expressions of αN-catenin and N-cadherin. In the brain, the expression patterns of the two molecules were similar, although some subtle regional differences were observed (Fig. 3A,B). The neural retina also showed similar staining for αN-catenin and N-cadherin (Fig. 3C,D). A similar correlated distribution of the two molecules was also observed in the spinal cord (data not shown). In the lens of 8-day embryos, N-cadherin expression occurred in both the epithelium and fiber cell layer, whereas αN-catenin was strongly expressed only in the epithelium (Fig. 4C,D), although the two molecules were equally expressed in both cell layers in the 4-day lens (Fig. 4A,B). Cells of peripheral nerves strongly expressed N-cadherin, but their αN-catenin expression was generally weak (Fig. 5C,D and also Fig. 2A,B). Concerning endodermal tissues, the lung and esophageal epithelia were devoid of N-cadherin, but these cell layers expressed αN-catenin (Fig. 5). The differential expression of these molecules was also observed in various regions of the epidermis in patterns similar to those found in 4-day embryos.

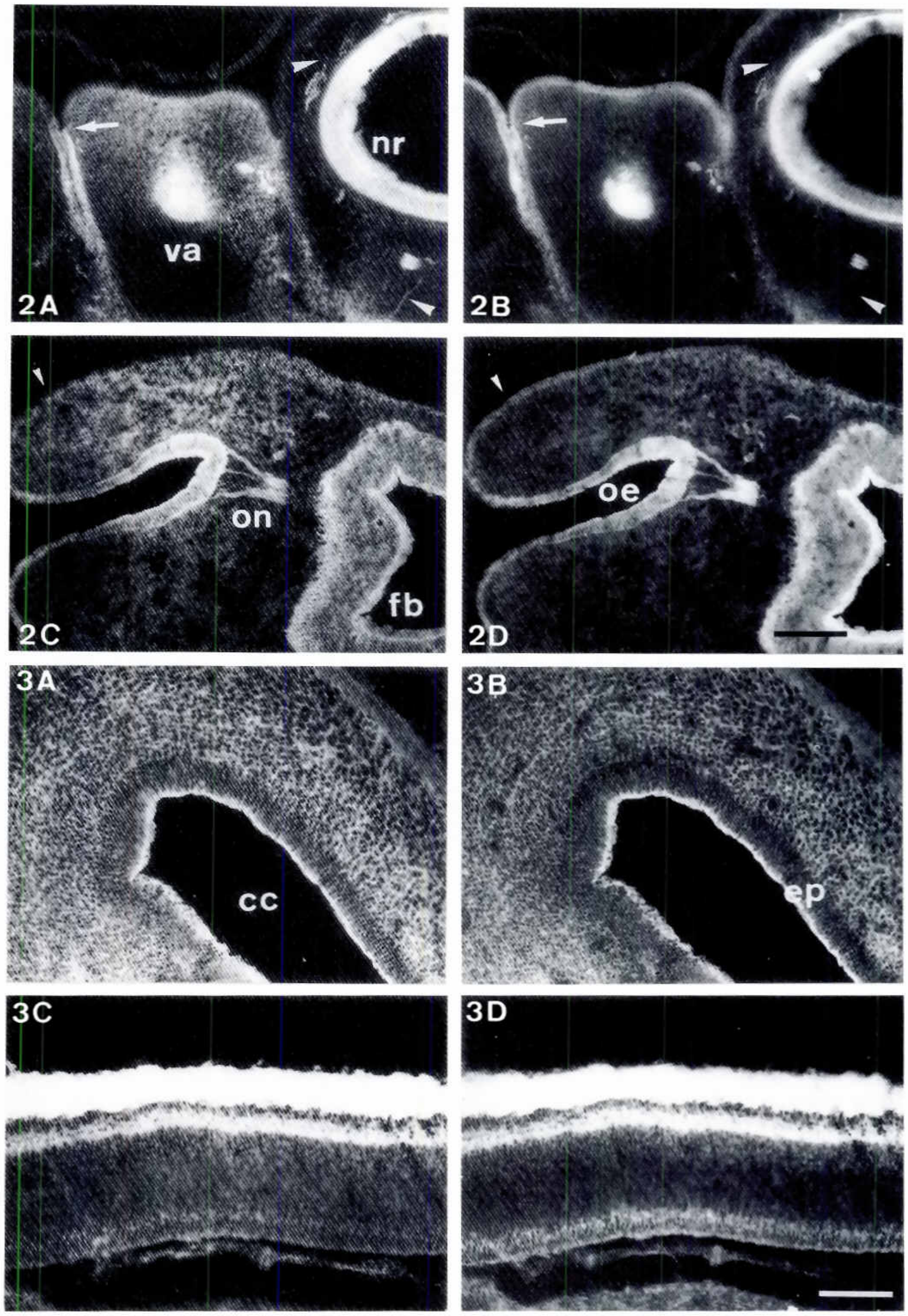

Fig. 2. Double-immunofluorescence staining for N-cadherin and αN-catenin in visceral arches (A,B) and olfactory organs (C,D) of a 4-day embryo at stage 23. (A and C) *N-cadherin;* **(B and D)**, *αN-catenin. In* **(A)**, *N-cadherin is expressed in the ectoderm only at the visceral furrow region where the ectodermal layers on two arches are in contact with one another. The boundary between N-cadherin-positive and -negative regions in the ectoderm are marked with an arrow. αN-catenin is expressed throughout the ectoderm in