

Molecular Cloning and Characterization of B-Cadherin, a Novel Chick Cadherin

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Abstract. Calcium-dependent cell-cell adhesion is mediated in large part by a set of homologous integral membrane glycoproteins termed cadherins. In this report, antibodies to conserved domains in previously described cadherins have been used to isolate cDNAs encoding a novel chick cadherin. The deduced primary structure of this novel molecule, assigned the name B-cadherin, contains 726 amino acid residues which include five extracellular domains characteristic of this class of adhesion molecules, a single putative transmembrane spanning region, and a cytoplasmic tail. In each domain, B-cadherin shares extensive ho-

mologies with other cadherins, but is more closely related to E-cadherin, P-cadherin, and L-CAM than to N-cadherin. It is expressed in a wide variety of chick tissues at embryonic day 13. In particular, immunohistochemical staining and in situ hybridization localize B-cadherin protein and mRNA to the epithelial lining of the choroid plexus and to cells in specific layers of the optic tectum in chick brain. Levels of the protein and RNA transcript change dramatically as development proceeds in chick brain. These results suggest that B-cadherin has important functions in neurogenesis, in at least some epithelia, and in embryogenesis.

ORGANIZATION of individual cells into distinct, well-defined tissues and organs involves a complex, coordinated series of developmental events. Cell-cell interactions are of paramount importance in the processes of aggregation, segregation, and migration which underlie these morphogenetic changes. It is well established that calcium-dependent cell adhesion systems are major components of cell sorting mechanisms. Cadherins are a conserved family of glycoproteins which are major mediators of calcium-dependent cell-cell adhesion (Takeichi, 1990). They are expressed on the cell surface and are present in specialized structures of cell-cell contact, such as the adherens-type junction in cardiac cells and intermediate junction of intestinal epithelia, where they associate with elements of the actin filament network (Gumbiner and Simons, 1986; Hirano et al., 1987; Ozawa et al., 1989, 1990; Kemler and Ozawa, 1989; Geiger, 1989; Nelson et al., 1990). During embryogenesis, individual cadherins have been shown to be required for compaction of blastomeres and somitomeres, the formation of tight junctions between epithelial cells, the segregation into layers of the diverse cell populations of the neuroretina, myoblast fusion, and axon outgrowth on surfaces of cells expressing these molecules (Hyafil et al., 1980; Boller et al., 1985; Behrens et al., 1985; Duband et al., 1987; Bixby et al., 1987; Matsunaga et al., 1988; Neugebauer et al., 1988; Tomaselli et al., 1988; Letourneau et al., 1990; Pouliot et al., 1990).

Despite their similar biochemical properties, each cadherin manifests a different spatiotemporal pattern of expression and cell-binding specificity. E-cadherin, also known as uvomorulin, is prominently expressed in a diversity of epithelial tissues and in subsets of neurons (Takeichi et al., 1990). P-cadherin, which was originally identified in mouse placenta, exhibits a widespread distribution in many other tissues, while N-cadherin is abundantly expressed in the nervous system, skeletal and cardiac muscle (Nose and Takeichi, 1986; Vestweber et al., 1987; Hatta et al., 1987; Hirai et al., 1989; Takeichi, 1990). Additional cadherins include L-CAM, identified in chick liver, prominently expressed in epithelia (Gallin et al., 1987) and proposed to be the avian homologue of E-cadherin (Miyatani et al., 1989); R-cadherin, a recently described adhesion molecule in neuroretina which is closely related to N-cadherin (Takeichi et al., 1990); and E/P-cadherin, a new protein identified in *Xenopus* oocytes (Ginsberg et al., 1991).

The molecular basis of cadherin-mediated adhesion appears to involve a homophilic binding mechanism whereby each cadherin binds to the same cadherin on adjacent cells. Expression of cadherins with distinct binding specificities in transfected cell lines results in efficient sorting of the different cell populations in cell aggregation assays (Chuong and Edelman, 1985a, b; Edelman et al., 1987; Nose et al., 1988; Hatta et al., 1988; Nose et al., 1987; Nagafuchi et al., 1987; Jaffe et al., 1990). In some circumstances, however, indi-

vidual cadherins also appear to engage in heterophilic associations where they bind weakly to other members of the cadherin family (Volk et al., 1987). Whereas E-, P-, and N-cadherin have each been shown to interact homophilically, R-cadherin has recently been demonstrated to bind N-cadherin and itself (Takeichi et al., 1990).

In the developing nervous system, N-cadherin is a major regulator of calcium-dependent cell adhesion. Inhibition of N-cadherin function with specific antibodies has been shown to disrupt formation of the neural tube, histogenesis of the neuroretina, and axon outgrowth on astroglia, Schwann cells, and skeletal myotubes (Hatta and Takeichi, 1986; Bixby et al., 1987; Matsunaga et al., 1988; Neugebauer et al., 1988; Tomaselli et al., 1988; Detrick et al., 1990). Despite the dramatic effects of such antibodies, they do not completely abolish calcium-dependent cell aggregation, suggesting that additional cadherins exist in neural tissues (Matsunaga et al., 1988).

To identify new members of the cadherin family expressed in brain, we screened for molecules that cross-react antigenically with described cadherins by using antibodies to conserved sequences in their cytoplasmic domains. In the present report, we describe the cloning, primary sequence, and localization of a novel cadherin, subsequently named B-cadherin since the cDNA encoding this protein was identified in a λ gt11 embryonic chick brain library. The primary sequence of each domain of B-cadherin has high homology to the same domains in previously characterized cadherins. In brain, B-cadherin is localized in discrete layers of the optic tectum as well as the cuboidal epithelium of the choroid plexus. Outside the nervous system, B-cadherin is detected in a wide variety of tissues. The expression pattern of this protein suggests that it has diverse functions in embryogenesis.

Materials and Methods

Reagents and Solutions

Restriction enzymes, T₄ polynucleotide kinase, reverse transcriptase, and Klenow fragment of DNA polymerase were from Boehringer Mannheim Diagnostics (Houston, TX). *Thermus aquaticus* (Taq) polymerase used in polymerase chain reactions was purchased from Perkin-Elmer Cetus Corp. (Emeryville, CA). Exonuclease III, pGEM plasmid vectors, SP6 and T7 polymerases were obtained from Promega Biotec (Madison, WI) and used according to the manufacturer's suggested protocols. Radioactive nucleotides ($[\alpha\text{-}^{35}\text{S}]$ dATP, $[\gamma\text{-}^{32}\text{P}]$ ATP, $[\alpha\text{-}^{32}\text{P}]$ dCTP) were supplied by Amersham Chemical Co. (Arlington Heights, IL). DNA sequencing kits were acquired from U.S. Biochemical Corp. (Cleveland, OH). Oligonucleotide primers and synthetic peptides were provided by facilities in the Howard Hughes Medical Institute at University of California at San Francisco. Protein A-Sepharose CL4B was procured from Pharmacia Fine Chemicals (Piscataway, NJ). RNA molecular weight standards were from Bethesda Research Laboratories, Inc. (Gaithersburg, MD). Vectastain ABC kits for immunohistochemical staining were from Vector Laboratories (Burlingame, CA). All other reagents, enzymes and chemicals not specified above were purchased from Sigma Chemical Co. (St. Louis, MO).

Blocking solution ("Blotto") for immunoblotting is 5% dry milk in PBS. Denhardt's solution (1 \times) is 0.02% Ficoll Type 400, 0.02% polyvinylpyrrolidone, and 0.02% BSA. SSC (1 \times) is 150 mM sodium chloride and 15 mM sodium citrate (pH 7.0). RNA hybridization buffer is 50% formamide, 5 \times SSC, 5 \times Denhardt's solution, 0.1% SDS, and 200 μ g/ml salmon sperm DNA.

Peptides and Antibodies

Peptides were synthesized at the Howard Hughes Medical Institute at Uni-

versity of California at San Francisco. The 27-residue synthetic peptide QDY-DLNEWGNRFKLAELYGGEDDE, designated CADCYTO-2, is derived from the deduced amino acid sequence of the carboxy terminus of L-CAM (Gallin et al., 1987). CADCYTO-1 is a 25-residue peptide derived from the deduced amino acid sequence of L-CAM at position 658-682 whose sequence is ENLKAADTDPTAPPYDSLVLVFDYEG. B-EC5 is a 23-residue peptide with the sequence TEPLEQNLVSYVYLRFLDRQKQDQ, and is derived from the deduced amino acid sequence of B-cadherin (residues 500-522) which is located in ectodomain 5 (EC5) of the peptide backbone of the cadherin family (Takeichi, 1990). A terminal cysteine was appended to each peptide to facilitate coupling to keyhole limpet hemocyanin (KLH) as a carrier for immunization. Briefly, the crosslinker N-hydroxysuccinimide ester (MBS) was mixed with 5 mg KLH for 30 min at room temperature. After this coupling reaction, 5 mg of peptide was added to KLH-MBS and conjugation of peptide to carrier allowed to proceed for 3 h at room temperature. The antigen was sent to Caltag Laboratories (South San Francisco, CA) for immunization of rabbits by standardized methods.

