A Novel Cadherin Subfamily, the CNR Family, is Associated with Fyn, and Concentrated in the Postsynaptic Density Fraction of the Mouse Brain

Naohiro Kohmura

DOCTOR OF PHILOSOPHY

Department of Physiological Sciences

School of Life Sciences

The Graduate University for Advanced Studies

1996

Contents

Abbreviations	1
Summary	2
Introduction	3
Materials and Methods	6
Results	15
Discussion	23
References	31
Figures	42

Abbreviations

AGPC : acid guanidium thiocyanate-phenol-chloroform

BCIP : 5-bromo-4-chloro-indolyl phosphate

BPB : bromophenol blue

BSA : bovine serum albumin

CBB : Coomassie Brilliant Blue

EDTA : ethylenediaminetetraacetic acid

ELISA : enzyme-linked immunoabsorbent assay

FISH : fluorescence *in situ* hybridization

GST : glutathione-*S* transferase

HEPES : N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]

IPTG : isopropyl-β-D-thiogalactopyranoside

LB : Luria broth

LTP : long-term potentiation

MAG : myelin associated glycoprotein

NBT : nitro-blue tetrazolium

NMDA : N-methyl-D-aspartate

OD : optical density

pABSF : 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride

PAGE : polyacrylamide gel electrophoresis

PBS : phosphate buffered saline

PCR : polymerase chain reaction

PNPP : p-nitrophenyl phosphate

PSD : postsynaptic density

RIPA : radio immuno protein assay

SDS : sodium dodecyl sulfate

SH2 : *src* homology 2

SH3 : *src* homology 3

SSC : saline-sodium citrate buffer

SSPE : saline-sodium phosphate-EDTA buffer

TBS : Tris (hydroxymethyl) aminomethane-buffered saline

Summary

The cadherin-related neural receptor 1 (CNR1) cDNA had been partially identified by Kai et al. by the yeast two-hybrid system using the Fyn unique-SH3-SH2 domain as bait protein to screen a postnatal day 0 mouse brain cDNA library. To obtain a full-length cDNA of the mouse CNR1 gene, I constructed a mouse cDNA library, and screened it using the cDNA obtained by the two-hybrid system as a probe. I thus succeeded in obtaining a full-length cDNA of CNR1. The CNR2 full-length cDNA, which contains 88.4% identity at nucleotide sequence level with CNR1, was also obtained. CNR1 and CNR2 contained an extracellular domain, a transmembrane domain, and a cytoplasmic domain. The extracellular domain could be divided into five typical cadherin repeat domains. The extracellular and transmembrane domains of CNR1 and CNR2 contained amino acid sequence homology to the protocadherin2, but no similar protein sequence was found in the cytoplasmic domain. Sequence comparisons of CNR1 and CNR2 revealed that they were well-conserved, and the two shared similar features: the RGD motif of the extracellular domain, cysteine residues that appeared at regularly-spaced intervals spanning 18, 9, 9, and 18 amino acid residues, five PXXP motifs (which is the SH3-binding minimal consensus motif), and a lysine-rich sequence. Using a probe containing sequences conserved between CNR1 and CNR2, genomic Southern blot analysis was performed and I found CNRs to constitute multi-gene family. RT-PCR using sequences conserved between CNR1 and CNR2 as primers, revealed 6 additional new sequences, CNR3 through CNR8. The deduced amino acid alignment of the eight CNRs revealed that these sequences were well-conserved. RT-PCR analysis using specific primers and genomic Southern blot analysis using specific probes for each CNR genes demonstrated that mRNAs corresponding to each of these eight CNRs were expressed in the brain, and that the genes were located at different loci on the mouse genome. In addition, I have produced CNR1 antiserum and monoclonal antibody against CNR1. Using these, I have demonstrated that the CNR1 protein is maximally expressed in mouse brains at postnatal day 7, and is concentrated in the PSD fraction. Furthermore, by immunoprecipitation, I have demonstrated that CNR1 is associated with Fyn *in vivo*. Utilising fusion proteins derived from the conserved sequences of the cytoplasmic domain in all CNRs, I have demonstrated via ELISA assays that molecules of the CNR family are associated with the regulatory region of Fyn *in vitro*.

Introduction

An important goal for understanding the development and function of the mammalian nervous system is to elucidate what genes regulate mammalian behaviors, such as movement, recognition, emotion, learning and memory. In recent years, gene targeting techniques have allowed us to approach these topics at a molecular level. For example, defects in spatial learning or impairment of synaptic formation and plasticity are produced by the disruption of genes encoding the following proteins: α-calmodulin kinase II (α-CaMK II) (Silva et al., 1992; Chen et al., 1994), protein kinase Cγ (PKCγ) (Abeliovich et al., 1993), N-methyl-D-aspartate receptor (NMDA-R) ε1 subunit (Sakimura et al., 1995) and δ2 subunit (Kashiwabuchi et al., 1992), cyclic-AMP responsive element binding protein (CREB) (Bourtchuladze et al., 1994), neural cell adhesion molecule (NCAM) (Cremer et al., 1994), and Fyn (Grant et al., 1992). In one particular case, the Fyn-deficient mouse also shows several behavioral defects in CNS such as abnormality in suckling behavior (Yagi et al., 1993a), hyper-responsiveness to fear-inducing stimuli (Miyakawa et al., 1994), enhanced susceptibility of audiogenic seizures (Miyakawa et al., 1995), and undulation in the hippocampal cell layers (Grant et al., 1992; Yagi et al., 1994). These defects suggest that Fyn plays critical roles in brain formation and in determining behaviors.

In the brain, Fyn is extensively expressed from post-embryonic to adult stages. It is concentrated in the oligodendrocytes, nerve growth cone membranes (Bare et al., 1993; Bixby and Jhabvala, 1993; Meyerson and Pahlman, 1993) and the postsynaptic density (PSD) fraction (Grant et al., 1992; Yasuda, in preparation). Fyn appears to be a proximal components in neural cell adhesion molecule signalings, as demonstrated by studies showing that NCAM inhibits tyrosine phosphorylation of tubulin and other substrates in nerve growth cones (Atashi et al., 1992). It is co-localized with NCAM on many axonal tracts in the developing central and peripheral nervous systems and in the olfactory system (Gennarini et al., 1986; Bare et al., 1993; Yagi et al., 1993b) and mediates NCAM-dependent neurite

growth (Beggs., 1994). NCAM-deficient mice also show reduction of spatial learning in the Morris water maze, similar to that of Fyn-deficient mice (Cremer et al., 1994).

Protein tyrosine kinases play important roles in mediating signaling pathways by cell to cell interactions. These pathways regulate cellular proliferation, differentiation and function in a variety of cell types. The protein tyrosine kinases are of two types: the receptor type and the non-receptor type. The Src family belongs to the non-receptor tyrosine kinases composed of Src, Fyn, Yes, Lyn, Lck, Fgr, Blk, and Yrk. They are localized to the cytoplasmic face of the plasma membrane via their myristoylated N-terminal glycine residues. Fyn, Src, Yes and Lyn are expressed in mammalian brain. Interestingly Src- or Yes-deficient mice have not been reported to exhibit behavioral defects.

The protein structure of the Src family consists of the N-terminal unique SH3, SH2, and kinase (SH1) domains. The SH3 and SH2 domains can mediate intramolecular and intermolecular binding that regulate the functional activity of these proteins in intracellular signal transduction pathways (Koch et al., 1991; Pawson, 1992). The SH3 and SH2 domains bind to the phosphotyrosines and proline-rich sequences of the target molecules, respectively. These domains of Fyn are known to associate with focal adhesion kinase (Fak) (Cobb et al., 1994), phospholipase Cγ (PLCγ), and p85 phosphatidylinositol 3-kinase (PI3-kinase) (Pleiman et al., 1993; Pleiman et al., 1994). Recently, other new molecules have been identified to associate with Fyn. These include embryonal Fyn-associated substrate (Efs) (Ishino et al., 1995), alpha- and beta- tubulin (Marie-Cardin et al., 1995), actin-filament associated protein-110 kD and 120 kD (AFAP110 and AFAP120) (Flynn et al., 1995), related adhesion focal tyrosine kinase (RAFTK) (Li et al., 1996), Cbl (Panchamoorth et al., 1996), and zeta-associated tyrosine kinase (ZAP70) (Fusaki et al., 1996). Fyn is also associated with receptor proteins, such as the CD3 component of the T-cell receptor complex in T cells (Samelson et al., 1990), membrane binding IgM complex in B cells (Burkhardt et al., 1991), Fc mu receptor (Fc mu R) in natural killer (NK) cells (Rabinowich et al., 1996), and Fas antigen (Apo 1/CD95) in hybridomas (Atkinson et al., 1996). In the nervous system,

Fyn is also associated with myelin-associated glycoprotein (MAG) , a myelin-specific protein of the immunoglobulin superfamily exclusively expressed in oligodendrocytes, during early myelin formation (Umemori et al., 1994). The nicotinic acetylcholine receptor (AchR), which mediates depolarization at the neuromuscular junction, has also been shown to associate with Fyn in the *Torpedo* electric organ (Swope and Huganir 1994; Swope et al., 1995). However, the identification of membrane proteins which can be demonstrated to directly bind to Fyn in the neuronal cells of the brain remains to be confirmed.

To obtain proteins that directly bind to Fyn in the neuronal cells, Kai et al. have screened approximately 1.7×10^5 clones of a postnatal day 0 mouse brain cDNA library by the yeast two-hybrid system using the Fyn unique-SH3-SH2 domain (non-catalytic domain) as bait protein (Kai et al., in press). After sequencing 154 different positive clones, it was shown that one candidate contained a hydrophobic amino acids cluster as well as a region of homology to cadherins. This clone was designated CNR1 (Cadherin-related Neural Receptor 1) (Figure 1A). The cadherins are a family of transmembrane glycoproteins involved in calcium dependent cell-cell adhesion (reviewed by Takeichi, 1990, 1991), and are localized in adhesion structures, such as adherence junctions (Gaiger et al., 1990) and synapses (Fannon et al., 1996). Recent studies revealed that a variety of cadherin-related proteins are expressed in different tissues of various organisms, and it is evident that these proteins constitute a large superfamily.

To gain new insight into Fyn signaling pathways, I have focused on characterizing this putative transmembrane molecule. In the present study, I have succeeded in identifying a novel cadherin subfamily (designated as the CNR family), whose members are associated with Fyn and highly concentrated in the PSD fraction of the mouse brain.

Materials and Methods

Library Construction and cDNA Screening

Total RNA was isolated from 6 week old C57BL/6 mouse brains by the AGPC method as described (Sambrook et al., 1989). Poly $(A)^+$ RNA was prepared using oligo d(T) cellulose columns (BRL). Reverse transcription was performed with *Xho*I site-linked poly d(T) primer and 10 mM each dNTPs by reverse transcriptase (Stratagene). The second-strand cDNA was synthesized with 10 mM dNTPs, RNaseH and DNA polymerase I. An *Eco*RI adapter was ligated to the double-stranded cDNAs, followed by digestion with restriction enzyme *Xho*I. The *Eco*RI-*Xho*I cDNA fragment was inserted to λZAPII vector (Stratagene). Packaging was performed with Giga pack II Gold (Stratagene). The constructed cDNA library had a titer of 9.5×10^6 plaque forming units (pfu) with an average insert size of 2.5 kb.

The 1.8 kb CNR1 cDNA fragment obtained from the two-hybrid system was labelled with [α-32P] dCTP by a random-primed labeling method. Using this labelled fragment as a probe, 6.5×10^5 recombinant phage clones were screened. Hybridization was performed at 65 °C in a solution containing 6 x SSC (1 x SSC is 0.15M NaCl, 0.015 M sodium citrate), 5 x Denhardt's solution, 0.1 % SDS, 100 ug/ml salmon testes DNA (Sigma) for 16 h. The membranes were washed in 0.1 x SSC-0.1 % SDS twice at room temperature, twice at 55 °C, and exposed to X-ray films at -70 °C. The recombinant DNAs of positive phage clones were subcloned into pBluescript SK(+) vector.