these tissues* **(B)**. *Large arrowheads indicate some peripheral nerves surrounding the eye, which express N-cadherin more strongly than αN-catenin. In* **(C)** *and* **(D)**, *both N-cadherin and αN-catenin are intensely expressed in the olfactory epithelium (oe), olfactory nerve (on) and forebrain (fb). However, N-cadherin, but not αN-catenin, is absent in the ectoderm at such regions as indicated by the small arrowheads. Also, note the tendency of intenser staining for N-cadherin than for αN-catenin in many regions of the mesenchyme. va, first visceral arch; nr, neural retina. Bar, 200 µm.*

Fig. 3. Double-immunofluorescence staining for N-cadherin and αN-catenin in part of the forebrain (A,B) and neural retina (C,D) of an 8-day embryo. (A and C) N-cadherin; **(B and D)** *αN-catenin. Note that the two molecules show a similar distribution in both neural tissues except for subtle differences. The ganglion layer of the neural retina is located on the top in* **(C,D)**. *cc, central canal; ep, ependymal layer. Bar, 100 µm.*

Discussion

αN-catenin was identified as an N-cadherin-associated protein (Hirano *et al.*, 1992). These two proteins, in fact, are colocalized in many tissues such as CNS and skeletal muscles. However, we found a number of exceptional cases.

During early development of chicken embryos, intense staining for αN-catenin was first detected in the closed somites and neural tube, in both of which N-cadherin is known to be expressed strongly (Hatta and Takeichi, 1986; Hatta *et al.*, 1987; Duband *et al.*, 1988). At later stages, however, non-correlative expression of the two molecules appeared in various tissues. For example, N-cadherin

