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Diversity of the cadherin family: evidence for eight new cadherins in nervous tissue

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To examine the diversity of the cadherin family, we isolated cDNAs from brain and retina cDNA preparations with the aid of polymerase chain reaction. The products obtained included cDNAs for two of three known cadherins as well as eight distinct cDNAs, of which deduced amino acid sequences show significant similarity with the known cadherin sequences. Larger cDNA clones were isolated from human cDNA libraries for six of the eight new molecules. The deduced amino acid sequences show that the overall structure of these molecules is very similar to that of the known cadherins, indicating that these molecules are new members of the cadherin family. We have tentatively designated these cadherins as cadherin-4 through -11. The new molecules, with the exception of cadherin-4, exhibit features that distinguish them as a group from previously cloned cadherins; they may belong to a new subfamily of cadherins. Northern blot analysis showed that most of these cadherins are expressed mainly in brain, although some are expressed in other tissues as well. These findings show that the cadherin family of adhesion molecules is much larger than previously thought, and suggest that the new cadherins may play an important role in cell-cell interactions within the central nervous system.

Introduction

Cadherins are integral membrane proteins that mediate calcium-dependent cell-cell adhesion and are thought to play an important role(s) in a wide range of cell-cell interactions. In particular, cadherins appear to play a key role in morphogenesis (for reviews, see McClay and Ettensohn, 1987; Takeichi, 1988, 1990; Anderson, 1990).

Thus far, three cadherins, E-, N-, and P-cadherin, which are also known by other names, have been isolated from various tissues and organisms (Damsky *et al.*, 1983; Gallin *et al.*, 1983; Vestweber and Kemler, 1984; Yoshida-Noro *et al.*, 1984; Behrens *et al.*, 1985; Hatta *et al.*, 1985; Nose and Takeichi, 1986; Volk and Geiger, 1986), and their properties have been characterized (for reviews, see Takeichi, 1988, 1990). Some of the properties of these cadherins are similar in a number of respects, such as the molecular weight, the Ca^{2+} requirement for their activity, and Ca^{2+} protection against protease (Shirayoshi *et al.*, 1986). Amino acid sequences deduced from cDNA cloning have clearly shown that cadherins belong to a protein family (Gallin *et al.*, 1987; Nagafuchi *et al.*, 1987; Nose *et al.*, 1987; Ringwald *et al.*, 1987; Hatta *et al.*, 1988; Miyatani *et al.*, 1989; Shimoyama *et al.*, 1989). The cadherin molecule is divided by a transmembrane domain into a large extracellular domain at the N-terminal side and a small cytoplasmic domain at the C-terminal side. The extracellular domain consists primarily of five repeats of a motif unique to cadherins (Hatta *et al.*, 1988) and appears to determine the specificity of the homophilic cell adhesion activity of the cadherin (Nose *et al.*, 1990). On the other hand, the amino acid sequence of the cytoplasmic domain is highly conserved among the cadherins, suggesting that this region serves an important shared function. Recent molecular biological experiments have shown that this domain is essential for cell adhesion activity (Nagafuchi and Takeichi, 1988), and it is likely that it exerts this effect through interacting with cytoskeletal proteins (Hirano *et al.*, 1987; Nagafuchi and Takeichi, 1989; Ozawa *et al.*, 1989).

Recent studies suggest that at least two, and possibly more, additional cadherins exist. Vascular endothelial cells appear to express a specific new cadherin (Heimark *et al.*, 1990), and another cadherin closely related to N-cadherin has been reported in chicken liver (Crittenden *et al.*, 1988). Matsunaga *et al.* (1988b) and Choi *et al.* (1990) found evidence of other cadherins

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in chicken retina and in *Xenopus* embryo, respectively; however, detailed identification of these molecules has not yet been reported.

Development of the central nervous system (CNS)[1] requires complex cell-cell interactions, and cell adhesion molecules appear to play a key role in this process. Many adhesion molecules, in fact, have been isolated from the CNS and various functions have been attributed to them, such as specific cell-cell recognition, cell movement, neurite fasciculation, and axon elongation (Grumet and Edelman, 1984; Cunningham *et al.*, 1987; Harrelson and Goodman, 1988; Moos *et al.*, 1988; Ranscht and Dours, 1988). The N-cadherin, the only well-characterized cadherin in the CNS, is of particular interest. Expression of the N-cadherin is regulated spatiotemporally, which implies an important role in morphogenesis (Hatta and Takeichi, 1986; Hatta *et al.*, 1987). Indeed, it has been reported that anti-N-cadherin antibody interferes with the development of neural retina (Matsunaga *et al.*, 1988b). Moreover, N-cadherin appears to have neurite outgrowth-promoting activity, suggesting a role in the guidance of axon elongation (Matsunaga *et al.*, 1988a; Bixby and Zhang, 1990).