Affinity purification of antibodies on the peptide conjugated to Thiol-Sepharose 4B (Pharmacia Fine Chemicals) and isolation of IgG fractions on protein A-Sepharose CL4B (Pharmacia Fine Chemicals) were carried out according to the manufacturer's instructions.

The rat mAb, NCD-2, which is specific for chicken N-cadherin, was the generous gift of Dr. Masatoshi Takeichi (Kyoto University, Kyoto, Japan).

Immunoscreening of cDNA Library

An embryonic day 13 chick brain λ gt11 cDNA library was kindly provided by Dr. Barbara Ranscht (La Jolla Cancer Research Institute, La Jolla, CA). Immunoscreening of the library with the α CADCYTO-2 antibody was carried out according to established procedures (Young and Davis, 1983). Primary positive phages were subsequently plaque purified and amplified. cDNAs from these clones were restricted and subcloned into double-strand plasmid and single-strand M13 sequencing vectors for further analysis.

DNA Sequencing

Two independent overlapping cDNA clones inserted into single-strand M13 vectors were sequenced on both strands by the dideoxy chain termination method (Sanger et al., 1977) using exonuclease III digestion for the generation of unidirectional deletions and specific oligonucleotide primers for extension on undigested clones.

Nucleic Acid and Amino Acid Analysis

The PCGENE series of programs (Intelligenetics Corp., Mountain View, CA) was used for nucleic acid and amino acid sequence analysis. Hydrophobicity plots were calculated according to the method of Kyte and Doolittle (1982). Multiple sequence alignments were derived from the series of programs designed by Sobel and Martinez (1985).

RNA Analysis

Total cellular RNA was isolated from developmentally staged chick embryos according to Chompczynski and Sacchi (1987). The RNA was electrophoresed on a denaturing agarose-formaldehyde gel, transferred to a Hybond N nylon membrane, and probed with a full-length B-cadherin cDNA insert (Maniatis et al., 1989). Hybridization probes were generated by random priming (Feinberg and Vogelstein, 1984) using hexanucleotides supplied by Pharmacia Fine Chemicals. Final blot wash conditions were: 0.5 \times SSC, 0.1% SDS at 65°C.

Membrane Preparations and Western Blotting

Various tissues (e.g., brain, liver, heart, eye, skin, intestine, bladder, kidney, muscle, and retina) were dissected and homogenized in 4 vol of ice-cold homogenization buffer (10 mM HEPES, pH 7.4., 0.32 M sucrose, 1 mM PMSF, 2 mM EDTA, 2 mM NEM, 1 mg/ml pepstatin, 1 mg/ml leupeptin) and centrifuged at 2,000 g for 10 min at 4°C. The supernatants from this spin were then sedimented at 16,000 g for 35 min at 4°C. The resultant pellets were subsequently resuspended in SDS sample buffer and sonicated to solubilize all protein. 100 μ g of protein per lane was electrophoresed on 7.5% acrylamide gels and subjected to antigen blotting using standard methods (Towbin et al., 1979). All antibodies were diluted in Blotto. Affinity-purified α CADCYTO-2 and the monoclonal NCD-2 were used at a 1:100 dilution; α B-EC5 serum was diluted 1:1,500. All blots were scanned

GATTGGGTGATCCCTCCCATTAAGTTCCCGAATGAGAGGGTCCCTTCCCAAAAACCTGGTTCAGATCAAATCCAACCGG 84
D W V I P P I K V P E N E R G P F P K N L V Q I K S N R 20

GACCGGGAGGCAAGATTTTACAGCATACCGGGCAGGGGGCGGATGCTCCCCCGAGGGCATCTTACCATTGAGAAGGAGACAGGCTGG 177
D R E A K I F Y S I T G Q G A D A P P E G I F T I E K E T G W 59

ATGAAGTGACGCAGCGCTGGACCGGGAGCACATCAACAAGTACCACCTTACTCCCATGCCGTGTCGAGAAATGGCAAACCGTGGAGGAG 270
M K V T Q P L D R E H I N K Y H L Y S H A V S E N G K P V E E 90

CCGATGGAGATCATAGTGACGGTGACGGACAAAATGACAACAAGCCCGAGTTCACACAGGAGGTGTTCCGGGGCTCCGTGCCTGAAGCGCGG 363
P M E I I V T V T D Q N D N K P Q F T Q E V F R G S V P E G A 121

CTGCCAGGCACTTCGGTGTGCGGGTGAACGCCACGGACCGCTGATGATGATGTTGGAGACCTACAACGGTGTATCGCTACTCCATCCTCAGC 456
L P G T S V M R V N A T D A D D D V E T Y N G V I A Y S I L S 152

CAGGAGCCGCGGGAGCCCCATCCCCACATGTTACCGTCAACCGGCCACCGGCCACCTCAGCGTATCGCCAGCGGCTCGACCGGGAGCGC 549
Q E P R E P H P H M F T V N R A T G T L S V I A S G L D R E R 183

GTGCGGAGTACACGCTGACCATGCACGGCGGTGACCTGGACGGGCGGGCTGACCACAACGGCGCTGGCTGTATTGAGATCACGGACGTC 642
V R E Y T L T M Q A A D L D G Q G L T T T A L A V I E I T D V 214

AATGACAACGCCCTGAGTTTACCCCAAAACGTATGAGGGCGGCTGTCCAGAGAATGAGGCCGAGCTGGAGTGGCCCGGTTGGCCACCACG 735
N D N A P E F D P K T Y E A A V P E N E A E L E V A R L A T T 245

GACCTGGATGAGCGCACACACCGCTGGCGTCCGCTACTCCATCGTGCCTGGCAATGAGGGCGGTGCCTTACCATCACCACTGACCCT 828
D L D E P H T P A W R A V Y S I V R G N E G G A F T I T T D P 276

GCCAGCAACGAGGGCGTCTGCGCACGGCAAGGGTCTGGACTACGAGGCCAAGCGGAGTTCGTGCTCCACGTGGCCGTTGTAATGAAGCT 921
A S N E G V L R T A K G L D Y E A K R Q F V L H V A V V N E A 307

CCCTTCGCCATCAAGCTGCCACAGCCACGGCCACGGTGTGGTGTGAGGATGTAATGAGGCCCGCTTTCGACCCGCGCTGCGG 1014
P F A I K L P T A T A T V M V S V E D V N E A P V F D P P L R 338

CTGGCCAGGTGCCGGAGGAGCTGCCGCTGGGGCAGCCCCCTCGCCTCCTATACAGCACAGGACCTGACAGGGCCAGCAGCAGCGCATCAAG 1107
L A Q V P E D V P L G Q P L A S Y T A Q D P D R A Q Q Q R I K 369

TACGTGATGGCAGTGACCCCGGGGCTGGCTGGCCGTGACCCCGAGAACGGCTCATCACAGCACGGGAGCAGCTGGACCGTGTGCTCCCG 1200
Y V M G S D P A G W L A V H P E N G L I T A R E Q L D R E S P 400

TTACCAAGAACAGCACCTACGTGGCGTGTCTGGTGTGGATGACGGATTGCCACCCGCCACAGGCACCGGCACGCTGCTCCTCACCCCTG 1293
F T K N S T Y V A V L L A V D D G L P P A T G T G T L L L T L 431

CTGGATGTCATGACCCAGCCCTGAGCCGGAGCCCCCGACATCGTCTGTAAACCGCAGCCCTGTGCCCAAGTGTGACCATCACCGAC 1386
L D V N D H G P E P E P R D I V I C N R S P V P Q V L T I T D 462

CGGGACCTGCCCCCAACACCGGGCCCTTCGCTGCCGAGCTGAGCCACGGCTCTGGGGACAGCTGGCGGTGGAGTTGGCAATGGAGGTGAC 1479
R D L P P N T G P F R A E L S H G S G D S W A V E V G N G G D 493