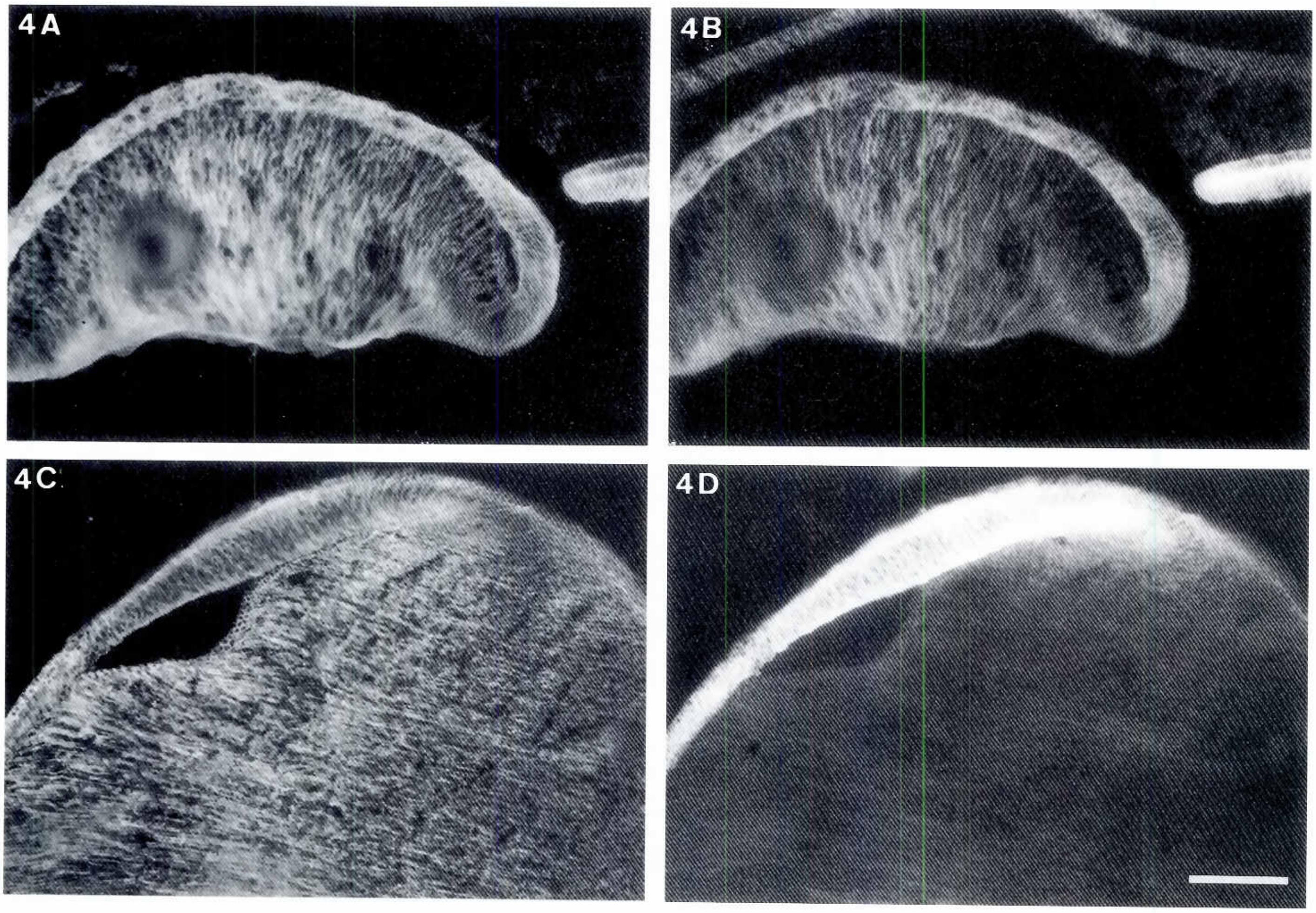

Fig. 4. Double-immunofluorescence staining for N-cadherin and αN-catenin in the lens of a 4-day (A,B) and 8-day (C,D) embryo. (A and C) *N-cadherin;* **(B and D)** *αN-catenin. αN-catenin staining is diminished in intensity in the secondary lens fiber cells of the 8-day embryo. Bar, 100 μm.*

was expressed in very restricted regions of the overlying ectoderm, while αN-catenin expression occurred more widely in the same tissue. Likewise, lung and esophageal epithelium did not express N-cadherin but did contain αN-catenin. On the other hand, we found opposite cases; the peripheral nervous system, lens fiber cells, and many mesenchymal tissues expressed N-cadherin, but their αN-catenin expression was relatively weak.

These results raise the question as to what molecule is associated with N-cadherin or αN-catenin when their original partner is absent. Cells forming solid tissues always express some type of cadherins. For example, most chicken epithelial cells express L-CAM (Thiery *et al.*, 1984; Crossin *et al.*, 1985), an E-cadherin-like molecule. Therefore, it is likely that αN-catenin binds to L-CAM or some other cadherins in the epithelia not expressing N-cadherin. Conversely, N-cadherin could bind to other α-catenins, such as αE-catenin, in the cells without αN-catenin. αE-catenin is known to be ubiquitously expressed in embryos except in the nervous system (Nagafuchi *et al.*, 1991); therefore, it could serve as a partner for N-cadherin in certain tissues. We also cannot rule out the possibility of the presence of other unidentified α-catenins.