Because N-cadherin appears to play an important role in the CNS, we initiated an investigation into the possibility that other, as yet unidentified cadherins might participate in cell-cell interaction in the CNS. To study this, we used polymerase chain reaction (PCR) and found a large number of new cadherins in brain and retina. Herein, we describe initial characterization of these cadherins and discuss their possible role in the development and function of nervous tissue.

Results

Isolation of cDNAs for possible new cadherins

A PCR method was applied for the isolation of cDNAs that correspond to new cadherins. Two highly conserved amino acid sequences from the cytoplasmic domain were chosen by comparing various cadherins, and the corresponding degenerate oligonucleotides were synthesized (Figure 1). PCR was carried out using the mixed oligonucleotides as primers and a rat brain and a rat retina cDNA preparations as templates. The resultant products, of ~160 nt in size, were

[1] Abbreviations used: CNS, central nervous system; PCR, polymerase chain reaction.

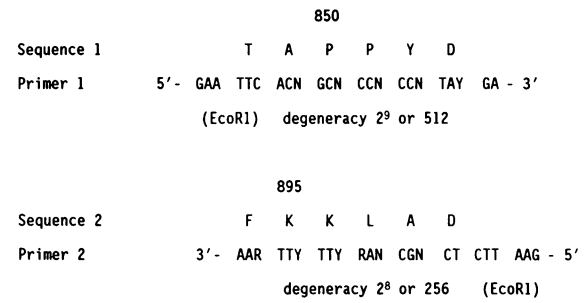

***Figure 1.* Amino acid sequences and corresponding nucleotide sequences for PCR primers.** Amino acid sequences 1 and 2 were chosen by comparing various cadherins. Numbering of sequences 1 and 2 was taken from that of the corresponding amino acid sequence of mouse N-cadherin (Miyatani *et al.*, 1989). The nucleotide sequence is as follows: A, deoxyadenosine; C, deoxycytosine; G, deoxyguanosine; T, deoxythymidine; R, either A or G; Y, either C or T; N, either A, C, G, or T.

isolated and subcloned into the M13 vector. Approximately 75 clones were then isolated and sequenced. The sequences revealed 10 different types of cDNA clones encoding distinctive amino acid sequences homologous with the cytoplasmic domain of the known cadherins (Figure 2). Attempts to isolate similar cDNAs from rat liver, lung, kidney, and skin yielded an additional distinctive clone (clone 3) from the skin cDNA. Homologous clones, except for clones 7 and 9, were isolated from human fetal and adult brain cDNA preparations. Judging from the deduced amino acid sequences, clones 1, 2, and 3 correspond to rat N-, E-, and P-cadherin, respectively. The molecules defined by the remaining 8 types of cDNAs may be new cadherins, because these sequences are strongly homologous with, but distinct from, those of the known cadherins.

To isolate larger cDNAs corresponding to the putative new cadherins, we screened a human fetal brain cDNA library using the short cDNAs obtained by PCR as probes, and then sequenced the resultant clones. cDNAs corresponding to clone 5 were isolated from a human placental cDNA library, because preliminary results had shown that the mRNA that hybridizes with the cDNA is expressed at a relatively high level in placenta. cDNAs corresponding to six possible cadherins were obtained, but the remaining two probes yielded no clones from these libraries.

Deduced amino acid sequences of the possible new cadherins

One of the human cDNAs corresponding to rat clone 5 appears to contain the entire coding

Clone 1 (N-cadherin)	S L L V F D Y E G S G S T A G S L S S L N S S S S G G D Q D Y D Y L N D W G P R
Clone 2 (E-cadherin)	S L L V F D Y E G S G S E A A S L S S L N S S E S D Q D Q D Y D Y L N E W G N R
Clone 3 (P-cadherin)	S L L V F D Y E G S G S D A A S L S S L T S S T S D Q D Q D Y N Y L T E W G S R
Clone 4	S L L V F D Y E G S G S T A G S V S S L N S S S S G - D Q D Y D Y L N D W G P R
Clone 5	T L H I Y G Y E G T E S I A E S L S S L S T N S S D S D I D Y D F L N D W G P R
Clone 6	S L A T Y A Y E G T G S V A D S L S S L E S V T T D G D Q D Y D Y L S D W G P R
Clone 7	S L Q T Y A F E G N G S V A E S L S S L D S N S S N S D Q N Y D Y L S D W G P R
Clone 8	S I Q I Y G Y E G R G S V A G S L S S L E S T T S D S D Q N F D Y L S D W G P R
Clone 9	S L A T Y A Y E G N D S V A N S L S S L E S L T A D C N Q D Y D Y L S D W G P R
Clone 10	S L A T Y A Y E G N D S V A E S L S S L E S G T T E G D Q N Y D Y L R E W G P R
Clone 11	S I Q I Y G Y E G R G S V A G S L S S L E S A T T D S D L D Y D Y L Q N W G P R