ACCGTTGCCTTGTGGCTGACGGAGCCCTGGAGCAGAACCTGTACAGCTCTACTCGGGCTCTTCGACCCGACGGGCAAGGACCAGGTACCC 1572
T V A L W L T E P L E Q N L Y S V Y L R L F D R Q G K D Q V T 524

GTTCATCAGGGCACAGGTGTGCGACTGCCAGGGCCGCTGGAGAGCTGTGCCAGAACCCAGCGTGGATACGGGTGTGCCATCGTGTGGCC 1665
V I R A Q V C D C Q G R V E S C A Q K P R V D T G V P I V L A 555

GTGCTGGTGTGTGCTGGCCCTG 1758
V L G A V L A L L L V L L L L L L L L V R R R K V V K E P L L L 586

CCTGAGGACGACAGGAGCAACATCTTCTACTATGGGAGGAAGTGGGGTGGAGGAGGACAGGACTACGACCTGAGCCAGCTGCACCCG 1851
P E D D T R D N I F Y Y G E E G G G E E D Q D Y D L S Q L H R 617

GGCCTGGACGCCCGCCGAGGTGATCCGCAATGATGTGGCCCCCGCTGATGGCCGCCCCAGTACCGGCCCGCCCGCAACCCCGAT 1944
G L D A R P E V I R N D V A P P L M A A P Q Y R P R P A N P D 648

GAGATCGGGAACCTCATTGACGAGAACCTGAAGGACAGCTGACACGGACCCCGCCCGCCCTACGACTCGTGTGTTGACTACGAG 2037
E I G N F I D E N L K A A D T D P T A P P Y D S L L V F D Y E 679

GGCGGGCTCGGAGGCCACCTCGCTCAGTCCCTCAACTCCTCCGCTCGGACAGGACAGGACTACGACTACCTCAACGAGTGGGCAAC 2130
G G G S E A T S L S S L N S S A S D Q D Q D Y D Y L N E W G N 710

CGCTTCAAGAAGCTGGCGGAGCTCTATGGCGGGGGAGGATGAAGAATAGCCCCCAGTGTGCTGCTGCTGCCAAAGCCTTACCCTGCCCCG 2223
R F K K L A E L Y G G G E D E E 726

GCCTTGGGGCCGCTGCCCTCCTGCACTCCCGAGCTCCCGAAGCAGCCTTGGCTTCTCCTGTGTGTTCAACTTTTCTTTTTTTTT 2326

ATTAAGAAACATGAATTTGAAGTGTGGATGGGACCGCTGGGCTGAGCTGGGTCTGTAGAAGTTTAAAGGAGTTTAAATAAGGATGTG 2419

GCTTCCAAAAAATAAAAAAAAAAAAAA 2446

Figure 1. Nucleotide and deduced amino acid sequence of chicken B-cadherin cDNA clone. The putative transmembrane domain is demarcated by the thick solid underline and the asterisks denote potential N-linked glycosylation sites (N_xT/S). Dashed line designates synthetic peptide B-EC5 which was used for generation of antisera. Poly(A) addition signal is indicated by a thin underline. These sequence data are available from EMBL/GenBank/DBJ under accession number 58518.

with a videodensitometer (model 620; Bio-Rad Laboratories, Cambridge, MA).

Immunohistochemical Staining

Polyester wax embedding and sectioning of embryonic day 13 chick brain tissue were performed according to Steedman et al. (1957) as modified by Sheppard et al. (1988). 10- μ m sagittal sections were blocked for 1 h at room temperature in Blotto and then incubated with 20 μ g/ml IgG of primary antibody diluted in Blotto overnight at 4°C. Samples were washed five times for 5 min with PBS, incubated successively with Vectastain ABC reagents (Vector Laboratories, Inc., Burlingame, CA), and developed to visualize HRP-catalyzed reaction product according to the supplier.

In Situ Hybridization

In situ hybridizations on paraffin-embedded sections of E13 chick brain were conducted according to the protocol of Angerer et al. (1985) as modified by Heuer et al. (1990). pGEM-3 plasmid constructs containing the entire coding region of the mature B-cadherin protein (pBCAD-1) were used to generate radiolabeled T7 and SP6 RNA transcripts which serve as sense and antisense probes. Final hybridization wash conditions were: 0.1 \times SSC at 65°C for 15 min followed by a 30-min rinse in 0.1 \times SSC at room temperature.

Results

Molecular Cloning of Chicken B-cadherin

α CADCYTO-2 is a polyclonal antibody raised to a COOH-terminal peptide of L-CAM that is highly conserved within the cadherin gene family (Takeichi, 1990). In preliminary experiments (see Fig. 3 a), this antibody was shown to react with several proteins of M_r between 120k and 130k, which were thus potential cadherin homologues. It did not, however, bind to any protein with the same M_r as N-cadherin. Moreover, the antibody did not bind in immunoblots to N-cadherin purified by immunoprecipitation with the N-cadherin-specific NCD-2 mAb (not shown). As it did not recognize the major identified cadherin in nervous tissue, it was a suitable reagent for the screening of a cDNA expression library to identify novel cadherins in developing chick brain. It was therefore used to screen an expression library prepared from embryonic chick brain poly A⁺ RNA. In 3 \times 10⁵ recombinants screened, several immunopositive clones were detected. These clones were then tested for reaction with a second antiserum, α CADCYTO-1, which recognizes another conserved COOH-terminal peptide common to all known cadherins (see Materials and Methods). Three clones that reacted with both antibodies were purified and characterized. Nucleotide sequencing of the largest of these cDNAs (2,446 bp) revealed a long open reading frame of 2,278 bp predicted to encode a mature 726 amino acid polypeptide (Fig. 1). The 168 bp of 3-untranslated sequence following the coding region contains a polyadenylation site (AATAAA) at nucleotide 2,405 which precedes the poly(A) tail beginning at nucleotide 2,427.

Since no initiation codon (ATG) or putative signal sequence was found in this truncated cDNA, these sequences must be contained within the additional 500 bases at the 5' end of the single mature mRNA (see Fig. 3 a). Other cadherins have been shown to have both signal peptides and poorly conserved prohormone-like sequences that are processed during cadherin maturation (Takeichi, 1990). These sequences are expected to be in the 5' end of the mRNA that is not present in the cDNAs described in this paper.

The first seven residues of the deduced amino acid sequence of B-cadherin correspond well with the NH₂-terminal consensus sequence of mature cadherins as they appear at the cell surface (Shirayoshi et al., 1986), therefore, it is likely that this cDNA encodes the mature, processed form of B-cadherin. A single transmembrane domain consisting of 26 amino acids starting at position 548 (Fig. 1, *thick underline*) was predicted by hydrophobicity analysis (Kyte and Doolittle, 1982). Notably, the five internally repeated extracellular sequences and putative Ca²⁺-binding sites characteristic of cadherins (Ringwald et al., 1987), are also conserved in B-cadherin (see below). The molecular mass of the 726 amino acid protein was calculated to be 79,742 D. Three potential N-linked glycosylation sites (N-x-T/S) are located along the polypeptide backbone of the extracellular domain (Fig. 1, *asterisks*). Even after accounting for these, though, the predicted molecular mass is considerably smaller than that estimated by SDS-PAGE (see Fig. 3). Anomalous migration of cadherins on acrylamide gels appears to be a common property of this class of proteins (Gallin et al., 1987; Hatta et al., 1988).

Homology with Other Cadherins

Comparison of the primary structure of B-cadherin with the deduced amino acid sequences of other cloned cadherins reveals a marked similarity along the entire length of the polypeptide chain (Fig. 2). The alignment scores in Table I reflect the degree of homology between various members of the cadherin family. B-cadherin bears a similarly strong resemblance to chicken L-CAM, mouse and human P-cadherin, and mouse E-cadherin, with which it shares 65, 65, 64, and 64% homology, respectively. Although both are derived from the same species, B-cadherin is less similar to N-cadherin, with the extent of homology between them calculated to be 44%. When conserved amino acid substitutions (i.e., A,S,T; D,E; N,Q; R,K; I,L,M,V; or F,Y,W) are considered in the determination of alignment scores, the degree of homology between each cadherin and other members increases on average between 10 and 12%.

Three contiguous NH₂-terminal stretches of 112 amino acids constituting the internally repeated extracellular domains EC1, EC2, and EC3, as well as the putative Ca²⁺-binding motifs imbedded within these segments (Ringwald et al., 1987), are all conserved in B-cadherin. Extracellular domain 5 (EC5), which resides just proximal to the transmembrane region, is least conserved between the cadherins. Consequently, a nonhomologous, hydrophilic peptide (B-EC5) from this location was chosen for use as an immunogen to produce B-cadherin-specific antibodies that would not cross react with other cadherins (described below).