RT-PCR

For the identification of new CNR family genes, total RNA was isolated from P0 mouse brains, and mRNA was isolated from P30 mouse brains. To test for the expression of each CNR mRNA in the mouse brain, total RNAs were extracted from the olfactory bulb, neocortex, paleocortex, hippocampus, diencephalon, cerebellum, and pons. First strand DNA synthesis was performed using 5 ug RNA and 200 units of reverse transcriptase (BRL;

SuperScript II) primed with oligo d(T) primer at 42 °C for 2 h. After treatment at 55 °C for 10 min, RNaseH (Stratagene) digestion was performed at 37 °C for 30 min. Second-strand DNA was synthesized by DNA polymerase I (Boehringer Mannheim) at 37 °C for 1 h. The products were diluted 50-fold with TE (pH8.0). Five ul of this first-strand cDNA dilute was utilized as template for PCR in a 50 ul reaction mixture containing 20 mM Tris-HCl (pH8.2), 2 mM $MgCl_2$, 10 mM KCl, 0.1% Triton X-100, 0.2 mM each dNTPs, 25 pmol each primers and 2.5 units of Pfu DNA polymerase (Stratagene). The primer sequences for the identification of new CNR family genes were: 5' consensus primer: 5'-CAAACACGGCACCTTCGTG- 3', 3' consensus primer: 5'-CGAGGCAGAGTAGCGCCAGT- 3'. The specific primer sequences for the detection of specific CNR mRNAs in the mouse brain are underlined in the Figure 5B. The amplifications were performed for 30 cycles; each cycle consisted of 45 s denaturation at 95 °C, 25 s annealing at 55 °C and 3 min elongation at 75 °C. The amplified fragments were separated on 1% agarose gels and an approximately 2.3 kb fragment was absorbed in glassmilk and purified. The 3' end of the isolated fragment was phosphorylated by T4 DNA polymerase (New England Biolabs) and cloned into pBluescript II SK(+) (Stratagene) for sequencing.

DNA Sequencing and Sequence Analysis.

Deletion mutants for sequencing full length cDNA and 2.3 kb RT-PCR cDNA fragments were prepared by a size-fractionated uni-directional deletion method (Henikoff, 1984). Briefly, digestions were performed using restriction enzymes yielding 3' protruding overhands and 5' protruding overhangs, whose recognition sites were located on one side of multi-cloning site of the cDNA-containing plasmid. With exonuclease III (Toyobo), 3' to 5' strand deletions were performed with sampling at 1 min intervals, and the samples were collected in within a range of 10 tubes. The samples were then treated with mung-bean nuclease (Toyobo) to produce blunt ends by the deletion of 5'- and 3-' overhangs. Each sample was re-ligated, hence producing a collection of deletion mutants for sequencing.

These plasmids were then introduced into *E. coli* JM109. Sequencing reactions were performed using the dideoxy chain termination method (SequiTherm Long-Read cycle Sequencing Kit-LC; Epicentre Technologies). IRD41 dye conjugated M13 forward (-29) and reverse primers were used. The sequence analyses were performed with an automated DNA sequencer (LI-COR).

Southern Blot Analysis.

Genomic DNA was extracted from C57BL/6 male mouse livers by the SDS-proteinase K method (McKnight, 1978). 10 ug DNA was digested with restriction enzymes *Eco*RI, *Bam*HI or *Sac*I, and separated by electrophoresis on a 0.8% agarose gel. The gels were treated with denaturation buffer (0.5 M NaOH, 1.5 M NaCl) for 45 min and neutralization buffer (0.5 M Tris-HCl [pH 7.5], 3 M NaCl) for 1 h, and the DNA was transferred to nitrocellulose membranes (Schleicher & Schuell; BA85). Hybridization was performed at 65 °C in a solution containing 6 x SSC, 5 x Denhardt's solution, 0.1% SDS, 100 ug/ml salmon testes DNA for 16 h, followed by washing twice in 2 x SSC-0.1% SDS at room temperature for 15 min and twice in 0.1 x SSC-0.1% SDS at 55 °C for 30 min. The *Msc*I-*Xmn*I fragment of CNR1 (nucleotide positions 98 to 491 in Figure 3B) was utilized as the 5' probe and the *Sph*I-*Pst*I fragment of CNR1 (nucleotide positions 2512 to 3030 in Figure 3B) was utilized as the 3' probe in Figure 5A. Sequences derived from the extracellular cadherin repeats 2 and 3 of each CNR comprised the specific probes in Figure 7. The fragments were labeled with [α-32P] dCTP by a random-primed labeling method. Hybridization was carried out at 65 °C in a solution containing 6 x SSC, 5 x Denhardt's solution, 0.1% SDS, 100 ug/ml salmon testes DNA for 16 h. The membranes were washed in 0.1 x SSC-0.1% SDS twice at room temperature, twice at 55 °C, and exposed to X-ray films at -70 °C.

Production of Monoclonal Antibodies and Antiserum

The protruding ends of the *Xho*I-*Nru*I fragment of the CNR1 RT-PCR product,

corresponding to the extracellular domain of CNR1 (amino acid residues 40-680 in Figure 3B), were trimmed and cloned in-frame into the *Sma*I site of pGEX-2T (for the production of GST-CNR1EC fusion protein) and into the *Eco*RV site of expression vectors pET32 (Novagen) (for the production of thioredoxinA(trxA)-CNR1EC fusion protein). The GST-CNR1EC fusion protein was expressed in *E. coli* JM109 and purified using glutathione-Sepharose 4B beads. The purification method using glutathione-Sepharose 4B beads is described in the last paragraph of Materials and Methods.

The trxA-CNR1EC fusion protein was expressed in *E. coli* AD494 and purified as follows. A single *E. coli* colony transformed with pET32-CNR1EC was inoculated into 3 ml of LB medium containing 50 ug ampicillin/ml and incubated overnight in a shaking incubator at 37 °C. The following morning, the bacterial culture was inoculated into 500 ml of LB medium containing 50 ug ampicillin/ml and incubated for 2 to 3 h at 37 °C with shaking at 190 rpm until an OD600 (optical density at 600 nm) reading of 1.0 was attained. Gene expression was induced by the addition of 1 mM IPTG, and incubation was continued for 5 h at 30 °C with shaking at 190 rpm. The bacterial culture was harvested by centrifugation at 5,000 x g for 10 min. The pellet was washed with PBS, and lysed by sonication at 0 °C in 30 ml of HisTag binding buffer (1% Triton X-100, 5 mM imidazole, 0.5 M NaCl and 20 mM Tris-HCl [pH7.9]). The cell lysate was pelleted by centrifugation at 35,000 x g for 15 min and lysed by sonication in 30 ml of HisTag binding buffer containing 6 M urea. The lysate was cleared by centrifugation at 35,000 x g for 15 min, and the supernatant was filtered through a 5 um pore-size syringe filter (Sartorius; Minisart). The filtrate was loaded onto a 5 ml Ni^{2+} -charged HisTag metal chelation resin affinity column. The column was washed with 50 ml (10 volumes) of 6 M urea- containing HisTag binding buffer, 30 ml (6 volumes) of wash buffer (6 M urea, 60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH7.9]), and the trxA-CNR1EC fusion protein was eluted with 30 ml (6 volumes) of elution buffer (6 M urea, 1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH7.9]).

For monoclonal antibody production, 100 ug of the purified trxA-CNR1EC fusion protein was suspended in Gerbu aduvant (Nakarai tesque), and was injected into the peritoneal cavities of pathogen-free 8-week-old female mice every 10 days. Three days after subsequent booster immunization, the spleen was harvested and transferred to serum-free RPMI-1640 medium, and teased into a single-cell suspension by squeezing with angled forceps. Following the removal of debris, cells were dispersed through a fine-mesh metal screen. The cells were centrifuged at 500 x g and were fused with freshly cultured P3UI mouse myeloma cells at a 5:1 ratio by the polyethylene glycol method (Bler et al., 1980). Fused cells were selected in HAT medium (RPMI-1640 containing 10% FCS, 100 uM hypoxianthine, 100 uM aminopterine, 10 uM thymidine, and recombinant mouse interleukin- 6) in five 96-well plates for 10 days in a humidified 37 °C, 5 % CO_2 incubator. 30 ul of supernatant from each well was removed for ELISA detection. The colonies of fusion protein- positive wells were placed into 24-well plates. Specificity was examined by analysis of Western blot staining patterns of GST-CNR1EC fusion protein and mouse brain extract. Using these procedures, anti-CNR1 monoclonal antibody 6-1B was obtained.

For antiserum production, 1 mg of the purified fusion protein was suspended in Gerbu aduvant, and the suspension was injected into the back of a New Zealand white rabbit. Four weeks after the priming immunization, the rabbit was given booster injections. Three days after the booster injections, antiserum for CNR1 was prepared from the blood of the rabbit.

Subcellular Fractionation of Brain

Brains of adult mice were homogenized with a Dounce homogenizer in 8 volumes of BS buffer (0.32 M sucrose, 10 mM HEPES [pH 7.4], 1 mM pABSF, 1 mM aprotinin, 1 mM pepstatin, 1 mM leupeptin). The homogenate was centrifuged at 1,000 x g for 10 min at 4 °C. The supernatant was collected and centrifuged at 13,800 x g for 20 min at 4 °C. The pellet (P2 fraction) was re-homogenized with a Dounce homogenizer in 3 vol of solution B (0.32 M sucrose, 1 mM $NaHCO_3$ [pH 8.3], 1 mM pABSF, 1 mM aprotinin, 1 mM pepstatin, 1 mM

leupeptin). The re-homogenized P2 fraction was further purified on a discontinuous sucrose density gradient. Eight ml of the re-homogenized P2 fraction was layered on top of tube containing 10 ml each of 0.85, 1.0 and 1.2 M sucrose solutions (all containing 1 mM $NaHCO_3$ [pH 8.3], 1 mM pABSF, 1 mM aprotinin, 1 mM pepstatin, 1 mM leupeptin), and the tube was centrifuged at 82,500 x g for 2 h at 4 °C (Beckman SW 27 swing rotor). The band between 1.0 and 1.2 M sucrose was collected as the synaptosome fraction and diluted with 5 volumes of ice-cold 80 mM Tris-HCl (pH 8.0). An equal volume of ice-cold 1% Triton X-100 was added, the diluted material was rotated for 15 min at 4 °C, and centrifuged at 32,000 x g for 20 min. The pellet was resuspended in 0.5% Triton X-100 buffer (0.5% Triton X-100, 40 mM Tris-HCl [pH 8.0], 1 mM pABSF, 1 mM aprotinin, 1 mM pepstatin, 1 mM leupeptin), and the suspension was centrifuged at 201,800 x g for 1 h. The pellet was used as the postsynaptic density (PSD) fraction.

SDS-PAGE

The *E. coli* samples were boiled with one-fourth volume of 5 x SDS sample buffer (50% sucrose, 15% SDS, 15% 2-mercaptoethanol, 0.1 M Tris-HCl [pH 6.8], and 1.5% BPB) for 3 min, and the lysates were applied to an SDS-polyacrylamide gel. To study the developmental profile of CNR1 protein, mouse brains were homogenized with a Dounce homogenizer in 8 volumes of BS buffer (0.32 M sucrose, 10 mM HEPES [pH 7.4], 1 mM pABSF, 1 mM aprotinin, 1 mM pepstatin, 1 mM leupeptin). The homogenates were centrifuged at 1,000 x g for 10 min at 4 °C. The supernatant was collected and centrifuged at 13,800 x g for 20 min at 4 °C. The pellets (P2 fraction) were re-homogenized with a Dounce homogenizer in 5 vol of RIPA buffer (0.15 M NaCl, 20 mM Tris-HCl [pH 7.5], and 1% Triton X-100), and the homogenates were centrifuged at 15,000 x g for 30 min at 4 °C. The protein concentrations of the supernatants were quantitated by the CBB protein assay reagent (Nakarai Tesque) using BSA as the standard. One-fourth volumes of 5 x SDS sample buffer was added and 10 ug of each sample was boiled for 3 min, and the samples were applied to a SDS-polyacrylamide

gel. For studying subcellular distribution of CNR1 protein, 10 ug of P2, synaptosome and PSD fractions were boiled in SDS sample buffer and applied to SDS-PAGE. SDS-PAGE was performed using a 7% or 7.5% gel in a discontinuous Tris-glycine buffer system (Laemmli, 1970). The size markers were obtained from Pharmacia. Gels were stained with CBB buffer (1.25% CBB, 40% methanol, 10% acetic acid).

Western Blotting

Proteins were electrophoretically transferred from an SDS- polyacrylamide gel to a nitrocellulose filter by the method of Towbin et al. (Towbin et al., 1979). The nitrocellulose filters were treated with 10% skim milk in TBS (150 mM NaCl, 50 mM Tris-HCl [pH 7.5]) for blocking, and incubated with 3% skim milk -TBS diluted antibody or antiserum for 1 h at room temperature, followed by treatment with biotinylated sheep anti-mouse IgG (Amersham), biotinylated sheep anti-rat IgG (Amersham) or biotinylated sheep anti-rabbit IgG (Vector) for 1 h at room temperature. After the immunoreaction, the filters were washed with TBS for 10 min 3 times and incubated for 30 min with streptoavidine-conjugated alkaline phosphatase (Amersham) in 3% skim milk-TBS. The filters were washed in TBS for 15 min 3 times, and were developed in alkaline phosphatase buffer (10 mM ethanolamine, 5 mM $MgCl_2$, pH9.5) containing 1.67 g/ml of NBT (Sigma) and 0.83 g/ml BCIP (Amresco).