Mouse cadherins

	860	880
N-cadherin	S L L V F D Y E G S G S T A G S L S S L N S S S S G G D Q D Y D Y L N D W G P R	
E-cadherin	S L L V F D Y E G S G S E A A S L S S L N S S E S D Q D Q D Y D Y L N E W G N R	
P-cadherin	S L M V F D Y E G S G S D A A S L S S L T T S A S D Q D Q D Y N Y L N E W G S R	

Figure 2. **Deduced amino acid sequences of rat cDNA clones isolated by PCR.** All clones were isolated from rat brain and retina cDNA preparations, with the exception of clone 3, which was isolated from a rat skin cDNA preparation.

sequence of a putative cadherin (Figure 3). The sequence contains possible initiation codon followed by signal sequence but lacks the prosequence that is present in the known cadherin precursors (Hatta *et al.,* 1988). The sequence also contains the well-conserved, postulated proteolytic cleavage site of cadherin precursor polypeptides (Ozawa and Kemler, 1990). The deduced amino acid sequence of putative mature clone 5 protein exhibits significant homology with those of the previously described cadherin sequences, and the overall molecular structure is essentially the same as that of the other cadherins. One putative transmembrane domain located near the C-terminal separates the molecule into a small cytoplasmic domain at the C-terminal side and a large extracellular domain at the N-terminal side. The extracellular domains of the mature cadherins consist of five repeats of a motif specific to cadherins (Hatta *et al.,* 1988). The new sequence contains the five repeats of the motif, and each repeat is closely related to the corresponding repeat of the previously cloned cadherins (Figure 4).

The clones for the remaining five putative cadherins contain only partial sequences of the molecules. However, despite the fact that the extracellular domain sequences are truncated, it is clear that these sequences contain the cadherin extracellular domain repeats in the same order as those of other cloned cadherins (Figure 4). Moreover, the cytoplasmic domains are similar in size to, and show high homology with, the cadherin sequences already reported (Figure 5). These features identify the molecules defined by the cDNAs as new members of the cadherin family.

Although we were unable to isolate large cDNAs for the remaining two species, the high homology of the cytoplasmic domain makes it quite likely that these molecules have topography similar to that of the other cadherins. We have tentatively termed these eight molecules, defined by the cDNAs corresponding to rat clones 4–11, as cadherin-4 through -11, respectively. The switch to numbering from the currently used alphabetic prefix for the known cadherins seems to be justified on the basis of the existence of the large number of cadherins.

Homology among cadherin sequences

Cadherin-4 shows particularly high homology with the N-cadherin. When the two sequences