The conserved tripeptide sequence HAV, believed to be a cell adhesion recognition sequence (Blaschuk et al., 1990), is also present in B-cadherin at position 78–80 in EC1 (Figs. 1 and 2). Amino acid residues flanking this conserved triplet have been determined to be of particular importance in influencing binding specificities of the cadherins (Nose et al., 1990). In this regard, it is interesting that the amino acids at these positions in B-cadherin suggest that it may have an E- and P-cadherin-like hybrid character. Possible implications of this will be addressed in the Discussion.

The cytoplasmic domains of cadherins, which have been

Table I. Summary of Scoring Matrix

	1	2	3	4	5
huP	—				
mP	622	—			
cB	467	469	—		
mE	416	416	466	—	
cL	407	400	471	470	—
cN	297	292	320	324	329

Homology between B-cadherin and other cadherins. Alignment scores are calculated by the formula: $M - (G \times L)$ where $M = n$ of identical matches, $G = n$ of gaps, and $L =$ length of gap. The maximum score for a protein perfectly homologous to B-cadherin is 726. Alignment scores for B-cadherin against the other members of the family are in boldface type.

shown to interact directly with a group of putative cytoskeletal-associated proteins dubbed catenins (Kemler and Ozawa, 1989), are the most highly conserved portions of these molecules. Strict conservation of primary sequence in this region

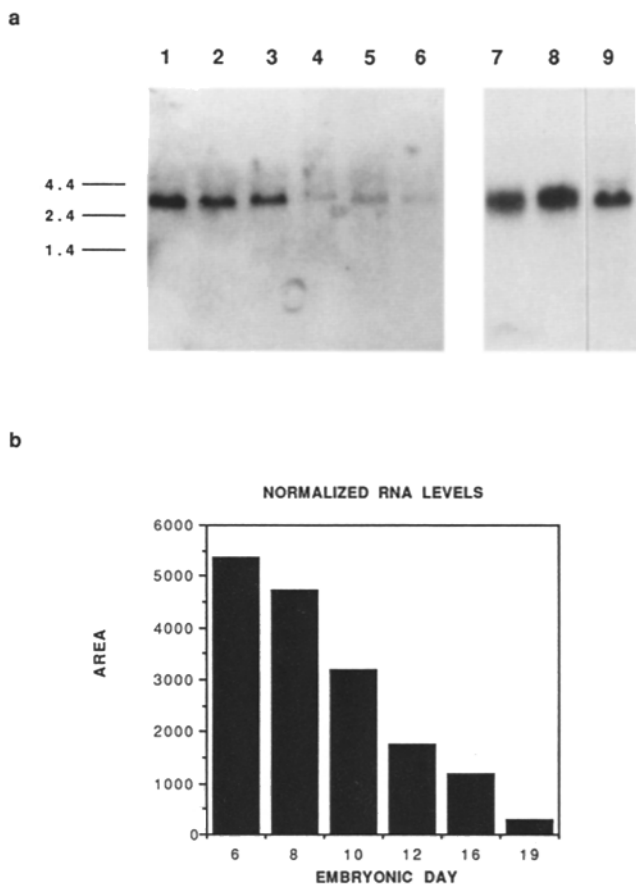


Figure 3. RNA blot analysis using B-cadherin cDNA as a probe. (a) Total RNA (10 µg/lane) isolated from whole chick brain at various stages of development was probed with a 2.4-kb full-length cDNA. (lane 1) Embryonic day 6 (E6); (lane 2) E8; (lane 3) E10; (lane 4) E12; (lane 5) E16; (lane 6) E19; (lane 7) E8 chick eye; (lane 8) E8 chick heart; and (lane 9) E8 chick liver. Note that the exposure time of lanes 7-9 is longer than that for lanes 1-6 to reveal the faint band at 4,000 nt in lane 9. Thus, the intensity of the bands in these lanes (7-9) cannot be directly compared to that observed in lanes 1-6. (b) Normalized RNA levels obtained from densitometric tracings of autoradiograms (lanes 1-6) and methylene blue-stained filters.

is also seen when examining B-cadherin, where no unique stretches of divergent residues are discernible.

RNA Blot Analysis

Expression of B-cadherin mRNA was examined in developing chick brains. Results in Fig. 3 *a* show that at all time points investigated, a single mRNA species of ~3,000 bases is detected. The level of this transcript changes dramatically during development. Densitometric scanning of the autoradiogram and normalization of the data to reflect equal amounts of RNA per lane (Fig. 3 *b*) reveals a progressive, ~14-fold diminution of message levels from E6 to E19. mRNAs estimated to be 3,000 nt in length were also observed in E8 heart, eye, and liver (Fig. 3 *a*, lanes 7-9, respectively). A second, fainter band at ~4,000 nt could also be detected in liver (Fig. 3 *a*, lane 9).

Protein Analysis

Membrane proteins prepared from staged chick brains (as above) were subjected to immunoblot analysis with a B-cadherin-specific peptide antibody (Fig. 4). To develop a monospecific antibody against B-cadherin, B-EC5 (see Materials and Methods), a hydrophilic peptide located in the least conserved region of the molecule, extracellular domain 5 (EC5), was selected for synthesis as an immunogen and subsequent injection into rabbits. This peptide is most divergent from chicken N-cadherin with which it shares 5 of 23 residues. It is most similar to mouse E-cadherin where 10 of 23 residues are identical. Only four amino acids in this region are common to all cadherins. The resultant B-cadherin-specific anti-peptide antibody (see Materials and Methods), α B-EC5, recognizes a single polypeptide of 120 kD in stages E6, E8, and E10 (Fig. 4 *a*, lanes 1-3, respectively) whereas, upon close inspection, a tight doublet at 120-122 kD emerges in lanes 4-6 (Fig. 4 *a*) corresponding to stages E12, E16, and E19. The upper band may represent a precursor or posttranslationally modified form of the faster migrating species since the RNA blot analysis suggests that B-cadherin is translated from one mRNA species (Fig. 3 *a*). However, the possibility that the epitope(s) recognized by this antiserum resides in a second, developmentally regulated protein of similar molecular mass cannot be excluded.

Expression of the α B-EC5-antigen exhibits a gradual elevation from E6 to E16. When blots were scanned with a densitometer, approximately fivefold more reaction product was seen at E16 compared to E6. Following maximal expression at the E16 plateau, the level of B-cadherin at E19 (Fig. 4 *a*, lane 6) declines to that observed at E8 (Fig. 4 *a*, lane 2).

B-cadherin also appears as a single 120-kD product in a diversity of nonneural tissues and is, therefore, not exclusively expressed in the nervous system (Fig. 5). In addition to brain (lane 1), expression of B-cadherin in E13 embryos was also detected in liver (lane 2), whole eye (lane 3), heart (lane 4), intestine (lane 6), bladder (lane 7), skeletal muscle (lane 8), skin (lane 9), and retina (lane 10). While the α B-EC5 antibody recognizes a tight doublet (described above) in brain and eye (Fig. 5, lanes 1 and 3, respectively), no observable signal was detected in kidney (Fig. 5, lane 5), the only nonreactive tissue of those tested.

Affinity-purified α CADCYTO-2, which is directed against a highly conserved COOH-terminal peptide present in all