Immunoprecipitation

Adult mouse brain was homogenized with a Dounce homogenizer in 8 volumes of BS buffer (0.32 M sucrose, 10 mM HEPES [pH 7.4], 1 mM pABSF, 1 mM aprotinin, 1 mM pepstatin, 1 mM leupeptin). The homogenate was centrifuged at 1,000 x g for 10 min at 4 °C. The supernatant was collected and centrifuged at 13,800 x g for 20 min at 4 °C. The pellet (P2 fraction) was re-homogenized with a Dounce homogenizer in 10 vol of RIPA buffer (0.15 M NaCl, 1% Triton X-100, 20 mM Tris-HCl [pH 7.5], 1 mM pABSF, 1 mM aprotinin, 1 mM pepstatin, 1 mM leupeptin, and 1 mM Na_3PO_4) and the homogenate was left on ice for 15

min, followed by centrifugation at 15,000 x g for 30 min at 4 °C. The supernatant was collected and incubated with 100 ul of a 50% suspension of protein G-Sepharose 4B beads for 2 h at 4 °C by gentle rotating to remove proteins bound to the beads non-specifically. The supernatant was collected and incubated overnight with γC3 anti-Fyn mouse monoclonal antibody bound to protein G-Sepharose 4B beads. The beads were centrifuged at 1,000 x g at 4 °C for 1 min and washed five times with 20 vol of RIPA buffer. The bound proteins were eluted by 50 ul of 2 x SDS sample buffer for SDS-PAGE analysis.

Bacterial Expression of GST Fusion Proteins and ELISA Binding Assay

The targeted Fyn DNA sequences were amplified by PCR. Each pair of oligonucleotide primers was tagged with *Bam*HI and *Eco*RV sites to facilitate oriented, in-frame cloning into the pGEX-2T expression vector (Pharmacia). A fragment of human Fyn cDNA, encoding amino acid residues 1-264 (Semba et al., 1986), corresponding to the unique-SH3-SH2 domain, and a fragment encoding amino acid residues 82-145, corresponding to the SH3 domain, were amplified by PCR with *Pfu* thermostable DNA polymerase (Stratagene), separated on 1% agarose gels and purified using glassmilk (Qiagen; Qia-EX). The DNAs were digested with *Bam*HI and *Eco*RV. The fragments were cloned between the *Bam*HI and *Sma*I sites of pGEX-2T to express glutathione-S transferase-Fyn unique-SH3-SH2 fusion protein (GST-Fyn-U32) and glutathione-S transferase-Fyn-SH3 protein (GST-Fyn-3). The protruding ends of the *Sph*I-*Spe*I fragment of CNR1 (nucleotide positions 2512 to 3384 in Fig 3B) were filled in and blunt end-ligated into the *Sma*I site of pGEX-2T to produce glutathione-S transferase-CNR1 cytoplasmic domain (GST-CNR-CP). The control GST protein was produced from pGEX-2T. *E. coli* JM109 was transformed with each plasmid, and the bacteria were grown in 500 ml of LB medium containing 100 ug ampicillin/ml at 37 °C with shaking at 190 rpm until an OD600 reading of 1.0 was attained. Gene expression was induced by the addition of 1 mM IPTG, and the incubations were continued for 2 h at 37 °C with shaking at 190 rpm. The bacterial cultures were harvested by centrifugation at 5,000 x g

for 10 min. The pellets were washed with PBS (0.14 M NaCl, 2.7 mM KCl, 1.4 mM KH_2PO_4 [pH 7.4]), and resuspended in 30 ml of *E. coli* lysis buffer (PBS containing 1% Triton X-100, 1 mM pABSF, 1 mM aprotinin, 1 mM pepstatin, and 1 mM leupeptin). The bacteria were lysed by sonication at 0 °C. The lysates were cleared by centrifugation at 35,000 x g for 15 min. The supernatants were incubated with 1 ml of 50% glutathione-Sepharose 4B beads (Pharmacia) for 30 min at 4 °C. The beads were extensively washed five times with the lysis buffer, and the fusion proteins were eluted by glutathione elution buffer (5 mM glutathione [reduced form], 0.5 M NaCl, 10 mM Tris-HCl [pH 8.0]). The eluted fusion proteins were dialyzed against PBS and concentrated by ultrafiltration.

Five ug/ml of GST-CNR-CP fusion protein and control GST protein in coating buffer (5 mM Na_2CO_3 35 mM $NaHCO_3$) were loaded on an ELISA plate (Nunc; Maxi-Sorp) at 100 ul per well. After incubation at room temperature for 1 h, the plates were washed four times in PBS, and blocked with 400 ul of 5% skim milk-PBS. After washing four times in PBS, serial 3:4 dilutions of GST-Fyn-U32 and GST-Fyn-3 proteins in 5% skim milk-PBS beginning at 20 ug/ml were added in duplicate rows at 100 ul per wells and allowed to bind for 1 h at room temperature. Plates were washed four times in PBS, and incubated with a rat anti-Fyn monoclonal antibody γC3 (Yasuda et at, in preparation) for 1 h at room temperature. After washing four times in PBS, the plates were incubated with biotinylated sheep anti-rat IgG (Amersham) for 1 h at room temperature. Plates were washed four times in PBS, and the colorimetric reaction was performed in substrate buffer containing 1 mg/ml PNPP (Sigma) for 20 min. The colorimetric reaction was stopped with 3 N NaOH, and the Abs 415 was determined using a microplate reader.

Results

Isolation of full-length cDNAs of new cadherin-related genes

Before obtaining the full-length cDNA of the CNR1 gene, we performed Northern blot analysis. A transcript of 5.5 kb was detected in the forebrain and cerebellum (Figure 2A). It was present from embryonic day 15 to postnatal day 60 in the total brain (Figure 2B). Expression of this mRNA in the brain was highest at postnatal day 10, and reduced at the adult stage.

To obtain a full-length cDNA of the mouse CNR1 gene, I constructed a C57BL/6 mouse brain cDNA library, and performed cDNA screening by probing with the 1.8 kb CNR1 fragment obtained by the two-hybrid system. I successfully isolated several clones (Figure 3A). Clones B6-1 and B6-3 contained sequences identical to that of CNR1. The longer B6-1 clone contained almost the entire length of CNR1 cDNA. The complete cDNA sequence and deduced amino acid sequence of CNR1 are shown in Figure 3B. Mouse CNR1 cDNA was 5323 bp in length. The amino acid sequences deduced from the nucleotide sequence revealed a single long open reading frame encoding a protein of 948 amino acids (a calculated molecular weight of 103,142 Da), starting at a site 78 bp downstream from the 5'-end of the cDNA. The coding region was followed by 2400 bp of 3'-untranslated region. A polyadenylation signal sequence was found 17 bp upstream from the polyadenylate tail. A signal sequence of 26 amino acids was located at the amino terminus of the CNR1 protein, and another hydrophobic stretch spans amino acid residues 693 to 721 of CNR1 protein, which presumably functions as a transmembrane region. There were 3 possible N-linked glycosylation sites in the putative extracellular domain of CNR1. The putative extracellular domains of CNR1 were divided into six subdomains comprised of approximately 100 amino acid residues forming internal repeats. These domain structures are characteristic of typical cadherin repeats. At the first cadherin repeat EC1, there was an RGD (Arg-Gly-Asp) sequence, which is known as an integrin binding motif (Piershbacher and Ruoslahti, 1984).

In the transmembrane and cytoplasmic domain, five cysteine residues appeared at regular intervals spanning 18, 9, 9, and 18 amino acid residues, respectively. In the intracellular domain, there were five PXXP proline-rich motifs for putative SH3 region-mediated protein interactions. A lysine-rich sequence was also found in the C-terminal cytoplasmic region.

Isolation and sequence analysis of CNR2 cDNA

Nucleotide sequences of clones B6-2 and B6-4 were different from those of clone B6-1 and clone B6-3 (Figure 3A). B6-2 and B6-4 both encoded the same protein. We named this protein CNR2. The complete cDNA sequence and deduced amino acid sequence of CNR2 are shown in Figure 3C. Mouse CNR2 cDNA was 5369 bp in length, and its translation revealed a single long open reading frame encoding a protein of 947 amino acid residues in length (a calculated molecular weight of 102,312 Da), starting at a site 127 bp downstream from the 5'-end of cDNA. The coding region was followed by 2400 bp of 3' untranslated region. A signal sequence of 26 amino acids was located at the N-terminus of the CNR2 protein, and another hydrophobic stretch spans amino acid residues 892 to 720 of CNR2, which presumably functions as a transmembrane region. CNR2 contained the same features as CNR1: The putative extracellular domains of CNR2 were divided into six subdomains comprised of approximately 100 amino acid residues forming internal repeats. These domain structures were characteristic of typical cadherin repeats. Three possible N-linked glycosylation sites were located in the putative extracellular domain. The first cadherin repeat EC1 contained an RGD sequence. In the transmembrane and cytoplasmic domain, five cysteine residues appeared at regular intervals spanning 18, 9, 9, and 18 amino acid residues. In the intracellular domain, there were five PXXP proline-rich motifs for putative SH3 region-mediated protein interactions. A lysine-rich sequence was also found in the C-terminal cytoplasmic region in CNR2. Hence, these features are strikingly similar to those of CNR1.

Comparison of CNR1 and CNR2

Figures 4A and 4B show the alignment of CNR1 and CNR2 at the nucleotide level and at the amino acid level, respectively. CNR1 and CNR2 were closely related (88.4% identity at the nucleotide sequence level, 78.4% identity and 87.0% similarity at the amino acid level between clones B6-1 and B6-4). There were many well-conserved sequences between CNR1 and CNR2. In particular, the cDNA sequence of CNR1 downstream from position 2463 was completely identical to that of CNR2 cDNA downstream from position 2518.

Comparison of CNR1 and CNR2 with other cadherins.

The deduced amino acid sequences of the internal cadherin repeats in the extracellular domains of CNR1 and CNR2 were aligned with those of protocadherin 2 (Sano et al., 1993), and N-cadherin (Figure 4C). The negatively charged amino acids containing the DXD, DRE, and DXNDNAPXF sequence motifs (all are underlined with thick lines in the consensus sequence shown at the bottom of Figure 4C), which are the major conserved motifs among typical cadherin families, were well-conserved in the internal repeats of CNR1 and CNR2. The repeats of CNR1 and CNR2 contained the more conserved motifs which are typically found in protocadherins. The DXDXGXN and AXDXGXPXL motifs (all are underlined with thin lines in the consensus sequence shown at the bottom of Figure 4C), and the glycine residue in the middle of repeat (except for repeats EC2 and EC4) were the additional conserved features among CNR1, CNR2 and the protocadherin family. The single leucine residue (indicated by # in the consensus sequence at the bottom) was the prominent conserved features between CNR1 and CNR2. Figure 4D shows the schematic structural comparison of CNR1 and CNR2 with protocadherins and classic cadherins. The locations of the RGD motif, five cysteine residues appearing at regular intervals spanning 18, 9, 9, and 18 amino acid residues, and five PXXP proline-rich motifs for putative SH3 region-mediated protein interactions corresponded well in CNR1 and CNR2. Of the known cadherin proteins, that which exhibited the highest identity to the extracellular domains of CNR1 and CNR2

was protocadherin 2 (~37% amino acid sequence identity). The amino acid sequence identity of the extracellular domain of CNR1 and CNR2 to classic cadherins was ~23%. No similar protein sequences were found in the CNR1 and CNR2 cytoplasmic domain.

CNRs constitute a new cadherin-related multi-gene family

The sequences of CNR1 and CNR2 were well-conserved. To investigate other potential gene homologues, I performed genomic Southern blot analyses using the conserved 5' and 3' sequences between CNR1 and CNR2 as probes (Figure 5A). The probes used are shown in Figure 4A. The 5' probe hybridized to approximately 15 genes, but the 3' probe hybridized to 2 or 3 genes after genomic DNA digestion with each restriction enzyme. Mouse genomic library screening using a 5' probe resulted in the detection of approximately 20 positive clones per genome (Data not shown). These results revealed that the CNR family consists of multi-genes, at least in the 5' region. To identify these putative members of the CNR family, I performed RT-PCR with two primers derived from the EC1 and cytoplasmic domains, containing highly conserved sequences between CNR1 and CNR2 (shown in Figure 4A). RT-PCR was performed using total RNA from postnatal day 0 (P0) brains and mRNA from P30 brains. The expected-length products of approximately 2.3 kb were cloned into the pBluescriptII SK(+) vector. Sequence analysis was performed on 40 clones from P0 brain and 32 clones from P30 brain. From these clones, I have found 6 new sequences designated CNR3, CNR4, CNR5, CNR6, CNR7, and CNR8 (Table 1). From P0 brains, 28 individual CNR8 clones were identified among the 40 clones analyzed, but none of the 32 P30 clones were CNR8. On the other hand, the CNR3, 4, 5, 6 and 7 clones were identified only from mRNA prepared from the P30 brain. Figures 5B and 5C show alignments of nucleotide and deduced amino acid sequences of CNR1 to CNR8, respectively. The similarity of each CNR at the amino acid level varied from 53% to 80%. The RGD motif in the EC1 domain and characteristic cysteine repeats of the transmembrane and cytoplasmic domains were well-conserved among all CNRs. Although CNR1 and CNR2 contain five PXXP amino acid

sequence motifs, in CNRs 3 through 7, the PXXP motif occurs six times, while in CNR8, four motifs are found. Specific sequences in each CNR were identified in the EC2, EC3, N- and C-termini of the EC6 and cytoplasmic domains. The sequences at the C-terminus of the cytoplasmic domain recur among the CNR. Figure 5D shows the phylogenetic tree of cadherin-related proteins deduced from amino acid sequence similarity (Saitou and Nei, 1987). The obtained eight CNRs form a new branch. Hence, the CNRs constitute a new cadherin-related multi-gene family.

mRNAs of eight CNR genes are expressed in the mouse brain, and their respective genes correspond to different loci of the mouse genome.