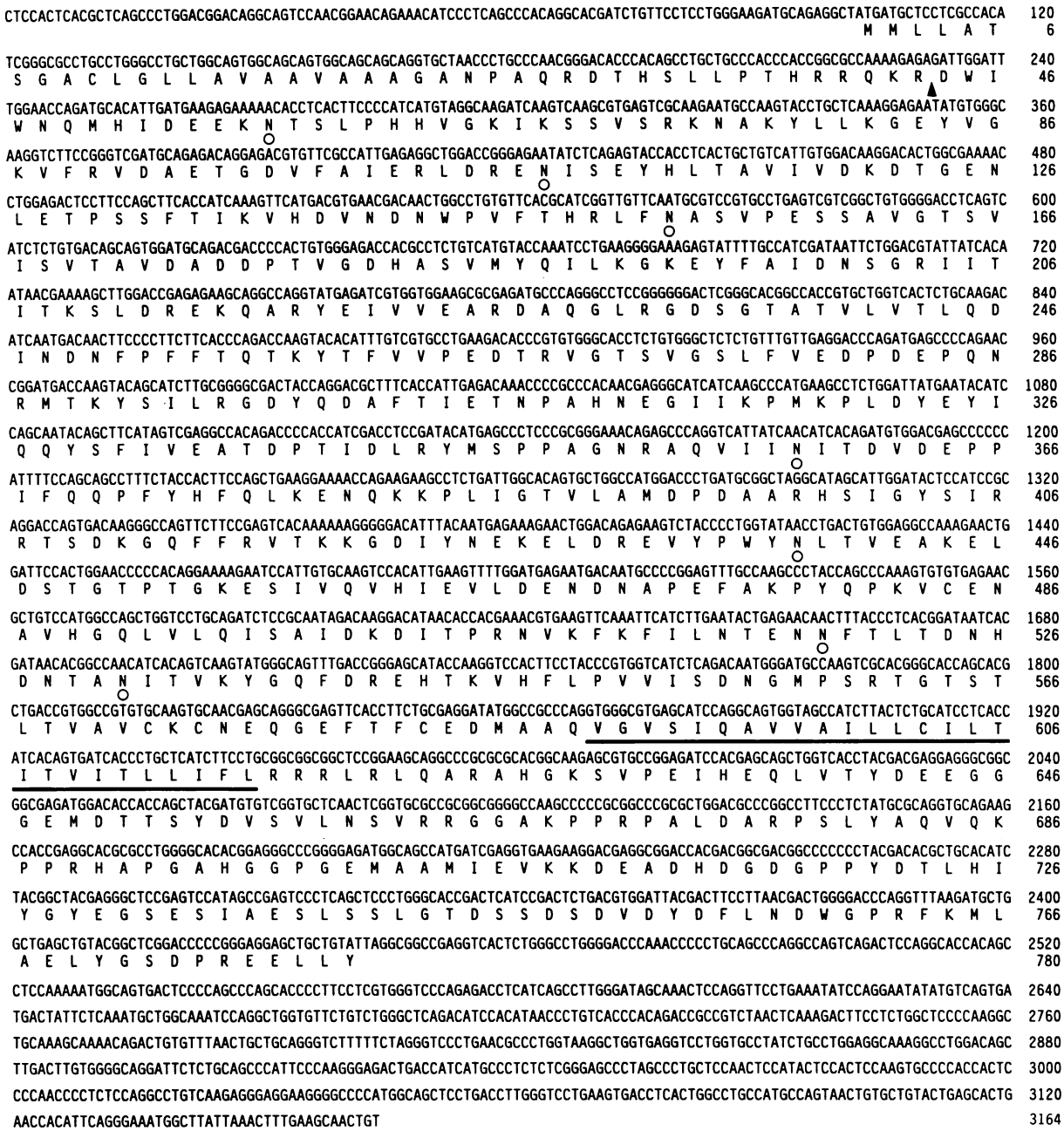

Figure 3. **Nucleotide sequence and deduced amino acid sequence of human cDNA corresponding to rat clone 5.** The possible proteolytic cleavage site of precursor protein is indicated by an arrowhead. The putative transmembrane domain is underlined and the possible N-glycosylation sites are marked by open circles.

are aligned, ~70% of the amino acids are identical, and only one amino acid deletion and one amino acid addition are present in the cytoplasmic domain (Figures 4 and 5). Cadherin-8 and cadherin-11 are also highly homologous to each other in the cytoplasmic and in the extracellular domains (Table 1). The cytoplasmic domains of cadherin-6 and cadherin-10 are very similar, but the extracellular domains could not be compared because of the lack of sequence information on cadherin-6. On the other hand, cadherin-5 is unique in that its cytoplasmic domain shows relatively low homology with the other cadherins.

Although the homologies among the extracellular domains are lower than those among

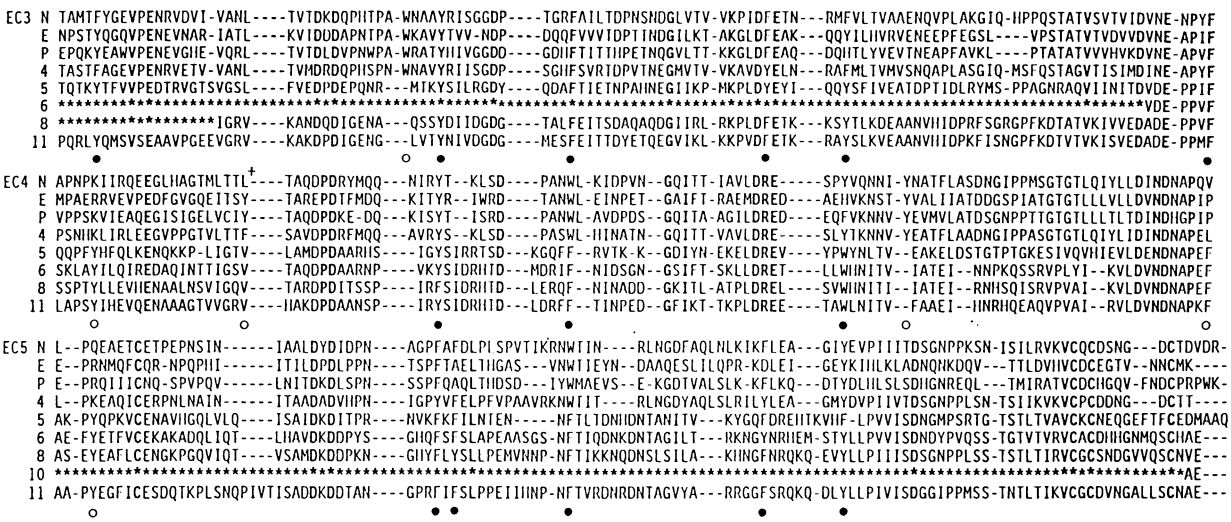

Figure 4. **Alignment of deduced amino acid sequences of possible cadherins.** Extracellular domains 3, 4, and 5 (EC3–5) of human cadherin-4 through -11 (4–11) and of mouse N-, E-, and P-cadherins (N, E, P) were aligned. ●, positions of aromatic amino acids conserved among cadherins. ○, positions of aromatic amino acids present only in the known cadherins or only in the new cadherins, with the exception of cadherin-4. -, gaps introduced to maximize homology, *, undetermined amino acids. + denotes an amino acid that in human N-cadherin is phenylalanine (unpublished observations).