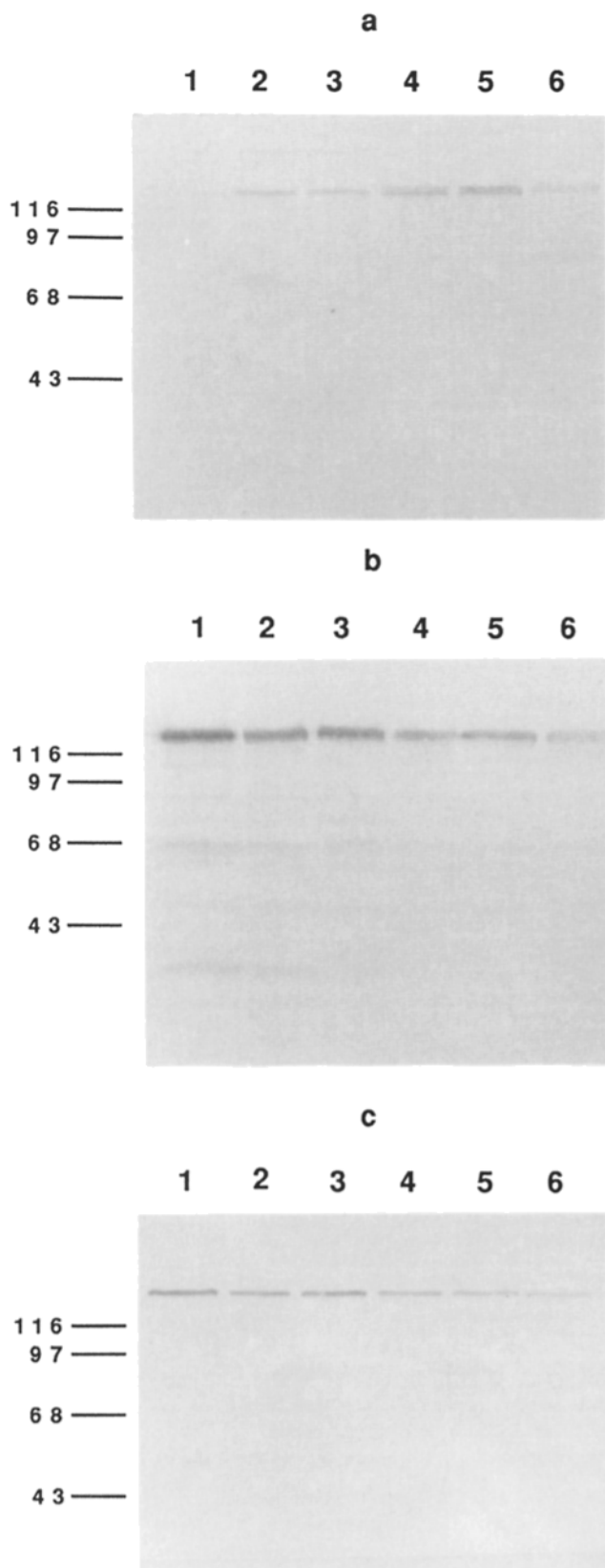


Figure 4. Immunoblot analysis of membrane proteins recognized by cadherin-specific antibodies. 100 μg /lane of crude membrane proteins isolated from staged embryonic chick brains were blotted with three different anticadherin antibodies. Loading patterns are identical for gels in *a-c*. (lane 1) E6; (lane 2) E8; (lane 3) E10; (lane 4) E12; (lane 5) E16; and (lane 6) E19. (*a*) α B-EC5; (*b*) α CADCYTO-2; (*c*) NCD-2 (anti-N-cadherin mAb). Molecular mass markers are in kilodaltons.

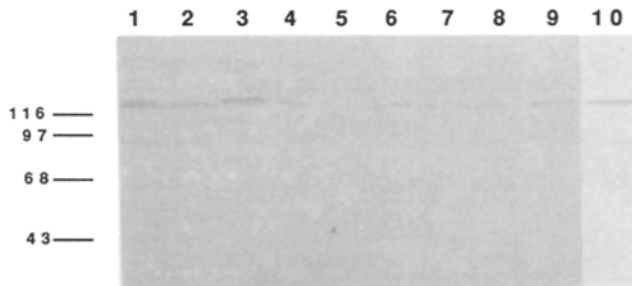


Figure 5. Distribution of B-cadherin in various chick tissues at embryonic day 13 as determined by immunoblotting with the B-cadherin-specific antibody, α EC-5. 100 μg of crude membrane proteins were loaded in each lane. (lane 1) Brain; (lane 2) liver; (lane 3) whole eye; (lane 4) heart; (lane 5) kidney; (lane 6) intestine; (lane 7) bladder; (lane 8) skeletal muscle; (lane 9) skin; and (lane 10) retina.

cadherins characterized to date, stains a prominent band at 120 kD in all ages tested (Fig. 4 *b*). This band, when scanned by densitometry, appears to progressively decrease in intensity by approximately sixfold in the developmental window chosen here. It most likely includes at least two, and possibly more, polypeptide antigens (see below).

N-cadherin expression in developing chick brain was examined with the NCD-2 mAb (Fig. 4 *c*). In contrast to the phasic wave of B-cadherin expression, levels of N-cadherin follow a pattern similar to that of the α CADCYTO-2 set of antigens (Fig. 4 *b*), displaying a steady fivefold reduction with increasing age. This profile has also been documented for the chick retina, where N-cadherin becomes highly restricted in distribution and limited in abundance during maturation (Matsunaga et al., 1988).

Immunohistochemical Staining

α B-EC5 was employed to delineate the distribution of B-cadherin in chick optic tectum at a time (E13) when robust expression of the antigen has been observed (see Fig. 4 *a*). At this stage of ontogeny, cell proliferation has ceased in the neuroepithelium and retinal fibers have interdigitated throughout all parts of the tectum (LaVail and Cowan, 1971). In addition, the 12 distinct layers forming the adult tectum can be identified and the only significant changes that occur after this period involve growth and differentiation of cells and their processes.

HRP staining with α B-EC5 has localized the antigen primarily to three cell-dense layers of the tectum: layers vi and viii (Fig. 6 *a*) and the ependyma (Fig. 6 *c*) as designated by LaVail and Cowan (1971). Layer vi is comprised of densely packed, small and medium-sized piriform cells, whereas layer vii, the most superficial cell-rich stratum, is characterized by its uniform population of small radially arranged cells. What was once a proliferative ventricular zone giving rise to cells in all superficial layers of the tectum has become, by E13, a definitive ependymal layer of columnar epithelial cells lining the ventricle. This epithelial layer is heavily labeled by the antibody (Fig. 6 *c*, EP). Also stained by the antibody are small numbers of cells located between the aforementioned layers (Fig. 6, *a* and *e*, arrows). These may represent postmitotic neuroblasts or glioblasts that are migrating from their birthplace in the ventricular zone to their final destinations in the outer layers of the tectum. Fu-