To confirm that a corresponding mRNA for each CNR gene identified by RT-PCR is expressed in the brain, I performed RT-PCR using specific primers. The primer sequences are shown in Figure 5B. Total RNA were prepared from the various regions of adult mouse brain, and RT-PCR reactions were performed. As shown in Figure 6A, all bands detected were of the expected size. Although the levels of CNR4 and CNR8 mRNA were very low in the adult brain, these results demonstrate that each CNR mRNA is expressed in the brain. Furthermore, these mRNAs are localized to various regions of the brain. To examine whether each CNR gene corresponds to a distinct locus on the genome, I performed genomic Southern blot analysis using non-cross-hybridizing probes for each CNR gene (Figure 6B). Each specific probe was derived from the EC2 and EC3 domains. Using these probes, one or two bands of distinct sizes were detected. These results confirm the existence of each CNR gene on the genome, and their correspondence to different loci. FISH (fluorescent *in situ* hybridization) analyses for several CNR genes indicated that all CNR genes mapped to the same region of mouse chromosome 18 (examined by Dr. Matsumoto). Therefore the CNRs are located closely to one another on the same region of the chromosome, but they map to different loci.

Characterization of anti-CNR1 antibodies

To investigate the function of CNRs, I have attempted to obtain antibodies that recognize the CNR proteins. Antigens to immunize mice and rabbit for monoclonal antibody and antiserum production, respectively, against CNR1 were produced by inserting the *Xho*I-*Nru*I fragment of the CNR1 RT-PCR product into the pET32 expression vector. This fragment corresponds to the extracellular domain of CNR1. This vector was used to transform *E. coli* AD494. Expression of the gene fragment was induced by the addition of IPTG, and the thioredoxinA (trxA)-CNR1EC fusion protein was purified using a Ni^{2+} -charged HisTag metal chelation resin affinity column (Figure 7A). The purified fusion protein was injected into the rabbit and into mice, and rabbit antiserum and mouse monoclonal antibodies were obtained as described in Materials and Methods. The same *Xho*I-*Nru*I DNA fragment of the CNR1 RT-PCR product was also inserted into the pGEX-2T expression vector for the production of GST-CNR1EC fusion protein. This fusion protein was used to screen the anti-CNR1 antibody. Figure 7B shows the reactivity of antiserum (lanes 3 and 4) and monoclonal antibody 6-1B (lanes 5 and 6). Although several non-specific bands were detected, a prominent band was visible from *E. coli* extract containing IPTG-induced GST-CNR1EC fusion protein (lanes 4 and 6), while merely a small, thin band was detectable in extract prepared from uninduced *E. coli* cells (lanes 3 and 5).

Developmental Change of CNR1 Protein Expression

To study the developmental profile of CNR1, I examined the developmental changes of CNR1 protein expression in the brain by Western blot analysis using monoclonal antibody 6-1B. As shown in Figure 7C, an approximately 160 kD protein was detected. The level of expression of this protein was very low in E17 brains, becoming more detectable after birth, and gradually increased in subsequent stages up to post natal day 7, at which time CNR1 protein was maximally expressed. CNR1 expression was reduced at postnatal day 30, whereas Fyn expression is highest after birth and gradually decreases. The CNR1 protein

levels were consistent with the mRNA levels detected in Figure 2B.

CNR1 protein concentrates in the PSD fraction.

It is known that Fyn is enriched in the postsynaptic density (PSD) fraction (Grant at al. 1992; Yasuda, in preparation). Since CNR1 was identified as a Fyn-associated protein, I next examined whether CNR1 was enriched in the PSD fraction. Figure 8A shows the SDS-PAGE analysis of the P2, synaptosome, and PSD fractions prepared from adult mouse brains. First, the purity of the fractions was quantitated using anti-NMDA receptor ε2 subunit antibody because NMDA receptor ε2 has been shown to be highly concentrated in the PSD fraction (Moon et al., 1994). In addition, Fyn protein levels were quantitated on the same Western blot (Figure 8B). Figures 8C and 8E shows Western blot analysis using anti-CNR1 antiserum and 6-1B monoclonal antibody. The 160 kD band corresponding to CNR1 protein was highly concentrated in the PSD fraction. The CNR1 band was not detectable using pre-immune serum (Figure 8D).

CNR1 associates with Fyn *in vivo* and *in vitro*

Because CNR1 was identified as a Fyn-associated protein using the yeast two-hybrid system, I performed immunoprecipitation analysis using the mouse anti-Fyn monoclonal antibody γC3, which recognizes the SH3 domain of Fyn (Yasuda et al., in preparation). The γC3 antibody was coupled to protein G beads and incubated with mouse brain extract. The immune complexes were resolved by SDS-PAGE and transferred to a nitrocellulose filter. The blot was incubated with anti-CNR1 antiserum or with 6-1B monoclonal antibody. As shown in Figure 9, the immune complexes contained a 160 kD protein which was detectable using both antiserum and 6-1B monoclonal antibody (lanes 3 and 6). As further confirmation, I performed ELISA assays using polypeptides containing the Fyn regulatory region and the CNR cytoplasmic domain, which are conserved in CNR types. These protein domains were produced in *E. coli* as GST fusion proteins, and were purified using Glutathione Sepharose

4B beads as described in Materials and Methods. Figure 10A shows the schematic representation of GST-Fyn-U32, which contains the unique, SH3, and SH2 domains of Fyn, and GST-Fyn-3, which contains only the SH3 domain of Fyn fused to GST. The GST-CNR-CP, containing the cytoplasmic domain conserved in all types of CNR, was coated onto each well. After washing the plate, serially-diluted GST-Fyn-U32 and GST-Fyn-3 proteins were incubated, and binding activities were evaluated using monoclonal antibody γC3. As shown in Figure 10B, GST-CNR-CP and GST-Fyn fusion proteins bound in a dose-dependent manner. The GST-Fyn-U32 bound two-fold more strongly than GST-Fyn-3 to GST-CNR-CP. Based on these results, I have demonstrated that CNR1 directly associates with Fyn *in vivo* and *in vitro*.

Discussion

Structure of the CNR family as a cadherin superfamily

CNR1 protein is a new member of the cadherin superfamily. The cadherin superfamily includes classical cadherins, numbered cadherins, desmocollins, desmogleins, protocadherins, ret proto-oncogene, the product of the *Drosophila* gene fat and others. To date, more than 30 cadherin-related genes have been identified and cloned. As a superfamily, the cadherins function in selective cell adhesion in a wide variety of systems (Takeichi, 1991; Gumbiner, 1996) and control the development, maintenance, and regeneration of tissues. In particular, cadherins are directly involved in morphogenic regulation, intercellular adhesion, the stratification of cells into layers within tissues, embryo compaction, and neural tube formation (Geiger and Ayalon, 1992; Kintner, 1992). The classical cadherins, which are the most well-studied subfamily, include N-cadherin (neural cadherin, ACAM), E-cadherin (epitherial cadherin, uvomorulin), P-cadherin (placental cadherin), R-cadherin (retinal cadherin) and some others. They mediate calcium-dependent cell adhesion through homophilic binding interactions. For example, E-, or N-cadherin-expressing cells bind to E-, or N-cadherin-expressing cells, respectively, but E-cadherin-expressing cells do not bind to N-cadherin-expressing cells. The extracellular domain can be divided into five repeat domains designated EC1 to EC5. Repeat domains EC1 through EC4 contain conserved DXNDNXPXF and DXD motifs which constitute putative calcium-binding sequences (Ringwald et al., 1987; Ozawa et al., 1990a). These calcium-binding motifs are conserved among the EC2 to EC5 domains of the CNR family. Thus, the CNR family may also function as calcium-dependent cell adhesion molecules.

Various studies of classical cadherins have revealed that binding specificity resides in the EC1 cadherin repeat domains (Nose et al., 1990; Shapiro et al., 1995a). Since the EC1 domains are well-conserved among the CNR family, members of this family may bind heterophilically with one another. On the other hand, the EC2 and EC3 domains of this

family are not conserved. It is therefore possible that the EC2 and EC3 domains of the CNR family may contribute to homophilic binding activity. This issue should be further examined. The HAV tripeptide between the DRE and DXNDNXPXF motifs of the EC1 domain, which is conserved in the classical cadherins, has been suggested to be responsible for cell adhesion activity (Blaschuk et al., 1990). Between the DRE and DXNDNXPXF motifs of the CNR family EC1 domain, an HLE tripeptide is conserved instead of the HAV motif characteristic of classical cadherins. This indicates that the cell adhesion activity of the CNR family may be different from that of classical cadherins. Interestingly, the RGD motif in the EC1 domain is conserved among members of the CNR family. The RGD tripeptide was first described as the cell adhesion site of fibronectin with integrins. The integrins, cell-surface extracellular matrix receptors (Piershbacher and Ruoslahti, 1984), are widely recognized among proteins involved in cell adhesion. Synthetic RGD tripeptides bind to integrin and inhibit cell adhesion and migration of cultured neuronal cells (Cardwell and Rome, 1988; Hatta et al., 1994). The RGD motif is not found among previously-identified cadherin-related proteins. The CNR family is the first known cadherin family containing the RGD motif. L1, one of the neuronal cell adhesion molecules that show calcium-independent homophilic binding, has an RGD motif and can bind to integrin subdomain VLA-5 (Ruppert et al., 1995). It has recently been reported that the $\alpha 8$ integrin subunit localizes to the synaptic junction (Einheber et al., 1996). Since the RGD motif of the CNR family is located in the N-terminal region of the extracellular domain, this motif might function in extracellular protein-protein interactions. Hence, the RGD motif of the CNR family may have the potential to bind to integrins, resulting in heterophilic binding of CNR and integrin in the developing synaptic junction.

It is known that the cytoplasmic domain of classical cadherins exhibit a high degree of homology, and the search for the biological function associated with this structural conservation led to the identification of catenins as cytoplasmic anchorage proteins (Ozawa et al., 1989; Nagafuchi and Takeichi, 1989). The catenins are mediators of intracellular signaling and associate with the cytoskeleton. Pulse-chase experiments and the analysis of

different non-ionic detergent cell lysates indicated that β-catenin binds directly to the cytoplasmic domain of E-cadherin (Ozawa and Kemler, 1992), and this binding is important for mediating the cell adhesion activity of classical cadherins (Nagafuchi and Takeichi, 1988; Ozawa et al., 1990b). The cytoplasmic domain of the CNR family is well-conserved, but is not homologous to that of classical cadherins, protocadherins or other known cadherins. Considering the unique structural features of the CNR family, the cell adhesion mechanism and cytoplasmic signal transduction of the CNR family may be different from those of classical cadherins.

Within the consensus sequence of the cytoplasmic domain of the CNR family, there are four PXXP motifs. This proline-rich motif is the minimal consensus sequence for SH3-binding sites and has been discovered in a number of proteins involved in signaling pathways (Ren, et al., 1994; Saksela et al., 1995; VanderNoot et al., 1995; Alexandropoulos et al., 1995). The SH3 domain was first discovered by cDNA cloning of PLC-γ and v-Crk, which contained domains of homology to the Src family (Mayer et al, 1988). The SH3 domain is present in many proteins that are important for signal transduction, and is the key domain for intracellular signal transduction (Koch, et al. 1991). Fyn also contains a SH3 domain. I have demonstrated using ELISA assays, that the SH3 domain of Fyn binds to the consensus sequence of the cytoplasmic region of the CNR family. In mouse brain extracts, CNR1 was also co-precipitated using anti-Fyn antibody. Therefore, it is likely that the CNR family associates with Fyn in the mammalian brain, and this interaction is mediated through the PXXP sequence of CNR and the SH3 domain of Fyn.