the cytoplasmic domains, the end regions of the repeats are conserved well (Figure 4). These sequences correspond to a portion of the Ca^{2+} binding sites postulated by Ringwald *et al.* (1987). The sequences of the cytoplasmic domains, on the other hand, tend to be highly conserved among the cadherins (Hatta *et al.,* 1988), but our results reveal more variability in them than previously thought. However, many of the changes are conservative.

The new cadherins, with the exception of cadherin-4, show lower homology with the previously cloned cadherins than had been observed among those cadherins (Table 1). More-

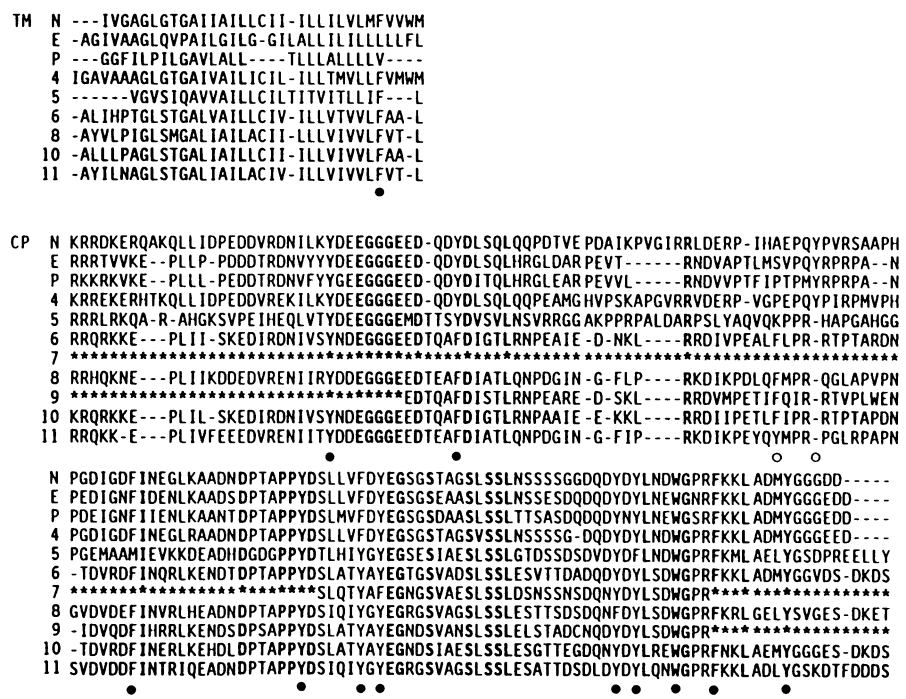

Figure 5. **Alignment of transmembrane domain and cytoplasmic domain of possible cadherins.** The transmembrane domain (TM) and cytoplasmic domain (CP) of cadherin-4 through -11 (4–11) and mouse N-, E-, and P-cadherins (N, E, P) were aligned. The sequences of cadherin-7 and cadherin-9 are from rat cDNAs. Symbols are the same as Figure 4.

Table 1. Homology of deduced amino acid sequences of cadherins

	N	E	P	4	5	6	7	8	9	10	11
N	—	37	41	65	27	(25)	-	29	-	-	30
E	62	—	46	35	24	(25)	-	27	-	-	25
P	56	80	—	36	27	(21)	-	25	-	-	27
4	82	61	57	—	27	(25)	-	28	-	-	30
5	39	36	35	49	—	(34)	-	37	-	-	39
6	51	50	52	49	37	—	-	(45)	-	-	(39)
7	(65)	(57)	(53)	(63)	(60)	(73)	—	-	-	-	-
8	46	45	43	46	38	59	(73)	—	-	-	59
9	(47)	(47)	(46)	(47)	(37)	(72)	(68)	(73)	—	-	-
10	50	40	50	48	57	83	(70)	57	(68)	—	-
11	50	47	45	48	39	62	(63)	76	(54)	59	—

Amino acid identity of extracellular domains 3, 4, and 5 (above the diagonal) and of the cytoplasmic domains (below the diagonal) of cadherins-4 through -11 (4–11) and N-, E-, and P-cadherins (N, E, P) was calculated. The amino acid identity values given in parentheses were calculated from the partial amino acid sequences available.