If different α-catenins can support the function of the same cadherin, why does an embryo requires multiple α-catenin subtypes and express them differentially? αN-catenin is conserved in amino acid sequence among vertebrate species (Claverie *et al.*, 1993; Uchida *et al.*, 1994), suggesting that it must have specific conserved functions. Although our *in vitro* transfection experiments have so far failed to discriminate between the functions of αE-catenin and αN-catenin, it is possible that they play some different roles *in vivo*. For example, different α-catenin subtypes might generate different cytoplasmic signals after extracellular cadherin-cadherin interactions, and this kind of mechanism could be used for some process of cell-cell recognition. Interestingly, recent results of ours indicated that, in the mouse, αN-catenin distribution was more specifically restricted to the nervous system than in the chicken (Uchida *et al.*,1994). This suggests that αN-catenin has a specific role in the nervous system, at least in the mouse. Moreover, there exist alternative splicing products of αN-catenin (Claverie *et al.*, 1993; Uchida *et al.*, 1994). Further analyses of α-catenin function based on these observations should eventually elucidate the role of the differential expression of these cadherin-associated proteins during embryogenesis.

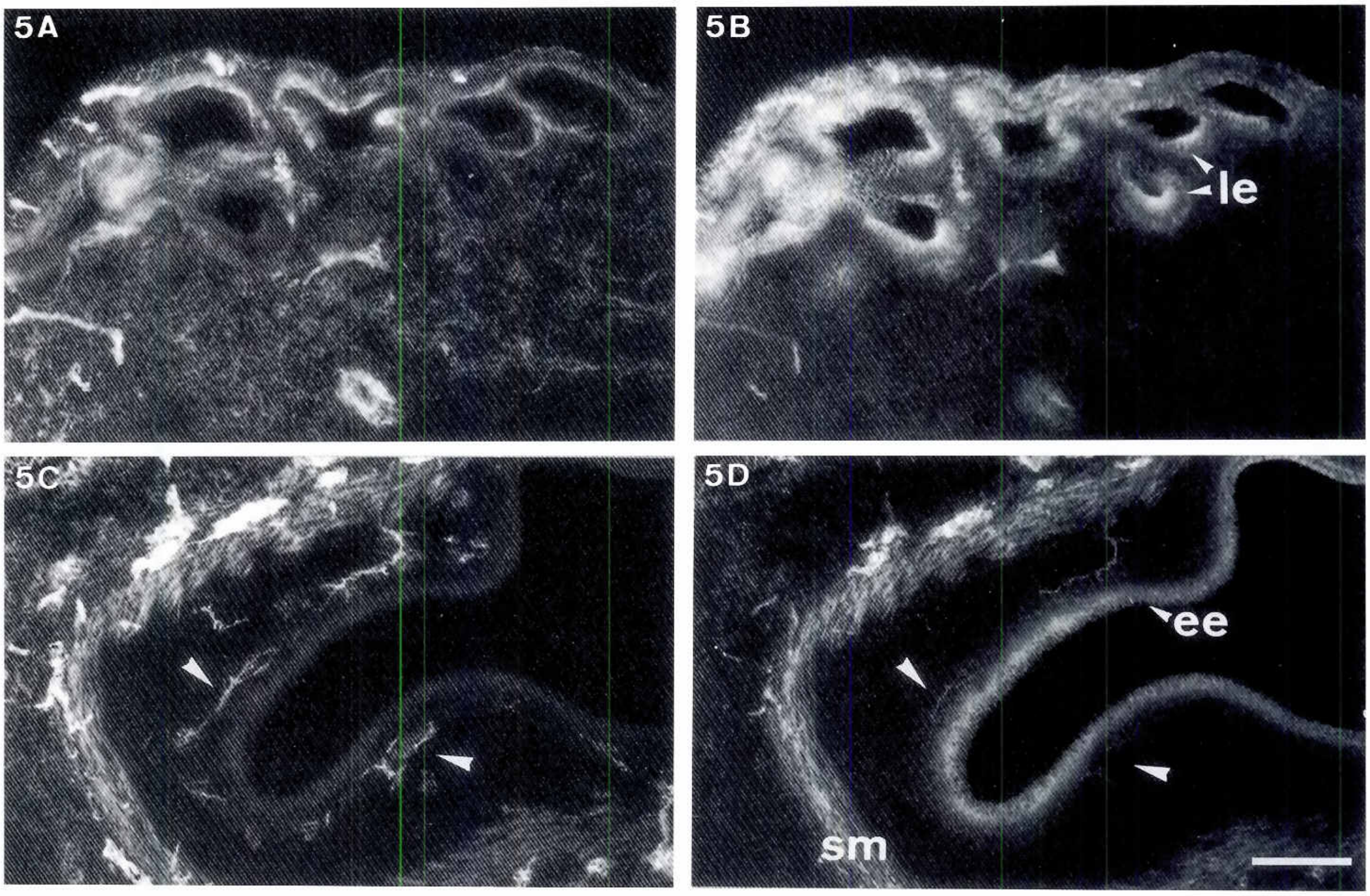

Fig. 5. Double-immunofluorescence staining for N-cadherin and αN-catenin in the lung (A,B) and esophagus (C,D) in an 8-day embryo. (A and C) *N-cadherin;* **(B and D)** *αN-catenin. Both lung (le) and esophageal (ee) epithelia express αN-catenin but not αN-cadherin. On the contrary, mesenchymal cells, and peripheral ganglia and nerves (indicated by arrowheads) express N-cadherin more strongly than αN-catenin in each tissue. Skeletal muscles (sm) express intensely both molecules. The mesothelium layer covering the lung also expresses these two molecules. Bar, 100 μm*

Materials and Methods

Animals