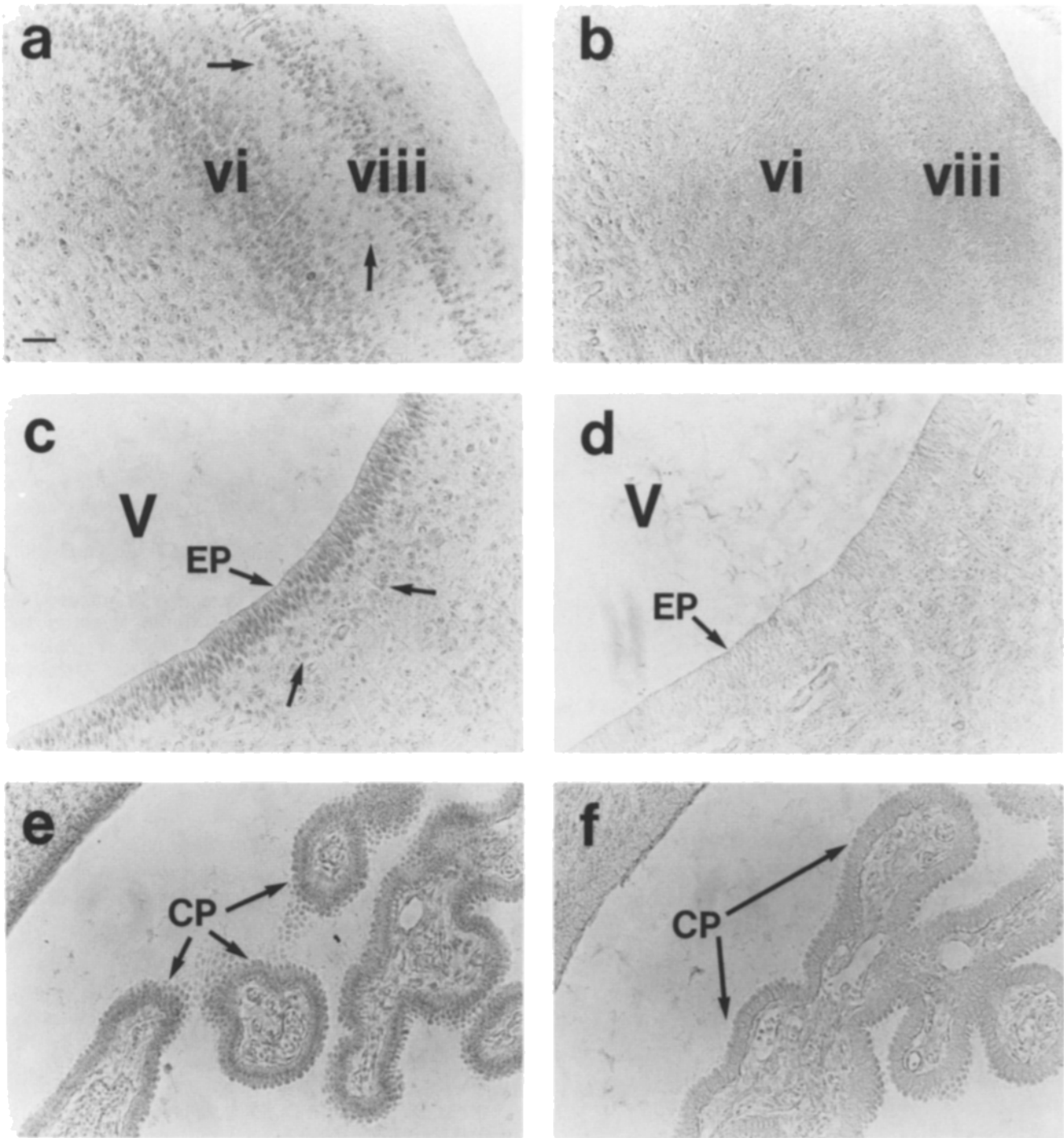
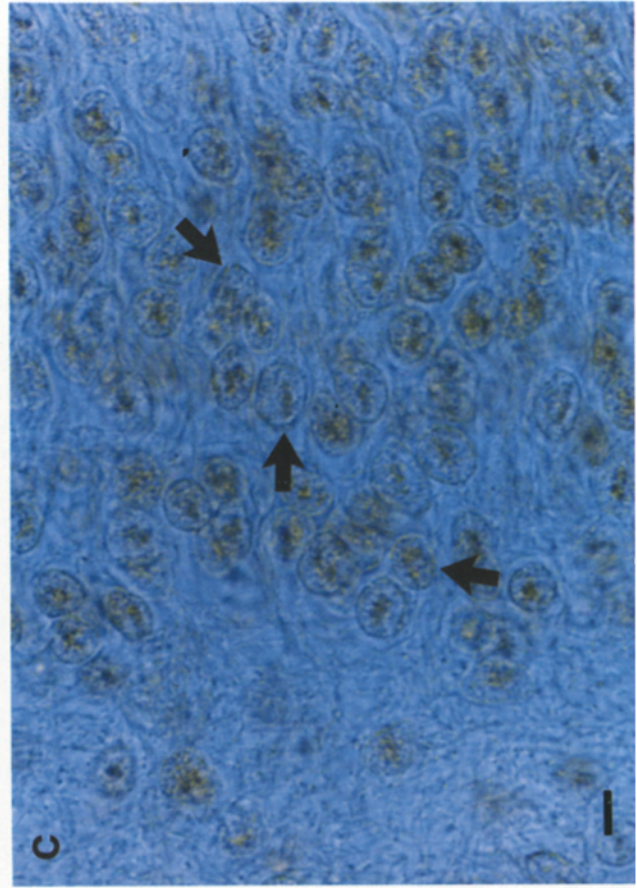
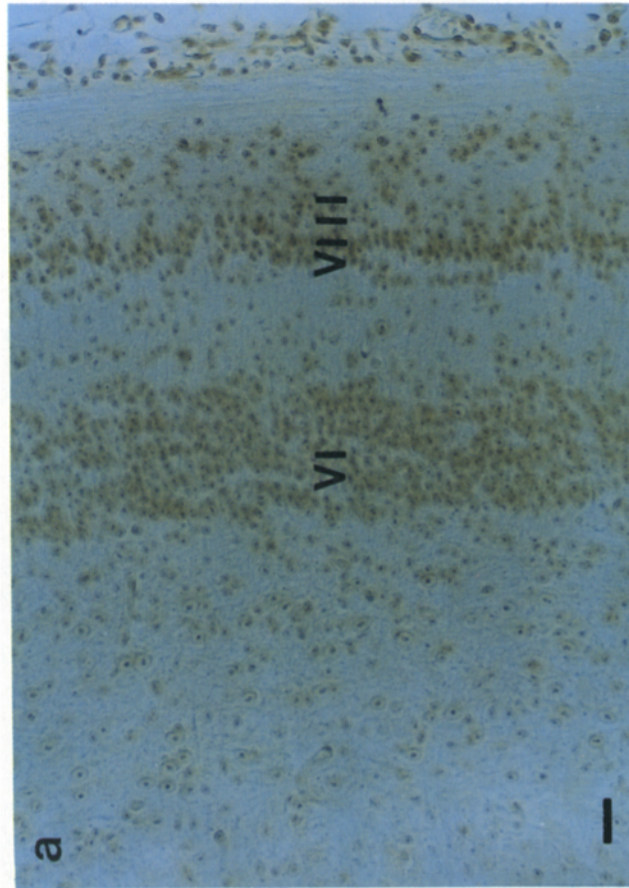
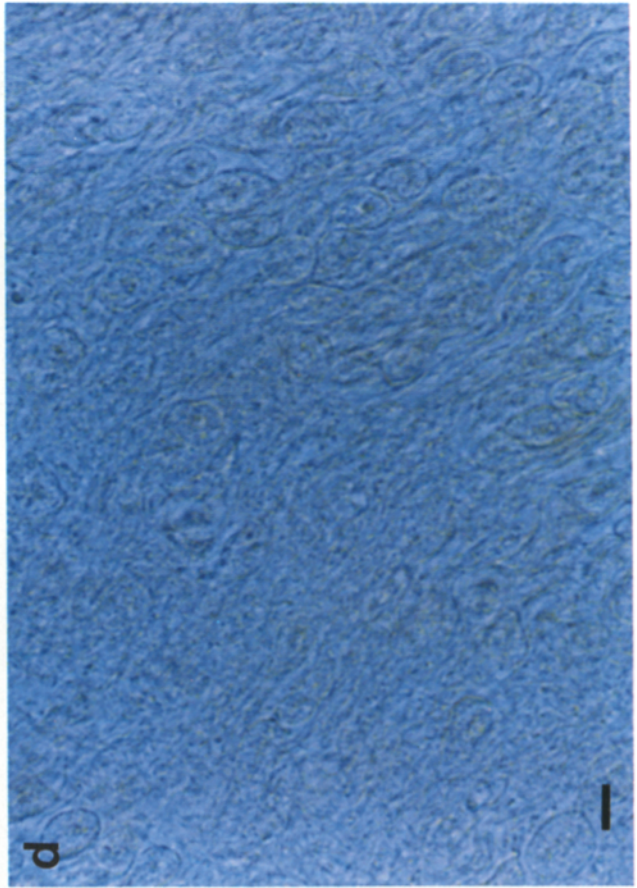
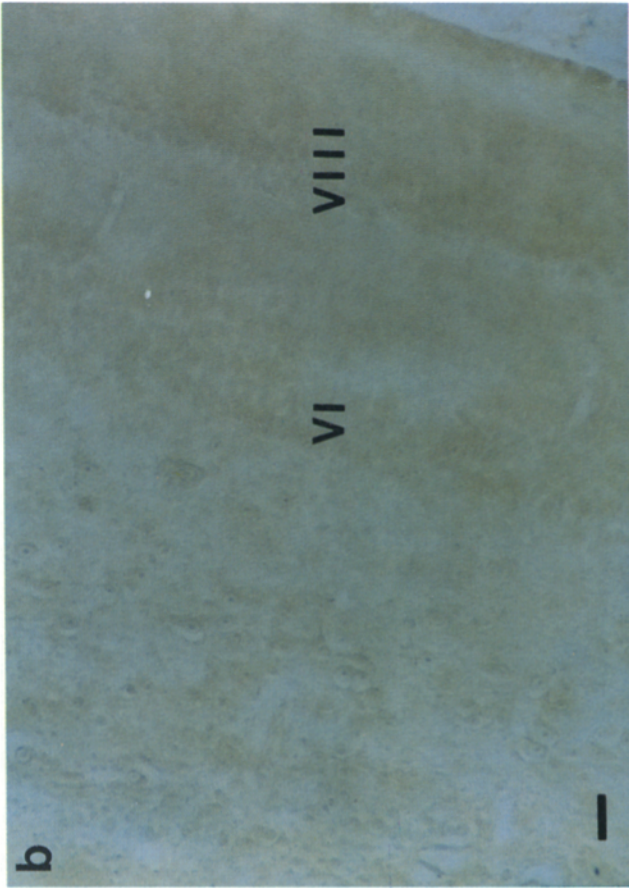


Figure 6. HRP staining of E13 chick brain sagittal sections using 20 $\mu\text{g/ml}$ IgG of $\alpha\text{B-EC5}$, the specific B-cadherin antipeptide antibody (*a*, *c*, and *e*) or 20 $\mu\text{g/ml}$ of rabbit preimmune serum (*b*, *d*, and *f*). (*a* and *b*) Section through optic tectum. Note specific staining of cell layers vi and viii; (*c* and *d*) section through ventricle (*V*) of optic tectum. Staining is localized to the ependymal layer (*EP*) lining the ventricle; (*e* and *f*) section through the choroid plexus. Note the intense outlining of the epithelial lining of the plexus by HRP-reaction product. Bar, 100 μm .