-, not done.

over, cadherins-5 through -11 have in common a number of distinctive features. For example, whereas most of the aromatic amino acids are shared by all cadherins, including the new cadherins, some of these amino acids are missing from cadherins-5 through -11 and several additional aromatic amino acids are present in the 5–11 group of cadherins (○ in Figures 4 and 5). Many similar cases are evident at the other amino acid positions. Taken together, these features suggest that the cadherin family may be classified into two subfamilies, 1–4 and 5–11, on the basis of these structural characteristics.

Expression of cadherin mRNAs

The expression of mRNAs for the new cadherins was examined by Northern blot analysis. Most of the mRNAs were detected in brain (Figure 6), as expected based on the results of PCR. However, the mRNAs for cadherin-6 and cadherin-7 gave very faint signals, even after prolonged exposure. The cadherin-8 probe hybridized with multiple mRNA species, possibly representing the products of alternative splicing. The sizes of the mRNAs were 3.5–5 knt, which are similar to those of reported cadherins.

The mRNAs of cadherins-8 through -10 were detected only in brain, whereas cadherins-4,

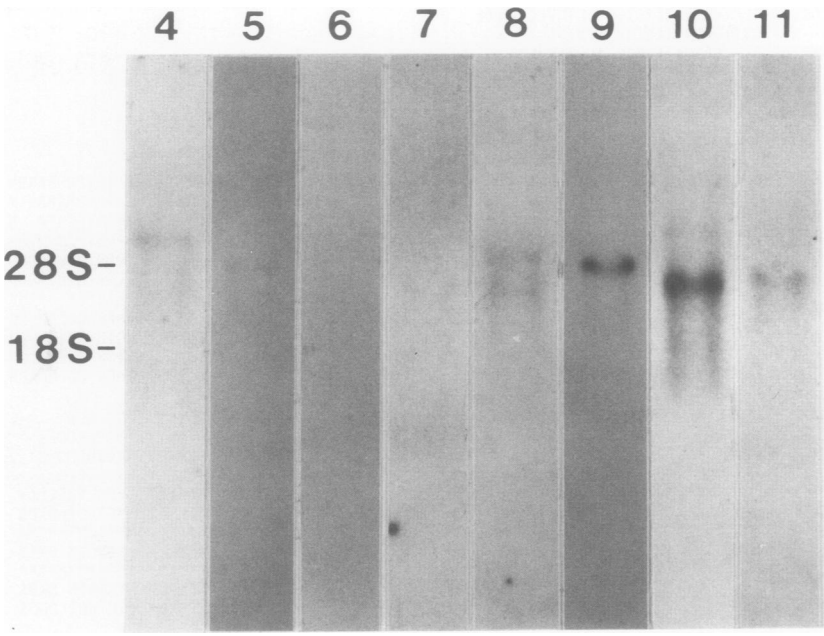

***Figure 6.* Expression of mRNAs for new cadherins in rat brain.** Poly(A)$^+$ RNA preparation prepared from rat brain (2.5 μg/lane) was separated electrophoretically on an agarose gel under denaturing conditions and transferred onto a nitrocellulose filter. The resultant filter was hybridized with respective ^{32}P-labeled rat PCR probes (4, cadherin-4; 5, cadherin-5; 6, cadherin-6; 7, cadherin-7; 8, cadherin-8; 9, cadherin-9; 10, cadherin-10; 11, cadherin-11). Calf liver ribosomal RNAs (28S and 18S) were used as size markers.

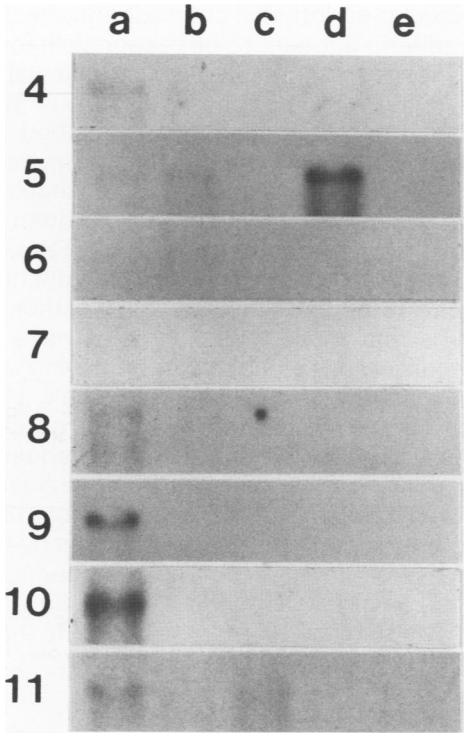

Figure 7. **Expression of mRNAs for new cadherins in rat tissues.** Expression of new cadherins in different rat tissues (a, brain; b, kidney; c, liver; d, lung; e, skin) was examined by Northern blot analysis. The experimental procedures are described in the legend for Figure 6. Symbols are the same as Figure 6.