Fertilized eggs of White Leghorn chicken were obtained from a local farm and incubated at 37°C. Staging of the embryos was performed according to Hamburger and Hamilton (1951).

Immunohistochemistry

For immunohistochemistry, we basically followed the method of Hatta *et al.* (1987). Briefly, embryos were fixed with 3.5% paraformaldehyde in a Hepes-buffered balanced salt solution (HBSS, pH 7.4) for 1 to 4 h at 4°C. After incubation in 12-18% sucrose in HBSS for several hours, the samples were embedded in Tissue-Tek (Miles Inc., USA) and frozen in liquid nitrogen. Cryostat sections (15 μm thick) were made, mounted on slides coated with gelatin, and dried in air. The samples were treated with ethanol at -20°C for 30 min, followed by treatment with 5% skim milk (Difco) in 50 mM Tris-buffered saline (pH 7.6) containing 1 mM Ca^{2+} (TBS-Ca) for blocking of nonspecific binding of antibodies. For double-immunostaining for αN-catenin and N-cadherin, the samples were incubated successively in TBS-Ca, with washing at each interval with TBS-Ca, containing (1) a mixture of the anti-αN-catenin monoclonal antibody NCAT-2 (Hirano *et al.*, 1992) and a rabbit anti-N-cadherin serum (Hatta *et al.*, 1988), (2) biotinylated anti-rat Ig (Amersham), and (3) a mixture of fluorescein-streptavidin (Amersham) and rhodamine-conjugated anti-rabbit Ig (Cappel). After mounting with 90% glycerol-10% TBS-Ca containing 0.1% p-phenylenediamine, photographs were taken with a Zeiss Axiophot microscope. To evaluate specific signals, we included an appropriate negative control for each staining. The whole-mount staining was performed according to Fujimori *et al.* (1990) with a slight modification; *i.e.*, HBSS was used instead of the balanced saline for *Xenopus*.

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References

CLAVERIE, J.M., HARDELIN, J.P., LEGOUIS, R., LEVILLIERS, J., BOUGUELERET, L., MATTEI, M-G. and PETIT, C. (1993). Characterization and chromosomal assignment of a human cDNA encoding a protein related to the murine 102 kD cadherin-associated protein (α-catenin). *Genomics 15:* 13-20.