Figure 7. Color micrographs of $\alpha\text{B-EC5}$ immunoperoxidase staining in E13 chick optic tectum (*a* and *c*) or preimmune serum (*b* and *d*). (*a* and *b*) Low power view of section as in Fig. 6; (*c* and *d*) high magnification of field in layer vi of optic tectum. Note in *c* the staining of cell surfaces as well as perinuclear regions of large neurons. Bars: (*a* and *b*) 50 μm ; (*c* and *d*) 10 μm .



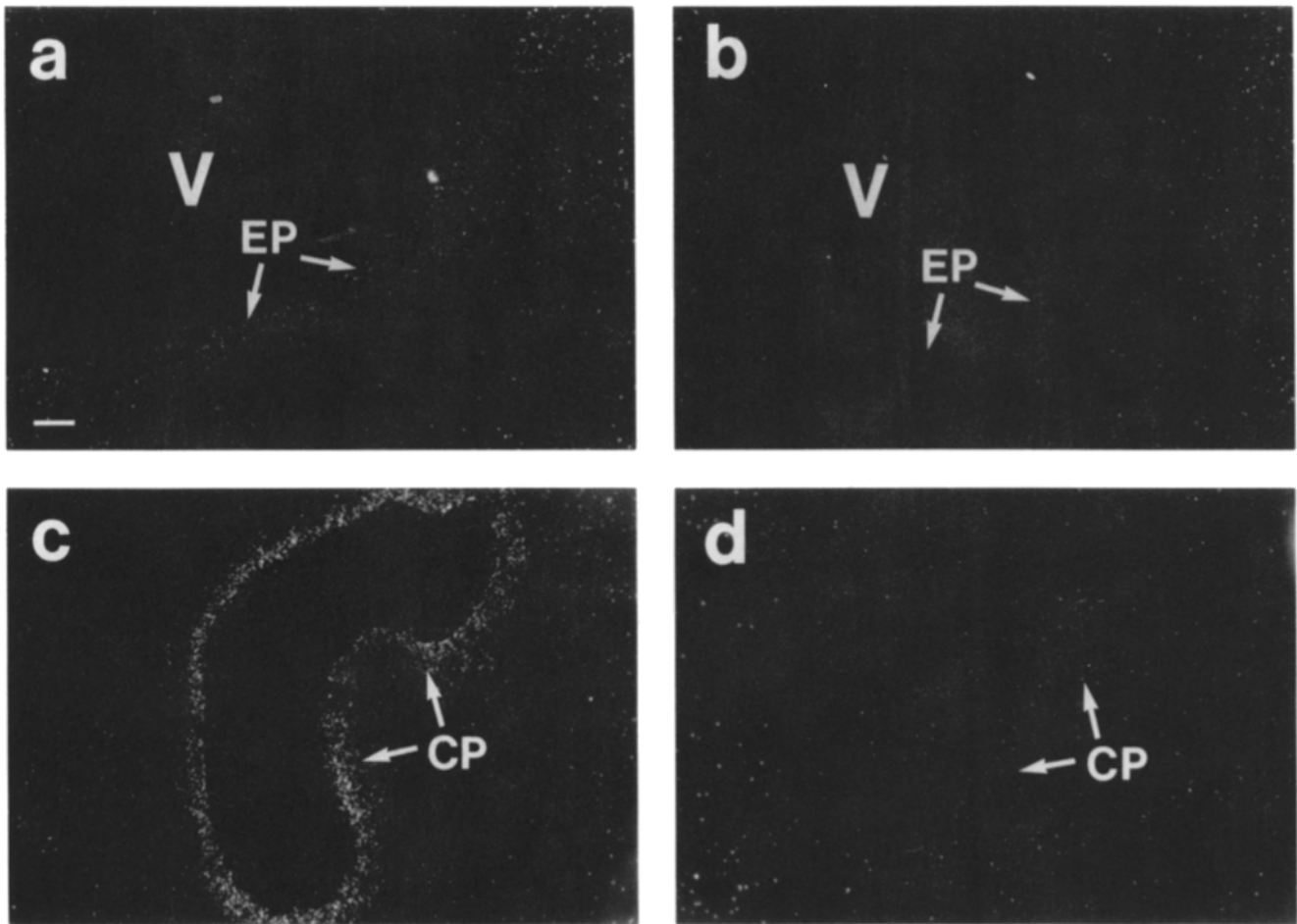


Figure 8. Darkfield micrograph showing in situ hybridization of brain tissue at E13. ^{35}S -labeled RNA transcripts generated from pGEM vectors containing the full-length cDNA were used as probes on paraffin-embedded sagittal sections of E13 chick brain. (a and c) Antisense probe; (b and d) control (sense) probe. (a and b) Ependyma (EP) and ventricle (V) of optic tectum. (c and d) Cross-section through choroid plexus (CP). Bar, 100 μm .

ture double-labeling experiments using neural- and/or glial-specific markers will be conducted to determine whether B-cadherin appears exclusively on neurons or glia.

Interestingly, specific staining of the antibody is also demonstrated in the choroid plexus where cells comprising the epithelial lining of this structure are intensely outlined (Fig. 6 e). That this adhesion molecule is expressed on cells of the choroid as well as ependymal cells suggests it may play a role in the formation of junctional complexes which function as selective barriers between components of the cerebrospinal fluid and brain. Subcellular localization of B-cadherin at the ultrastructural level may shed some light on this possibility.

Higher magnification of a field in layer vi of the optic tectum reveals a cell surface (several examples denoted by arrows in Fig. 7 c) as well as possible perinuclear distribution for the antigen. The appearance of B-cadherin within cell bodies may reflect an intracellular pool of this protein in the ER or Golgi network. Note that at about this age (E13) a doublet is seen in immunoblots (Fig. 5, lane I), the upper band of which may be an unprocessed precursor form of this molecule resident in one of the aforementioned membranous compartments within the cell.

In Situ Hybridization

B-cadherin mRNAs, detected by antisense probes and visu-

alized as light spots in darkfield microscopy, were concentrated in the ependymal layer of the optic tectum (Fig. 8 a). This distribution pattern is coincident with that seen by immunostaining, indicating that the site of synthesis of the RNA transcript is the same as that of the protein (Fig. 6 c). More superficial layers of the tectum, including layers vi and viii, are not included in this section. No specific labeling of periventricular structures was observed when the sense (control) strand was used for hybridization (Fig. 8 b). RNAs encoding B-cadherin were also highly abundant in the choroid lining, as judged by the intense deposition of silver grains along the entire circumference of the plexus (Fig. 8 c). Once again, this pattern is very similar to that observed using the B-cadherin-specific antibodies in immunohistochemistry, demonstrating colocalization of mRNA and protein (Fig. 6 e). The perimeter of the choroid plexus on sections hybridized with the sense (control) strand were unlabeled (Fig. 8 d).

Discussion

In this paper, we describe the cloning, primary structure analysis, and distribution of B-cadherin, a novel member of the cadherin family of cell adhesion molecules. At the primary sequence level, cadherins exhibit the highest degree of

homology in their cytoplasmic tails (Takeichi, 1990). In an effort to prepare reagents capable of recognizing all cadherins, two polyclonal antisera, α CADCYTO-1 and α CADCYTO-2, were raised to conserved peptides in the intracellular domain of L-CAM (see Materials and Methods). Both antibodies were shown by immunoblot analysis of various tissues to react with a group of proteins of M_r between 120 and 130k, which represent previously characterized as well as potentially novel cadherins (not shown). In immunoblots of retina and brain, the antibodies recognized several proteins in the same molecular weight range, suggesting that brain contains cadherins in addition to N-cadherin, the only cadherin identified in brain at the time these experiments were initiated. One of these antibodies, α CADCYTO-2, did not recognize immunopurified N-cadherin, even though it is an abundant cadherin in the chick nervous system. This result prompted the use of α CADCYTO-2 as a probe to search for novel cadherins in chick brain. Subsequent screening of an embryonic day 13 chick brain cDNA library resulted in the isolation of a cDNA encoding an apparently new cadherin, named B-cadherin.