-5, and -11 mRNAs were found in other tissues also (Figure 7). The mRNA of cadherin-5 was detected in the endothelial cells from human umbilical cord vein, but not in A431 human epidermoid carcinoma cells, IMR 90 human fibroblast cells, or SK-N-SH human neuroblastoma cells (data not shown). The mRNAs for some of the new cadherins examined were detected in nerve cell lines (Figure 8). SK-N-SH human neuroblastoma cells express cadherin-8 and cadherin-11, and U251 human glioma cells and Y79 human retinoblastoma cells weakly express cadherin-8, suggesting that at least some of the newly identified molecules are expressed in neurons and glial cells and/or their precursor cells.

Discussion

We have isolated cDNAs for 8 putative new cadherins (cadherin-4 through -11) from brain and retina cDNA preparations. We believe that the molecules defined by the cDNAs are new members of the cadherin family because they appear to have the same overall molecular structure as that of the known cadherins. Our findings bring the number of known cadherins to 11, indicating that the cadherin family is probably as large as is the integrin family of adhesion proteins (Ruoslahti and Pierschbacher, 1987). It is quite likely that additional new members of the cadherin family will be identified, because we used only one set of primers, one of which proved not to correspond to a completely conserved sequence (Figure 5). In fact, we have recently isolated several candidate cDNAs for additional new cadherins by using other primers (unpublished observations). The apparent role of the cadherins in the development of body structure makes it important to determine the complement of cadherins expressed in various tissues and at different developmental stages. The sequence data gath-

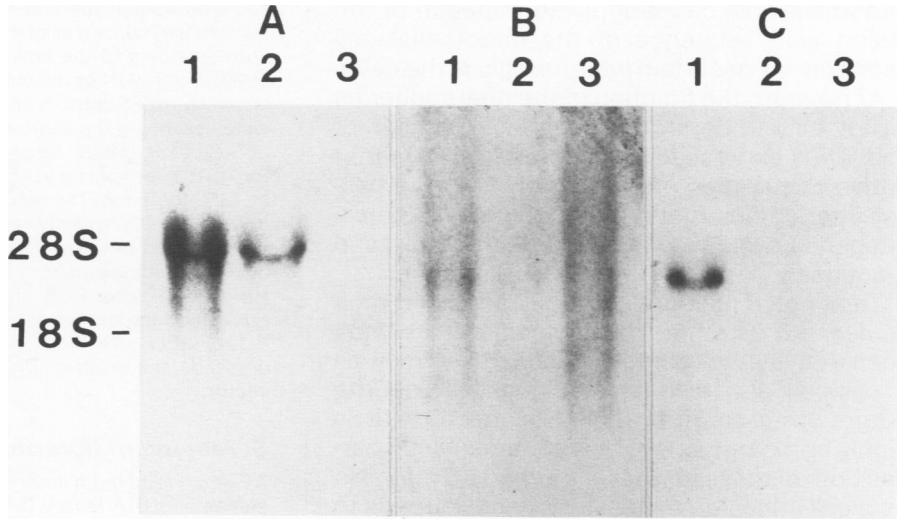

Figure 8. **Expression of cadherin-8 and cadherin-11 in cultured cells.** Expression of N-cadherin (A), cadherin-8 (B), and cadherin-11 (C) in cultured cells was examined by Northern blot analysis. cDNAs for human N-cadherin, cadherin-8, and cadherin-11 were used as probes. Experimental procedures are described in the legend for Figure 6. 1, SK-N-SH human neuroblastoma cells; 2, U251 human glioma cells; 3, Y79 human retinoblastoma cells.

ered thus far should prove to be a valuable resource in the search for new cadherins and in the analysis of cadherin expression.

An interesting feature of the new cadherins described here is that, with the exception of cadherin-4, they have specific features in common that distinguish them from the known cadherins. They show less homology with the reported cadherins than among themselves. Cadherin-5, for which we determined the entire putative coding sequence, is a prime example. Although it might not be a typical representative of the new cadherins because of its unique cytoplasmic domain, its extracellular domain differs so much from the E-, N-, and P-cadherins (Table 1) that it clearly demonstrates the existence of a new type of cadherin. These findings suggest that the new cadherins, with the exception of cadherin-4, belong to a new subfamily of cadherins that has not been recognized before. The distinct features of these new cadherins may explain in part why, despite various attempts, they had not been identified previously (Crittenden *et al.,* 1988; Choi *et al.,* 1990; Liaw *et al.,* 1990).