The cytoplasmic and the transmembrane regions of the CNR family contain five conserved cysteine residues appearing at regular intervals spanning 18, 9, 9, and 18 amino acid residues. Two cysteines can function in protein-protein interactions by forming a covalent an ionic bond with a cation. Regularly spaced cysteine residues may form homo or hetero polymers of the CNR family via lateral interactions. In C-cadherin, it has been reported that lateral dimerization is required for homophilic binding activity (Brieher, et al.,

1996). The crystal structure of the N-terminal domain of N-cadherin revealed that the cadherin repeat domains form parallel dimers (Shapiro et al., 1995). The dimers are predicted to form through the lateral interaction of two cadherins extending from the same cell surface. In this model, the lateral dimer interfaces are formed through hydrophobic interactions between the two monomers. In the CNR family, dimerizations producing homo- or hereto-dimers may occur through the cytoplasmic cysteine residues. If a hetero-multimer can form and be recognized specifically for cell adhesion, twenty individual types of CNR molecules can result in the production of 400 or 8000 specific recognition units through the formation of dimers or trimers, respectively.

A lysine-rich sequence is also conserved in the cytoplasmic C-terminal domain of the CNR family. It has been reported that lysine-rich sequences can bind to actin or to DNA promoter sequences (Yamamoto, 1989). Since members of the CNR family have a membrane-protein structure, this region may bind to actin filaments. It has been demonstrated that the catenin-mediated interaction of cadherin with actin filament is necessary to exhibit cell adhesion activity (Ozawa et al., 1990). Therefore, this lysine-rich sequence of the CNR family may be necessary for exhibiting cell adhesion activity through cytoskeletal interaction.

Presence of CNR1 in the PSD fraction

In this study, I have established anti-CNR1 antiserum and anti-CNR1 monoclonal antibody, 6-1B. These antibodies detected a 160 kD protein which was concentrated in the PSD fraction of the adult mouse brain. It is known that the PSD contains submembranous cytoskeletal elements of the postsynaptic structure of central synapses, observed under electron microscopy. Studies of the proteins in the PSD have been aided by the development of cell fractionation techniques (Cohen et al., 1977; Carlin et al., 1980; Gurd et al., 1982). The PSD fraction contains many proteins important for synaptic functions, such as NMDA receptor ε1 and ε2 subunits (Moon et al., 1994; and Figure 8B), αCaM kinase II (Kennedy et

al., 1983), PKCγ (Suzuki et al., 1993), Fyn (Grant et al., 1992; Yasuda, in preparation; and Figure 8B), and others (reviewed by Kennedy, 1993). Furthermore, mice in which each of these gene is disrupted shows reduction of LTP, and abnormalities in learning and behaviors, indicating that those proteins present in the PSD fraction are critical in synaptic plasticity. Since CNR1 is concentrated in the PSD fraction together with Fyn, this is a candidate cell adhesion molecule that is relevant to synaptic plasticity and behavioral regulation. The mechanisms contributing to synaptic forms of plasticity are still a topic of intense debate. One proposed hypothesis is that these specific patterns of activity could lead to modifications of synapse structures (Geinisman, 1993; Edwards, 1995), and eventually, changes in synaptic connectivity (Bailey and Kandel, 1993; Weiler et al., 1995). In support of this idea, numerous morphological studies have provided evidence that neuronal activity triggered through environmental stimulation, kindling, or electrical stimulation, is associated with modifications of dendritic arborizations, spine densities, or synapse morphology. In particular, the possibility has been raised that one of the initial steps following LTP induction involves a perforation of the postsynaptic receptors and presynaptic active zones (Geinisman, 1993; Buchs and Muller, 1996). In line with the hypothesis of structural re-organization of synapses by synaptic activity are the recent findings that NCAM, a cell adhesion molecule, and NCAM-linked polysialic acid (PSA) also contribute to synaptic plasticity (Mayford et al., 1992; Muller et al., 1996). Recent reports indicate antibodies against L1 also interfere with LTP induction in the hippocampus (Luthi et al., 1994). These indicate that members of the cell adhesion molecule families, in particular the immunoglobulin superfamily, may be critically important for, and contribute to the mechanisms of synaptic plasticity. Structural studies (Shapiro et al., 1995b; Overduin et al., 1995) have suggested that the five EC domains of classical cadherins share in common a folding topology that is remarkably similar to that of immunoglobulin-like molecules, despite the absence of primary sequence relationships between the cadherins and immunoglobulin gene superfamily members.

The synaptic complex is first and foremost built around an adhesive junction, and the

complex is quite similar to the desmosome and the adherence junction of epithelia (Peters et al., 1991), Both the desmosome and the adherence junction mediate adhesion via cadherins. A model is proposed whereby the cadherins function as primary adhesive moieties between pre- and post-synaptic membranes in the synaptic complex (Fannon and Colman, 1996). Similarities in ultrasrtructure between the adherence junction and the synaptic complex of nervous tissue are noted in electron microscopical studies, and when it becomes clear that the adherence junction is a classical cadherin-mediated organelle, the synaptic complex is also cadherin-based. Actually, Fannon and Colman have reported that N- and E-cadherin are localized to synaptic complexes in a mutually exclusive distribution, i.e., their distributions do not co-localize or overlap at the same synaptic complexes. The differential distribution of cadherins may lock in nascent synaptic connections. The researchers speculate that the cadherins may in part fulfill many of the conditions of the Sperry hypothesis, which postulates the existence of matching chemical specificities for neurons that link up with one another during development (Sperry, 1963). They report that many synaptic junctions in which these two cadherins are not expressed are still present, implying that other cadherins may be functioning at these synaptic junctions. Each classical cadherin family and many different cadherins, such as the protocadherin family (Sano et al., 1993; Sago et al., 1995), have been described to be expressed in brain tissue. It has been reported that at least 10 cadherins are expressed in the developing vertebrate brain (Redies, 1995). These cadherins are locally expressed: in particular, in developing brain nuclei, fiber tracts, and neural circuits. Since CNR1 is concentrated in the PSD fraction with Fyn, CNR1 may function as an adhesion molecule in synaptic junctions. Diversity within the CNR family may induce specific synaptic connections, and the connections may function in neural circuits. Therefore, members of the CNR family may also be candidate molecules based on the Sperry hypothesis.

The diversity of CNR gene family

Gene cloning and Southern blot analysis of genomic DNA has provided an estimate of the size of the CNR family. Mouse genomic library screening using a 5' probe derived from sequences well-conserved between CNR1 and CNR2 (see Figure 4A) detected approximately 20 positive clones per genome. This value is in accord with the results of genomic Southern blot analysis using the same probe which identified approximately 15 discrete genes (Figure 5A). I have been able to clone eight individual CNR cDNAs. It is likely that there are more CNR genes that remain to be identified.

Recent studies have demonstrated the existence of a large degree of diversity among genes encoding olfactory receptors and pheromone receptors in mammals. The olfactory receptors in mammals are encoded by large multi-gene families of about 1000 genes (Buck and Axel, 1991; Permentier et al., 1992; Ben- Arie et al., 1994; Dulac and Axel, 1995). Each of the different receptor genes are expressed in different neurons. Neurons producing olfactory receptors, although randomly distributed among the domains of olfactory epithelium and therefore responsive to a given odorant, project their axons to one or a small number of discrete loci of glomeruli within the olfactory bulb (Vassar et al., 1994; Ressler et al., 1994) and generate synaptic formation. A swapping of the odorant receptor genes by gene targeting techniques shows that odorant receptors converge at one glomerular location in the olfactory bulb (Mombaerts P, 1996). This indicates that diverse odorant receptors binding to different odors function as axonal guidance receptors to find the appropriate target. These results suggest that the diversity of connections on neural networks might be generated and organized by the diversity of some neuronal receptors. Thus, diversity among homologous receptor molecules may be involved in specified or selective cell to cell recognition, including cell migration, pathfinding, synaptogenesis, sprouting and regeneration. Since CNR1 is concentrated in the PSD fraction, diversity of the CNR family may function in differential synaptogenesis, sprouting or regeneration.

Association with Fyn in the brain

Since CNR1 is co-immunoprecipitated with anti-Fyn antibody (Figure 9), and the cytoplasmic consensus sequences of the CNR family can bind to the SH3 domain of Fyn, within the CNR family CNRs1 through 8 (and possibly other yet to be discovered members) might be associated with Fyn in vivo. Studies of Fyn-deficient mice revealed that Fyn plays critical roles in brain formation and in determining mice behavior (Grant et al., 1992; Yagi et al., 1993a ;Miyakawa et al., 1994). In particular, it has been demonstrated that Fyn-deficient mice are impaired in synaptic function (Grant et al. 1992). I therefore speculate that physiological coupling between molecules of the CNR family and Fyn results in the regulation of behavior through the generation or regeneration of neural circuits in mammals.

Conclusions

I have demonstrated that CNR1 is a new receptor protein that associates with Fyn, and CNR1 belongs to a novel CNR family, whose members exhibits structural homology to members of the cadherin superfamily. CNR1 is found to be enriched in the PSD fraction with Fyn. I speculate that the CNR family contributes to synaptic formation or plasticity. I believe that future studies of the CNR family will reveal new insights into synaptic formation related to behavioral controls, learning and memory, emotion, recognition, or locomotion through the elucidation of the molecular mechanisms for neural circuit and synapse formation.

References

Abeliovich, A., Chen, C., Goda, Y., Silva, A. J., Stevens, C. F., and Tonegawa, S. (1993). Modified hippocampal long-term potentiation in PKC gamma-mutant mice. Cell 75, 1253-62.

Alexandropoulos, K., Cheng, G., and Baltimore, D. (1995). Proline-rich sequences that bind to Src homology 3 domains with individual specificities. Proc Natl Acad Sci U S A 92, 3110-3114.

Atashi, J. R., Klinz, S. G., Ingraham, C. A., Matten, W. T., Schachner, M., and Maness, P. F. (1992). Neural cell adhesion molecules modulate tyrosine phosphorylation of tubulin in nerve growth cone membranes. Neuron 8, 831-42.

Atkinson, E. A., Ostergaards, H., Kanes, K., Pinkoski, M. J., Caputo, A., Olszowy, M. W., and Bleackley, R. C. (1996). A physical interaction between the cell death protein Fas and the tyrosine kinase p59fynT. J. Biol. Chem. 271, 5968-5971.

Bailey, C. H., and Kandel, E. R. (1993). Structural changes accompanying memory storage. Annu. Rev. Physiol. 1993 ;55:397-426.

Bare, D. J., Lauder, J. M., Wilkie, M. B., and Maness, P. F. (1993). p59fyn in rat brain is localized in developing axonal tracts and subpopulations of adult neurons and glia. Oncogene 8, 1429-36.

Beesley, P. W., Mummery, R., and Tibaldi, J. (1995). N-cadherin is a major glycoprotein component of isolated rat forebrain postsynaptic densities. J. Neurochem. 64, 2288-94.

Beggs, H. E., Soriano, P., and Maness, P. F. (1994). NCAM-dependent neurite outgrowth is inhibited in neurons from Fyn-minus mice. J Cell Biol 127, 825-833.

Ben-Arie, N., Lancet, D., Taylor, C., Khen, M., Walker, N., Ledbetter, D. H., Carrozzo, R., Patel, K., Sheer, D., Lehrach, H., and et al. (1994). Olfactory receptor gene cluster on human chromosome 17: possible duplication of an ancestral receptor repertoire.

Hum Mol Genet 3, 229-35.

Bixby, J. L., and Jhabvala, P. (1993). Tyrosine phosphorylation in early embryonic growth cones. J Neurosci 13, 3421-32.

Blaschuk, O. W., Sullivan, R., David, S., and Pouliot, Y. (1990). Identification of a cadherin cell adhesion recognition sequence. Dev Biol 139, 227-9.

Bourtchuladze, R., Frenguelli, B., Blendy, J., Cioffi, D., Schutz, G., and Silva, A. J. (1994). Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. Cell 79, 59-68.

Brieher, W. M., Yap, A. S., and Gumbiner, B. M. (1996). Laleral dimerization is required for the homophilic binding activity of C-cadherin. J. Cell Biol. 135, 487-496.

Buchs, P.-A., and Muller, D. (1996). Induction of long-term potentiation is associated with major ultrastructural chaanges of activated synpses. Proc. Natl. Acad. Sci. USA 93, 8040-8045.

Buck, L., and Axel, R. (1991). A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell 65, 175-87.

Burkhardt, A. L., Brunswick, M., Bolen, J. B., and Mond, J. J. (1991). Anti-immunoglobulin stimulation of B lymphocytes activates src-related protein-tyrosine kinases. Proc Natl Acad Sci U S A 88, 7410-4.

Cardwell, M. C., and Rome, L. H. (1988). RGD-containing peptides inhibit the synthesis of myelin-like membrane by cultured oligodendrocytes. J Cell Biol 107, 1551-9.

Carlin, R. K., Grab, D. J., Cohen, R. S., and Siekevitz, P. (1980). Isolation and characterization of postsynaptic densities from various brain regions: enrichment of different types of postsynaptic densities. J Cell Biol 86, 831-45.