The deduced amino acid sequence of B-cadherin displays a high homology with known cadherins in all domains of the molecule. Specifically, the five externally repeated segments of ~ 110 amino acids, which include regions implicated in Ca^{2+} -binding and homophilic interactions and are found in all previously described cadherins, are also contained in B-cadherin. Virtually all amino acid residues that are conserved in this extracellular domain in other cadherins are also present in B-cadherin. Homology is highest in the two most NH_2 -terminal repeats, where sequence identity with other identified cadherins has a range between 74 and 58%. The homology is lower in the three more $COOH$ -terminal repeats but, again, essentially all amino acids conserved in other cadherins are found in B-cadherin. The cytoplasmic domains of cadherins mediate binding to the cytoskeleton and are also required for normal functioning of these adhesion molecules (Ozawa et al., 1989; 1990; reviewed in Takeichi, 1990). Sequence identity between B-cadherin and other cadherins is particularly high in this domain, ranging from 93 to 71%, where again, virtually all amino acids conserved between other cadherins are also present in B-cadherin. The extent of sequence identity in the single transmembrane domain is lower (27–73%), but the level of homology is similar to that seen between other cadherins. To summarize, B-cadherin is homologous to previously characterized cadherins in all domains and contains every motif implicated in the function of this family of adhesion molecules.

While the specific binding properties of B-cadherin have not yet been elucidated, analysis of chimeric cadherins has shown that the NH_2 -terminal 113 amino acids are required for cadherin function and specificity (Nose et al., 1990). The epitopes recognized by function-blocking antibodies to N-, E-, and P-cadherins have also been localized to this region (Nose et al., 1990). All cadherins sequenced to date contain the tripeptide HAV within this domain. It has been proposed that this peptide is a cell adhesion recognition sequence which may stabilize homophilic associations between cadherins. Synthetic peptides containing HAV and flanking residues have been shown to inhibit cadherin-mediated cellular interactions (Blaschuk et al., 1990). Recent studies conducted by Nose and co-workers have indicated that specific amino acid residues surrounding this conserved triplet exert a sig-

nificant influence on the binding specificity of cadherins (Nose et al., 1990). In cells transfected with E-cadherin constructs, changing amino acids 78 and 83 to those present in P-cadherin generates a modified protein that appears to bind P- in addition to E-cadherin. These neighboring residues are highly, but not perfectly conserved between cadherins of the same subclass isolated from different species (reviewed in Takeichi, 1990). With respect to B-cadherin, the serine (S) at residue 78 is also found in the homologous position (amino acid 77) in E-cadherin and L-CAM, but is not shared by N-, P-, or R-cadherins. The glutamic acid at residue 82 in B-cadherin is found in P-cadherin, but not in E-, N-, or R-cadherin. Intriguingly, it does share these residues with the recently characterized *Xenopus* E/P-cadherin (Ginsberg and Geiger, 1990). Additional experiments using cell lines transfected with B-cadherin and other members of the family will be useful in deciphering the binding capabilities of B-cadherin.

The primary structure and distribution pattern of B-cadherin distinguish it from other cadherins. N-cadherin, R-cadherin, and L-CAM, all previously identified in chick, are clearly different in primary structure and distribution from B-cadherin (Takeichi, 1990). A chick homologue for mammalian P-cadherin has not been identified, so it is possible that B-cadherin represents this entity; however, this does not seem likely since chick B-cadherin is not more closely related to mammalian P-cadherin than to E-cadherin or L-CAM. In addition, the distribution of B-cadherin in chick embryos is quite different from that described for P-cadherin, which is not a prominent cadherin in the murine embryonic brain (reviewed in Takeichi, 1990). Recently, another distinct cadherin present in *Xenopus* oocytes, named E/P-cadherin, has been cloned, sequenced, and also been shown to be more closely related to L-CAM, E-, and P-cadherins than to N- or R-cadherins (Ginsberg and Geiger, 1990). Sequence comparison has indicated that it is also $\sim 63\%$ identical to B-cadherin, essentially the same degree of homology as to L-CAM, E- and P-cadherins. Its distribution in *Xenopus* embryos is distinct from that of B-cadherin in chick embryos. On balance, B-cadherin seems unlikely to be the chick homologue of this molecule.

mRNA analysis using the entire B-cadherin cDNA as a probe reveals the presence of a single mRNA of $\sim 3,000$ bases in most tissues. The appearance of a second, minor 4,000-base transcript of B-cadherin in total liver RNA (Fig. 3a, lane 9) raises the possibility that alternative splicing or alternative use of polyadenylation sites may play a role in the regulation B-cadherin in this organ. However, since stringency conditions for washing of the blot were approximately $T_m - 5^\circ C$ for a nick-translated 3' fragment of the B-cadherin probe and its corresponding mRNA and $T_m - 2^\circ C$ for this probe and L-CAM mRNA, this band likely represents cross-hybridization of conserved regions of the radiolabeled probe (e.g., 3' end of the cDNA which encodes the cytoplasmic tail) with L-CAM mRNA which is abundantly expressed in liver at E8. Single mRNAs for L-CAM, E-cadherin, and P-cadherin appear to be encoded by separate genes (Takeichi, 1990). When the genomic structure of the L-CAM gene was analyzed, no evidence for alternatively spliced exons was found (Sorkin et al., 1988). Recently, multiple RNAs were detected in bovine and human brain using cDNAs that encode N-cadherin homologues (Liaw et al., 1990; Walsh et al., 1990).

In studies of tissue distribution, the relative abundance of B-cadherin protein and mRNA were measured using antigen and RNA blots. B-cadherin protein was detected by antibodies raised to a sequence in the fifth extracellular repeated domain (EC5) that is not present in other cadherins. The antibodies recognized a doublet at 120–122 kD in chick brain and a single band at 120 kD in other tissues. Outside of the brain, both B-cadherin protein and mRNA were detected in a variety of locations suggesting that B-cadherin has functions in many developing tissues. This is consistent with observations on other cadherins which are also found in many different organs, but are differentially distributed (Takeichi, 1990).

When the levels of B-cadherin mRNA and protein were examined in embryonic chick brain at different stages of maturation, both were found to be developmentally regulated. Surprisingly, however, the expression pattern of mRNA and protein differed significantly from each other during the interval assayed. Levels of B-cadherin mRNA decreased steadily between E6 and E19 while, in contrast, protein levels increased to maximal values at E12–E16 before declining to lower levels. Among many possibilities, these observations may be attributed to a change in protein turnover. Recently, Volk et al. (1990) have presented evidence that during the early stages of sclerotome dissociation, a truncated derivative of N-cadherin is formed. They suggest that endogenous proteases are responsible for the rapid loss of intact N-cadherin and the generation of N-cadherin fragments which persist for longer periods in the disaggregating regions of the somites. Considering these results, it would not be surprising if turnover of B-cadherin in brain should also prove to be developmentally regulated. Clearly, it will be important in the future to determine which posttranscriptional mechanisms are key regulators of its level and function.

Within the chick brain, B-cadherin mRNA and protein were localized to the choroid epithelium and ependymal cells lining the ventricle by in situ hybridization and immunohistochemistry, respectively. To date, no other cadherins have been localized to these areas (reviewed in Takeichi, 1990). The concentration of these molecules in structures that line the ventricle and are therefore interposed between a fluid and cellular environment, suggests that B-cadherin may be important in organizing calcium-dependent junctional complexes which function as selective filters between the cerebrospinal fluid and brain.

In addition to labeling epithelial cell layers in the embryonic brain, B-cadherin-specific antibodies also labeled selected cellular layers in the embryonic E13 optic tectum. The cytoarchitecture of cells in these layers has been described in detail by LaVail and Cowan (1971). Comparison of the antigen distribution in the present paper to the cell types described by LaVail and Cowan (1971) indicates that the larger cells stained by the antibodies are clearly neurons. The identities of the smaller cells cannot be ascertained with the same confidence. N-cadherin has also been shown to be preferentially localized to specific layers of the embryonic retina (Matsunaga et al., 1988), where inhibitory antibodies disrupt histogenesis. By analogy, the expression of B-cadherin in the cell-dense laminae of the optic tectum suggests that this cell adhesion molecule may be important in the formation of discrete neural cell layers.

The restricted expression pattern and dramatic regulation of B-cadherin makes it a strong candidate to have several different functions in development. In the future, it will be important to obtain specific function-blocking antibodies to use in tests of these possibilities.

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