CROSSIN, K.L., CHUONG, C.M. and EDELMAN, G.M. (1985). Expression sequences of cell adhesion molecules. *Proc. Natl. Acad. Sci. USA 82:* 6492-6496.

DUBAND, J.L., VOLBERG, T., SABANAY, I., THIERY, J.P. and GEIGER, B. (1988).

Spatial and temporal distribution of the adherens-junction-associated adhesion molecule A-CAM during avian embryogenesis. *Development 103:* 325-344.

FUJIMORI, T., MIYATANI, S. and TAKEICHI, M. (1990). Ectopic expression of N-cadherin perturbs histogenesis in *Xenopus* embryos. *Development 110:* 97-104.

HAMBURGER, V. and HAMILTON, H. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol. 88:* 49-92.

HATTA, K. and TAKEICHI, M. (1986). Expression of N-cadherin adhesion molecule associated with early morphogenetic events in chick development. *Nature 320:* 447-449.

HATTA, K., NOSE, A., NAGAFUCHI, A. and TAKEICHI, M. (1988). Cloning and expression of cDNA encoding a neural calcium-dependent cell adhesion molecule: its identity in the cadherin gene family. *J. Cell Biol. 106:* 873-881.

HATTA, K., TAKAGI, S., FUJISAWA, H. and TAKEICHI, M. (1987). Spatial and temporal expression pattern of N-cadherin cell adhesion molecules correlated with morphogenetic processes of chicken embryos. *Dev. Biol. 120:* 215-227.

HERRENKNECHT, K., OZAWA, M., ECKERSKORN, C., LOTTSPEICH, F., LENTER, M. and KEMLER, R. (1991). The uvomorulin-anchorage protein α-catenin is a vinculin homologue. *Proc. Natl. Acad. Sci. USA 88:* 9156-9160.

HIRANO, S., KIMOTO, N., SHIMOYAMA, Y., HIROHASHI, S. and TAKEICHI, M. (1992) Identification of a neural α-catenin as a key regulator of cadherin function and multicellular organization. *Cell 70:* 293-301.

KNUDSEN, K.A. and WHEELOCK, M.J. (1992). Plakoglobin, or an 83-kD homologue distinct from α-catenin, interacts with E-cadherin and N-cadherin. *J. Cell Biol. 118:* 671-679.

McCREA, P.D., TURCK, C.W. and GUMBINER, B. (1991). A homolog of the armadillo protein in *Drosophila* (plakoglobin) associated with E-cadherin. *Science 254:* 1359-1361.

NAGAFUCHI, A. and TSUKITA, S. (1994). The loss of the expression of α catenin, the 102 kDa cadherin-associated protein, in central nervous tissues during development. *Dev. Growth Differ. 36:* 59-71.

NAGAFUCHI, A., TAKEICHI, M. and TSUKITA, S. (1991). The 102 kD cadherin-associated protein: similarity to vinculin and posttranscriptional regulation of expression. *Cell 65:* 849-857.

SHIMOYAMA, Y., NAGAFUCHI, A., FUJITA, S., GOTOH, M., TAKEICHI, M., TSUKITA, S. and HIROHASHI, S. (1992). Cadherin dysfunction in a human cancer cell line: possible involvement of loss of α-catenin expression in reduced cell-cell adhesiveness. *Cancer Res. 52:* 1-5.

THIERY, J.P., DELOUVEE, A., GALLIN, W.J., CUNNINGHAM, B.A. and EDELMAN, G.M. (1984). Ontogenic expression of cell adhesion molecules: L-CAM is found in epithelia derived from the three primary germ layers. *Dev. Biol. 102:* 61-78.

UCHIDA, N., SHIMAMURA, K., MIYATANI, S., COPELAND, N.G., GILBERT, D.J., JENKINS, N.A. and TAKEICHI, M. (1994). Mouse αN-catenin: two isoforms, specific expression in the nervous system, and chromosomal localization of the gene. *Dev. Biol.* (In press).