Chen, C., Rainnie, D. G., Greene, R. W., and Tonegawa, S. (1994). Abnormal fear response and aggressive behavior in mutant mice deficient for alpha-calcium-calmodulin kinase II. Science 266, 291-4.

Cobb, B. S., Schaller, M. D., Leu, T. H., and Parsons, J. T. (1994). Stable association of pp60src and pp59fyn with the focal adhesion- associated protein tyrosine kinase, pp125FAK. Mol Cell Biol 14, 147-55.

Cohen, R. S., Blomberg, F., Berzins, K., and Siekevitz, P. (1977). The structure of postsynaptic densities isolated from dog cerebral cortex. I. Overall morphology and protein composition. J Cell Biol 74, 181-203.

Cremer, H., Lange, R., Christoph, A., Plomann, M., Vopper, G., Roes, J., Brown, R., Baldwin, S., Kraemer, P., Scheff, S., and et al. (1994). Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. Nature 367, 455-9.

Dulac, C., and Axel, R. (1995). A novel family of genes encoding putative pheromone receptors in mammals. Cell 83, 195-206.

Edwards, F. A. (1995). LTP--a structural model to explain the inconsistencies. Trends Neurosci. 18, 250-255.

Einheber, S., Schnapp, L., Salzer, J., Cappiello, Z., and Milner, T. (1996). Regional and ultrastructural distribution of the a8 integrin subunit in developing and adult rat brain suggests a role in synaptic function. J. Comp. Neuro. 370, 105-134.

Fannon, A. M., and Colman, D. R. (1996). A model for central synaptic junctional complx formation based on the differential adhesive specificities of cadherins. Neuron 17, 423-434.

Flynn, D. C., Koay, T. C., Humphries, C. G., and Guappone, A. C. (1995). AFAP-120. A variant form of the Src SH2/SH3-binding partner AFAP-110 is detected in brain and contains a novel internal sequence which binds to a 67-kDa protein. J Biol Chem 270, 3894-3899.

Fusaki, N., Matsuda, S., Nishizumi, H., Umemori, H., and Yamamoto, T. (1996). Physical and functional interactions of protein tyrosine kinases, p59fyn and ZAP-70, in T cell signaling. J. Immunol. 156, 1369-1377.

Geiger, B., Volberg, T., Ginsberg, D., Bitzur, S., Sabanay, I., and Hynes, R. O. (1990). Broad spectrum pan-cadherin antibodies, reactive with the C-terminal 24 amino acid residues of N-cadherin. J Cell Sci 97, 607-14.

Geiger, B., and Ayalon, O. (1992). Cadherins. Annu. Rev. Cell. Biol. 8, 307-332.

Geinisman, Y. (1993). Perforated axospinous synapses with multiple, completely paritioned transmission zones: probable structural intermediates in synaptic plasticity. Hippocampus 3, 417-434.

Gennarini, G., Hirsch, M. R., He, H. T., Hirn, M., Finne, J., and Goridis, C. (1986). Differential expression of mouse neural cell-adhesion molecule (N-CAM) mRNA species during brain development and in neural cell lines. J Neurosci 6, 1983-90.

Grant, S. G., O'Dell, T. J., Karl, K. A., Stein, P. L., Soriano, P., and Kandel, E. R. (1992). Impaired long-term potentiation, spatial learning, and hippocampal development in fyn mutant mice [see comments]. Science 258, 1903-10.

Gumbiner, B. (1993). Proteins associated with the cytoplasmic surface of adhesion molecules. Neuron 11, 551-564.

Gumbiner, B. M. (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. cell 84, 345-357.

Gurd, J. W., Gordon-Weeks, P., and Evans, W. H. (1982). Biochemical and morphological comparison of postsynaptic densities prepared from rat, hamster, and monkey brains by phase partitioning. J Neurochem 39, 1117-24.

Hatta, T., Tanaka, O., and Otani, H. (1994). Contribution of RGD sequence to neuronal migration in developing cerebral cortex. Neuroreport 5, 2261-2264.

Henikoff, S. (1984). Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene 28, 351-9.

Ishino, M., Ohba, T., Sasaki, H., and Sasaki, T. (1995). Molecular cloning of a cDNA encoding a phosphoprotein, Efs, which contains a Src homology 3 domain and associates with Fyn. Oncogene 11, 2331-2338.

Kashiwabuchi, N., Ikeda, K., Araki, K., Hirano, T., Shibuki, K., Takayama, C., Inoue, Y., Kutsuwada, T., Yagi, T., Kang, Y., and et al. (1995). Impairment of motor coordination, Purkinje cell synapse formation, and cerebellar long-term depression in GluR delta 2 mutant mice. Cell 81, 245-252.

Kennedy, M. B., Bennett, M. K., and Erondu, N. E. (1983). Biochemical and immunochemical evidence that the "major postsynaptic density protein" is a subunit of a calmodulin-dependent protein kinase. Proc Natl Acad Sci U S A 80, 7357-61.

Kennedy, M. B. (1993). The postsynaptic density. Curr Opin Neurobiol 3, 732-7.

Kintner, C. (1992). Regulation of embryonic cell adhesion by the cadherin cytoplasmic domain. Cell 69, 225-36.

Koch, C. A., Anderson, D., Moran, M. F., Ellis, C., and Pawson, T. (1991). SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. Science 252, 668-74.

Köhler, G., Schreier, M., Hengartner, H., Berek, C., Trucco, M., Forni, L., Staehelin, T., Stocker, J., and Takass, B. (1980). Hybridona Techniques. Cold Spring Harbor Laboratory, Cold Harbor, NY., 65pp.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-5.

Larue, L., Ohsugi, M., Hirchenhain, J., and Kemler, R. (1994). E-cadherin null mutant embryos fail to form a trophectoderm epithelium. Proc Natl Acad Sci U S A 91, 8263-8267.

Li, J., Avraham, H., Rogers, R. A., Raja, S., and Avraham, S. (1996). Characterization of RAFTK, a novel focal adhesion kinase, and its integrin-dependent phosphorylation and activation in megakaryocytes. Blood 88, 417-428.

Luthl, A., Laurent, J. P., Figurov, A., Muller, D., and Schachner, M. (1994). Hippocampal long-term potentiation and neural cell adhesion molecules L1 and NCAM. Nature 372, 777-9.

Marie-Cardine, A., Kirchgessner, H., Eckerskorn, C., Meuer, S. C., and Schraven, B. (1995). Human T lymphocyte activation induces tyrosine phosphorylation of alpha-tubulin and its association with the SH2 domain of the p59fyn protein tyrosine kinase. Eur. J. Immunol. 25, 3290-3297.

Mayer, B. J., Hamaguchi, M., and Hanafusa, H. (1988). A novel viral oncogene with structural similarity to phospholipase C. Nature 332, 272-5.

Mayford, M., Barzilai, A., Keller, F., Schacher, S., and Kandel, E. R. (1992). Modulation of an NCAM-related adhesion molecule with long-term synaptic plasticity in Aplysia. Science 256, 638-644.

McKnight, G. S. (1978). The induction of ovalbumin and conalbumin mRNA by estrogen and progesterone in chick oviduct explant cultures. Cell 14, 403-13.

Miyakawa, T., Yagi, T., Watanabe, S., and Niki, H. (1994). Increased fearfulness of Fyn tyrosine kinase deficient mice. Brain-Res-Mol-Brain-Res 27, 179-82.

Miyakawa, T., Yagi, T., Taniguchi, M., Matsuura, H., Tateishi, K., and Niki, H. (1995). Enhanced susceptibility of audiogenic seizures in Fyn-kinase deficient mice. Brain-Res-Mol-Brain-Res 28, 349-52.

Mombaerts, P., Wang, F., Dulac, C., Chao, S. K., Nemes, A., Mendelsohn, M., Edmondson, J., and Axel, R. (1996). Visualizing an olfactory sensory map. Cell 87, 675-686.

Moon, I. S., Apperson, M. L., and Kennedy, M. B. (1994). The major tyrosine-phosphorylated protein in the postsynaptic density fraction is N-methyl-D-aspartate receptor subunit 2B. Proc Natl Acad Sci U S A 91, 3954-8.

Muller, D., Wang, C., Skibo, G., Toni, N., Cremer, H., Calaora, V., Rougon, G., and Z., K. (1996). PSA-NCAM is required for activity-induced synaptic plasticity. Neuron 17, 413-422.

Nagafuchi, A., and Takeichi, M. (1988). Cell binding function of E-cadherin is regulated by the cytoplasmic domain. Embo J 7, 3679-84.

Nagafuchi, A., and Takeichi, M. (1989). Transmembrane control of cadherin-mediated cell adhesion: a 94 kDa protein functionally associated with a specific region of the cytoplasmic domain of E-cadherin. Cell Regul 1, 37-44.

Nose, A., Tsuji, K., and Takeichi, M. (1990). Localization of specificity determining sites in cadherin cell adhesion molecules. Cell 61, 147-55.

Overduin, M., Harvey, T. S., Bagby, S., Tong, K. I., Yau, P., Takeichi, M., and Ikura, M. (1995). Solution structure of the epithelial cadherin domain responsible for selective cell adhesion [see comments]. Science 267, 386-389.

Ozawa, M., Baribault, H., and Kemler, R. (1989). The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. Embo J 8, 1711-7.

Ozawa, M., Engel, J., and Kemler, R. (1990a). Single amino acid substitutions in one Ca2+ binding site of uvomorulin abolish the adhesive function. Cell 63, 1033-8.

Ozawa, M., Ringwald, M., and Kemler, R. (1990b). Uvomorulin-catenin complex formation is regulated by a specific domain in the cytoplasmic region of the cell adhesion molecule. Proc Natl Acad Sci U S A 87, 4246-50.

Ozawa, M., and Kemler, R. (1992). Molecular organization of the uvomorulin-catenin complex. J Cell Biol 116, 989-96.

Panchamoorthy, G., Fukazawa, T., Miyake, S., Soltoff, S., Reedquist, K., Druker, B., Shoelson, S., Cantley, L., and Band, H. (1996). p120cbl is a major substrate of tyrosine phosphorylation upon B cell antigen receptor stimulation and interacts in vivo with Fyn and Syk tyrosine kinases, Grb2 and Shc adaptors, and the p85 subunit of phosphatidylinositol 3-kinase. J. Biol. Chem. 271, 3187-3194.

Parmentier, M., Libert, F., Schurmans, S., Schiffmann, S., Lefort, A., Eggerickx, D., Ledent, C., Mollereau, C., Gerard, C., Perret, J., and et al. (1992). Expression of members of the putative olfactory receptor gene family in mammalian germ cells. Nature 355, 453-5.

Pawson, T., and Gish, G. D. (1992). SH2 and SH3 domains: from structure to function. Cell 71, 359-62 issn: 0092-8674.

Peters, A., Palay, S. L., and Webster, H. d. F. (1991). The fine structure of the nervous system: Neurons and their supporting cells. New York: Oxford University Press.

Pierschbacher, M. D., and Ruoslahti, E. (1984). Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature 309, 30-3.

Pleiman, C. M., Clark, M. R., Gauen, L. K., Winitz, S., Coggeshall, K. M., Johnson, G. L., Shaw, A. S., and Cambier, J. C. (1993). Mapping of sites on the Src family protein tyrosine kinases p55blk, p59fyn, and p56lyn which interact with the effector molecules phospholipase C-gamma 2, microtubule-associated protein kinase, GTPase-activating protein, and phosphatidylinositol 3-kinase. Mol-Cell-Biol 13, 5877-87 issn: 0270-7306.

Pleiman, C. M., Hertz, W. M., and Cambier, J. C. (1994). Activation of phosphatidylinositol-3' kinase by Src-family kinase SH3 binding to the p85 subunit. Science 263, 1609-12.

Rabinowich, H., Manciulea, M., Metes, D., Sulica, A., Herberman, R. B., Corey, S. J., and Whiteside, T. L. (1996). Physical and functional association of Fc mu receptor on human natural killer cells with the zeta- and Fc epsilon RI gamma-chains and with src family protein tyrosine kinases. J. Immunol. 157, 1485-1491.

Redies, C. (1995). Cadherin expression in the developing vertebrate CNS: from neuromeres to brain nuclei and neural circuits. Exp Cell Res 220, 243-256.

Ren, R., Mayer, B. J., Cicchetti, P., and Baltimore, D. (1993). Identification of a ten-amino acid proline-rich SH3 binding site. Science 259, 1157-61 issn: 0036-8075.

Ressler, K. J., Sullivan, S. L., and Buck, L. B. (1994). A molecular dissection of spatial patterning in the olfactory system. Curr Opin Neurobiol 4, 588-596.

Ringwald, M., Schuh, R., Vestweber, D., Eistetter, H., Lottspeich, F., Engel, J., Dolz, R., Jahnig, F., Epplen, J., Mayer, S., and et al. (1987). The structure of cell adhesion

molecule uvomorulin. Insights into the molecular mechanism of Ca2+-dependent cell adhesion. Embo J 6, 3647-53.