Two of the new cadherins we described here possibly may have been described before. The deduced amino acid sequence for cadherin-4 is quite homologous with that of the N-cadherin. Crittenden *et al.* (1988) reported a possible N-cadherin-like molecule in chicken liver; cadherin-4 may correspond to that molecule. Our results show that the mRNA for cadherin-5, which we identified in rat brain, is expressed in various other tissues, including endothelial cells from the umbilical cord vein. Recently, a possible new cadherin has been isolated from vascular endothelial cells of bovine aorta (Heimark *et al.,* 1990), and cadherin-5 may correspond to this endothelial cell cadherin. Determination of the amino acid sequence of the endothelial cell cadherin will ascertain whether this is the case.

At present, the function of the new cadherins is not fully understood. The large number of cadherins expressed in the brain suggests that in this tissue they may be involved in the cell-cell interactions that are required for CNS morphogenesis and may maintain its structure and function.

The known cadherins are cell adhesion molecules, but whether the new cadherins exhibit such activity is not known. Cadherin-4 may be a cell-cell adhesion molecule and/or neurite outgrowth-promoting molecule, because it is highly homologous with N-cadherin, which carries both of these activities. Cadherin-5 may also be a cell adhesion molecule if it corresponds to the vascular endothelial cell cadherin, because that cadherin appears to be responsible for the Ca^{2+}-dependent cell adhesion of vascular endothelial cells (Heimark *et al.,* 1990). It may be that the other new cadherins described here are not cell adhesion molecules but have a different role in cell-cell interactions. Regulation of cell adhesion and neurite outgrowth-promoting activity, as suggested by Takeichi's group (Matsunaga *et al.,* 1988a; Nagafuchi and Takeichi, 1988), is one possibility. Another possibility is that these molecules may have cell-cell repulsion or cell adhesion modifying activities (for review, see Keynes and Cook, 1990). Currently, we are in the process of obtaining cDNAs that cover the entire coding sequences of the new cadherins. Once the full-length cDNAs are obtained, it will be possible to examine the function of these molecules by gene transfer experiments, such as have been done with the known cadherins (Nagafuchi *et al.,* 1987; Matsunaga *et al.,* 1988a; Jaffe *et al.,* 1990).

Methods

mRNA preparation and cDNA synthesis

Total RNAs were prepared from various cells and tissues by the use of a guanidium isothiocyanate method (Maniatis *et al.,* 1982). Poly(A)$^+$ RNAs were isolated by the use of an Invitrogen (San Diego, CA) FastTrack kit. Poly(A)$^+$ RNA preparations from human adult and fetal brains were obtained from S. Taketani (Kansai Medical University, Japan), and rat retina poly(A)$^+$ RNA was purchased from Clonetech (Palo Alto, CA). cDNAs were synthesized from poly(A)$^+$ RNA preparations using a cDNA synthesis kit obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN).

PCR

PCR was carried out in a manner similar to that described previously (Suzuki and Naitoh, 1990). We chose two highly conserved amino acid sequences by comparing various cadherin sequences and used them to make degenerate primers that contain all of the possible nucleotide sequences corresponding to the amino acid sequences (Gould *et al.,* 1989) (Figure 1). For the subsequent subcloning, *Eco*RI linkers were added at the 5′ end of the primers. PCR conditions were essentially the same as those described in the method of Saiki *et al.* (1988). Denaturation was performed at 94°C for 1.5 min, annealing at 45°C for 2 min, and polymerization at 72°C for 3 min. The reaction was initiated by adding two units of Taq DNA polymerase, after which 35 reaction cycles were carried out. Taq DNA polymerase and the reaction buffer were obtained from International Biotechnology (New Haven, CT). After PCR, the reaction products were separated by agarose gel electrophoresis and DNA bands of ~160 nt in size were extracted. Subsequent to *Eco*RI digestion, the resultant DNAs were subcloned into the M13 vector.

Screening of libraries and DNA sequencing

A human fetal brain library (Stratagene, La Jolla, CA) and a placental cDNA library (Millán, 1986) were screened with

^{32}P-labeled cDNA probes, obtained by PCR, using a plaque hybridization method (Suzuki and Naitoh, 1990). The positives were plaque-purified and the inserts were cut out using an in vivo excision method, then subcloned into the M13 vector for sequencing.

Sequencing of DNAs was carried out according to the dideoxynucleotide chain termination method of Sanger *et al.* (1977), using a sequenase DNA sequencing kit from United States Biochemicals (Cleveland, OH). DNA and amino acid sequence analyses were carried out using the Beckman (Fullerton, CA) Microgenie program.

Other procedures

RNA preparations were electrophoresed in 0.8% agarose gel under denaturing conditions and transferred onto a nitrocellulose filter using a capillary method. Northern blot analyses were performed according to the method of Thomas (1980). The final wash was in 0.2× standard saline citrate containing 0.1% sodium dodecyl sulfate at 65°C for 10 min.

Endothelial cells from human umbilical cord vein were obtained from Clonetics (San Diego, CA) and grown in accordance with the manufacturer's instructions. Other cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

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