Ruppert, M., Aigner, S., Hubbe, M., Yagita, H., and Altevogt, P. (1995). The L1 adhesion molecule is a cellular ligand for VLA-5. J. Cell Biol. 131, 1881-91.

Sago, H., Kitagawa, M., Obata, S., Mori, N., Taketani, S., Rochelle, J. M., Seldin, M. F., Davidson, M., St. John, T., and Suzuki, S. T. (1995). Cloning, expression, and chromosomal localization of a novel cadherin- related protein, protocadherin-3. Genomics 29, 631-640.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4, 406-25.

Sakimura, K., Kutsuwada, T., Ito, I., Manabe, T., Takayama, C., Kushiya, E., Yagi, T., Aizawa, S., Inoue, Y., Sugiyama, H., and Mishina, M. (1995). Reduced hippocampal LTP and spatial learning in mice lacking NMDA receptor epsilon 1 subunit. Nature 373, 151-5.

Saksela, K., Cheng, G., and Baltimore, D. (1995). Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4. Embo J 14, 484-491.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Lboratory Press, Cold Spring Harbor, N. Y.

Samelson, L. E., Phillips, A. F., Luong, E. T., and Klausner, R. D. (1990). Association of the fyn protein-tyrosine kinase with the T-cell antigen receptor. Proc Natl Acad Sci U S A 87, 4358-62.

Sano, K., Tanihara, H., Heimark, R. L., Obata, S., Davidson, M., St John, T., Taketani, S., and Suzuki, S. (1993). Protocadherins: a large family of cadherin-related molecules in central nervous system. Embo J 12, 2249-56.

Semba, K., Nishizawa, M., Miyajima, N., Yoshida, M. C., Sukegawa, J., Yamanashi, Y., Sasaki, M., Yamamoto, T., and Toyoshima, K. (1986). yes-related protooncogene,

syn, belongs to the protein-tyrosine kinase family. Proc Natl Acad Sci U S A, 5459-63. .

Shapiro, L., Fannon, A. M., Kwong, P. D., Thompson, A., Lehmann, M. S., Grubel, G., Legrand, J. F., Als-Nielsen, J., Colman, D. R., and Hendrickson, W. A. (1995a). Structural basis of cell-cell adhesion by cadherins [see comments]. Nature 374, 327-337.

Shapiro, L., Kwong, P. D., Fannon, A. M., Colman, D. R., and Hendrickson, W. A. (1995b) f. Considerations on the folding topology and evolutionary origin of cadherin domains. Proc Natl Acad Sci U S A 92, 6793-6797.

Silva, A., J,, Stevens, C. F., Tonegawa, S., and Wang, Y. (1992). Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice. Science 257, 201-6.

Sperry, R. W. (1963). Chemoaffinity in the orderly growth of nerve fiber patterns and connections. Proc. Natl. Acad. Sci. USA 50, 703-710.

Suzuki, T., Okumura-Noji, K., Tanaka, R., Ogura, A., Nakamura, K., Kudo, Y., and Tada, T. (1993). Characterization of protein kinase C activities in postsynaptic density fractions prepared from cerebral cortex, hippocampus, and cerebellum. Brain Res 619, 69-75.

Swope, S. L., and Huganir, R. L. (1994). Binding of the nicotinic acetylcholine receptor to SH2 domains of Fyn and Fyk protein tyrosine kinases. J Biol Chem 269, 29817-29824.

Swope, S. L., Qu, Z., and Huganir, R. L. (1995). Phosphorylation of the nicotinic acetylcholine receptor by protein tyrosine kinases. Ann N Y Acad Sci 1995 May 10;757:197-214.

Takeichi, M. (1990). Cadherins: a molecular family important in selective cell-cell adhesion. Annu Rev Biochem 59, 237-52.

Takeichi, M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulator.

Science 251, 1451-5.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 76, 4350-4.

Umemori, H., Sato, S., Yagi, T., Aizawa, S., and Yamamoto, T. (1994). Initial events of myelination involve Fyn tyrosine kinase signalling. Nature 367, 572-6.

VanderNoot, V. A., and Fitzpatrick, F. A. (1995). Competitive binding assay of src homology domain 3 interactions between 5-lipoxygenase and growth factor receptor binding protein 2. Anal. Biochem. 230, 108-114.

Vassar, R., Chao, S. K., Sitcheran, R., Nunez, J. M., Vosshall, L. B., and Axel, R. (1994). Topographic organization of sensory projections to the olfactory bulb. Cell 79, 981-91.

Weiler, I. J., Hawrylak, N., and Kiss, J. Z. (1995). Morphologenesis in memory formation: synaptic and cellular mechanisms. Behav. Brain Res. 66, 1-6.

Yagi, T., Aizawa, S., Tokunaga, T., Shigetani, Y., Takeda, N., and Ikawa, Y. (1993a). A role for Fyn tyrosine kinase in the suckling behaviour of neonatal mice. Nature 366, 742-5.

Yagi, T., Shigetani, Y., Okado, N., Tokunaga, T., Ikawa, Y., and Aizawa, S. (1993b). Regional localization of Fyn in adult brain; studies with mice in which fyn gene was replaced by lacZ. Oncogene 8, 3343-51.

Yagi, T., Shigetani, Y., Furuta, Y., Nada, S., Okado, N., Ikawa, Y., and Aizawa, S. (1994). Fyn expression during early neurogenesis in mouse embryos. Oncogene 9, 2433-40.

Yamamoto, K. (1989). Binding manner of actin to the lysine-rich sequence of myosin subfragment 1 in the presence and absence of ATP. Biochemistry 28, 5573-7.

Figure 1.

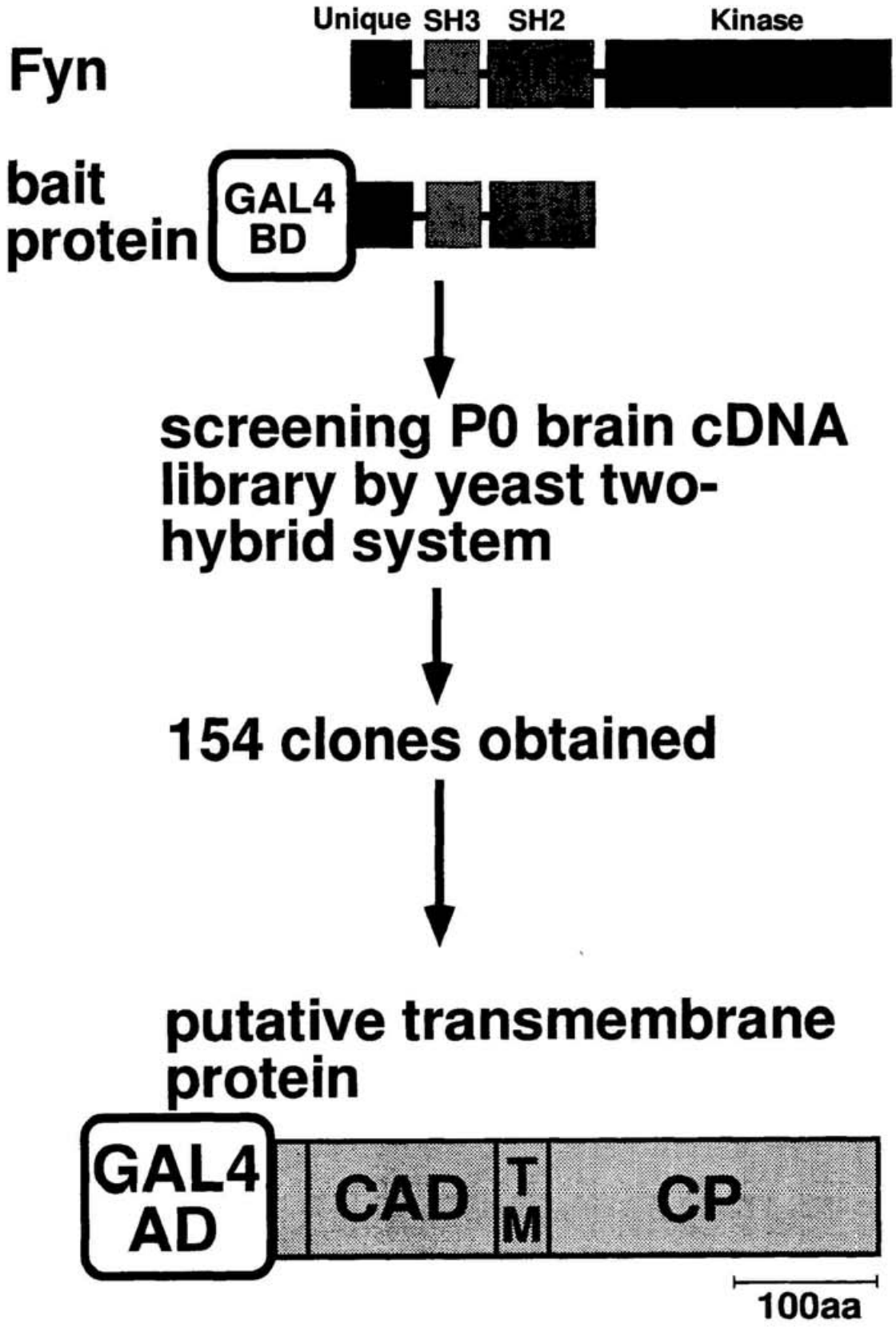

Unique
SH3
SH2
Kinase
Fyn
bait protein
GAL4 BD
screening P0 brain cDNA library by yeast two-hybrid system
154 clones obtained
putative transmembrane protein
GAL4 AD
CAD
TM
CP
100aa

Figure 1. Strategy for the Identification of Putative Fyn-associated Transmembrane Proteins by the Yeast Two-Hybrid System.

The Fyn unique-SH3-SH2 domain was cloned under the control of the GAL4 binding domain (BD) to yield bait protein for the yeast two-hybrid system. A postnatal day 0 brain cDNA library was screened using this bait protein, and 154 independent clones were obtained. Sequencing analysis was performed, and one 1.8 kb cDNA encoding a putative transmembrane protein was obtained that was co-expressed with the GAL4 activation domain (AD). The encoded protein, which was named CNR1, contained a cadherin-repeat (CAD), a putative transmembrane region (TM) and a putative cytoplasmic region (CP).

Figure 2.

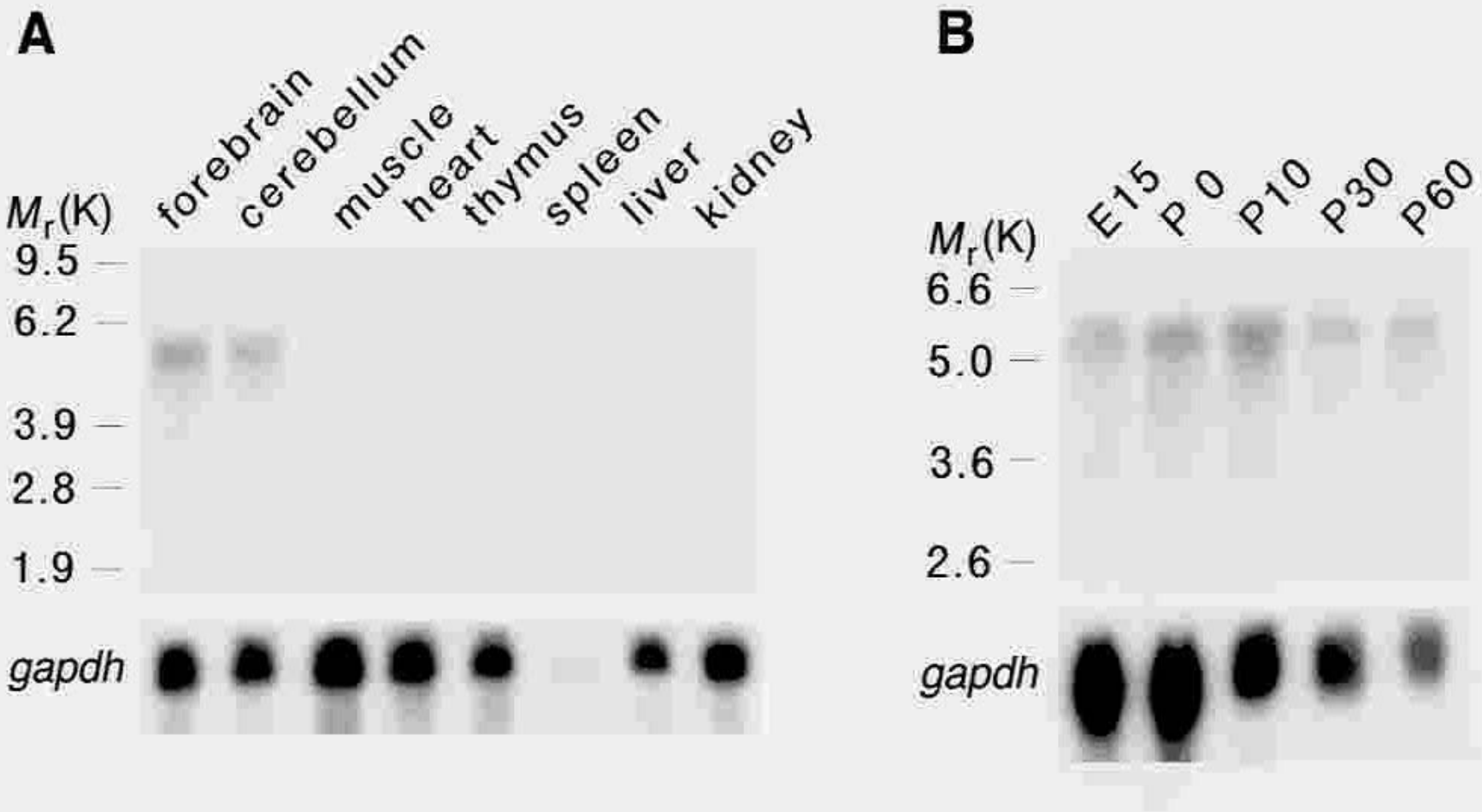

A
forebrain
cerebellum
muscle
heart
thymus
spleen
liver
kidney
M_r(K)
9.5
6.2
3.9
2.8
1.9
gapdh
B
E15
P 0
P10
P30
P60
M_r(K)
6.6
5.0
3.6
2.6
gapdh

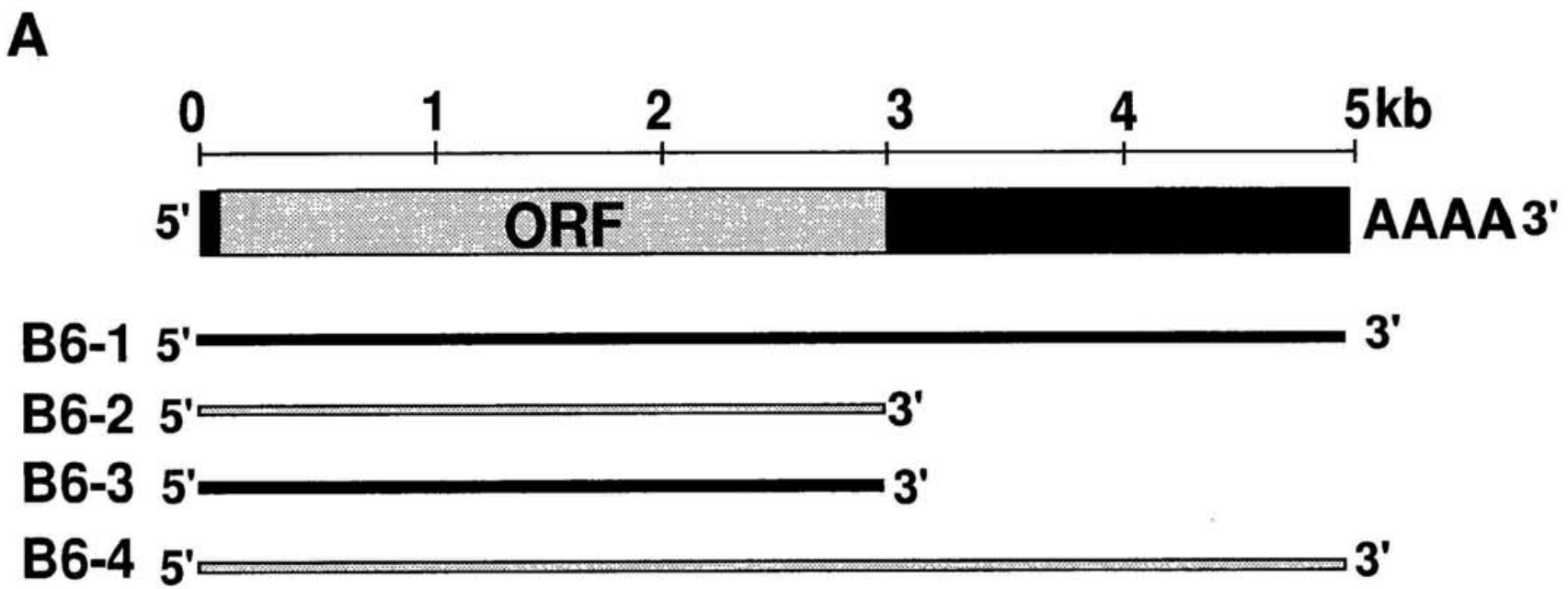
Figure 2. Northern Blot analysis of CNR1 Expression.

The 1.8 kb cDNA fragment obtained from the two-hybrid system was used as a probe.

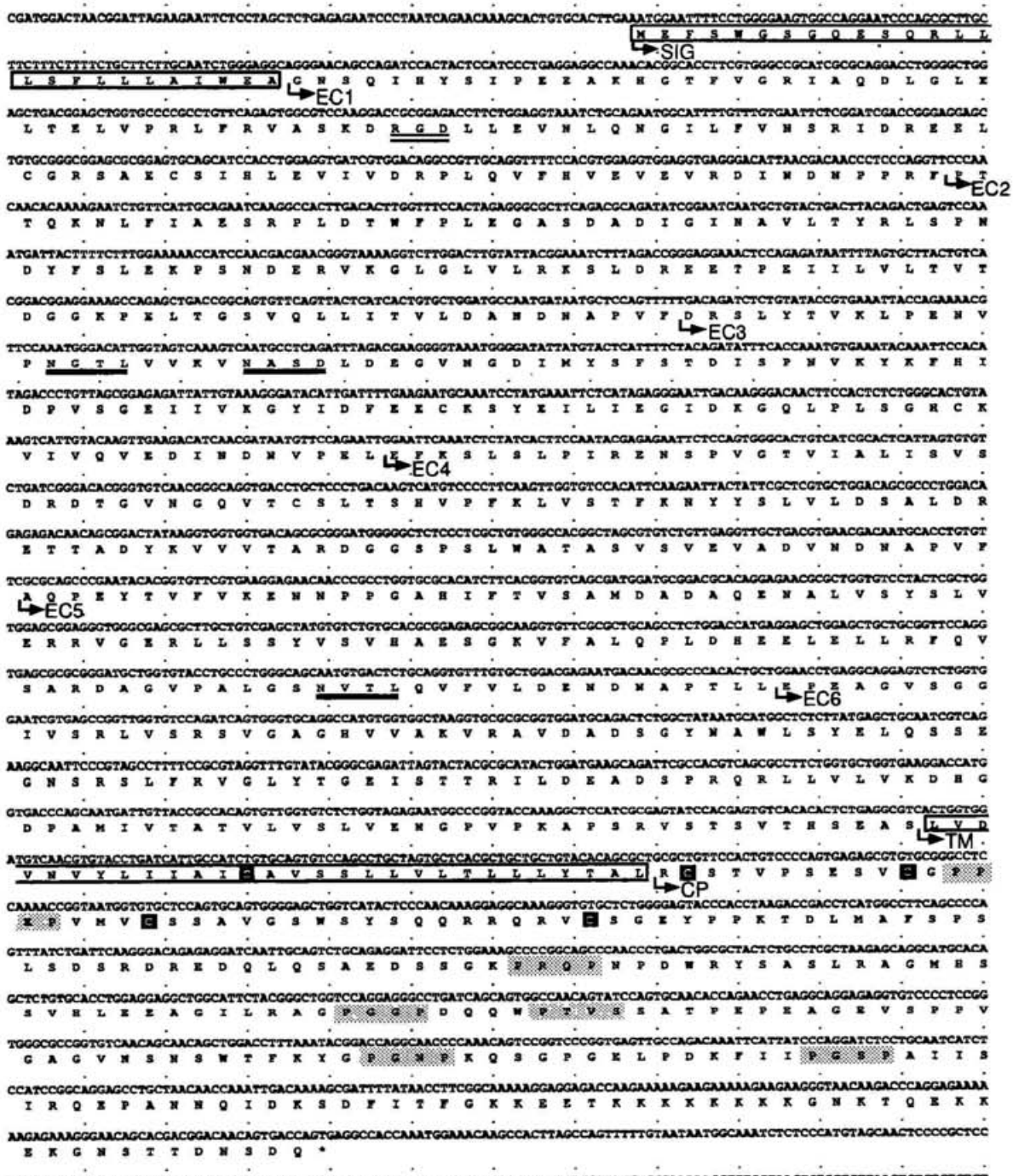
(A) CNR1 expression in adult mouse tissues. Thirty ug of total RNA from each tissue of P60 mouse was loaded in each lane. The lower panel represents the same Northern blot hybridized with a gapdh cDNA probe.

(B) CNR1 expression in brain at several developmental stages. Thirty ug of total RNA was loaded in each lane. The lower panel shows the same Northern blot hybridized with a gapdh cDNA probe.

Figure 3.



B



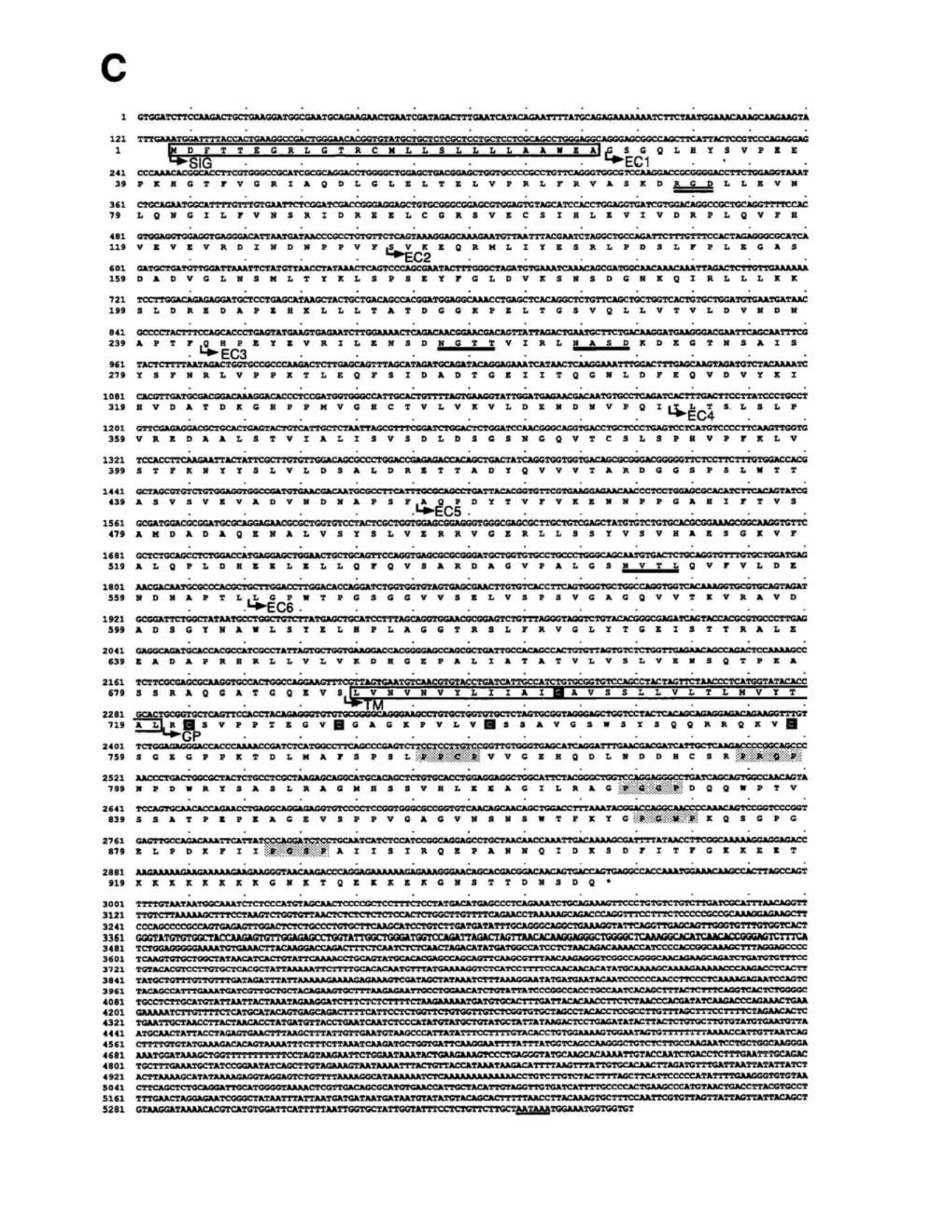

C
SIG
EC1
EC2
EC3
EC4
EC5
EC6
TM
GP

Figure 3. Cloning and the Nucleotide Sequence of CNR1 and CNR2.

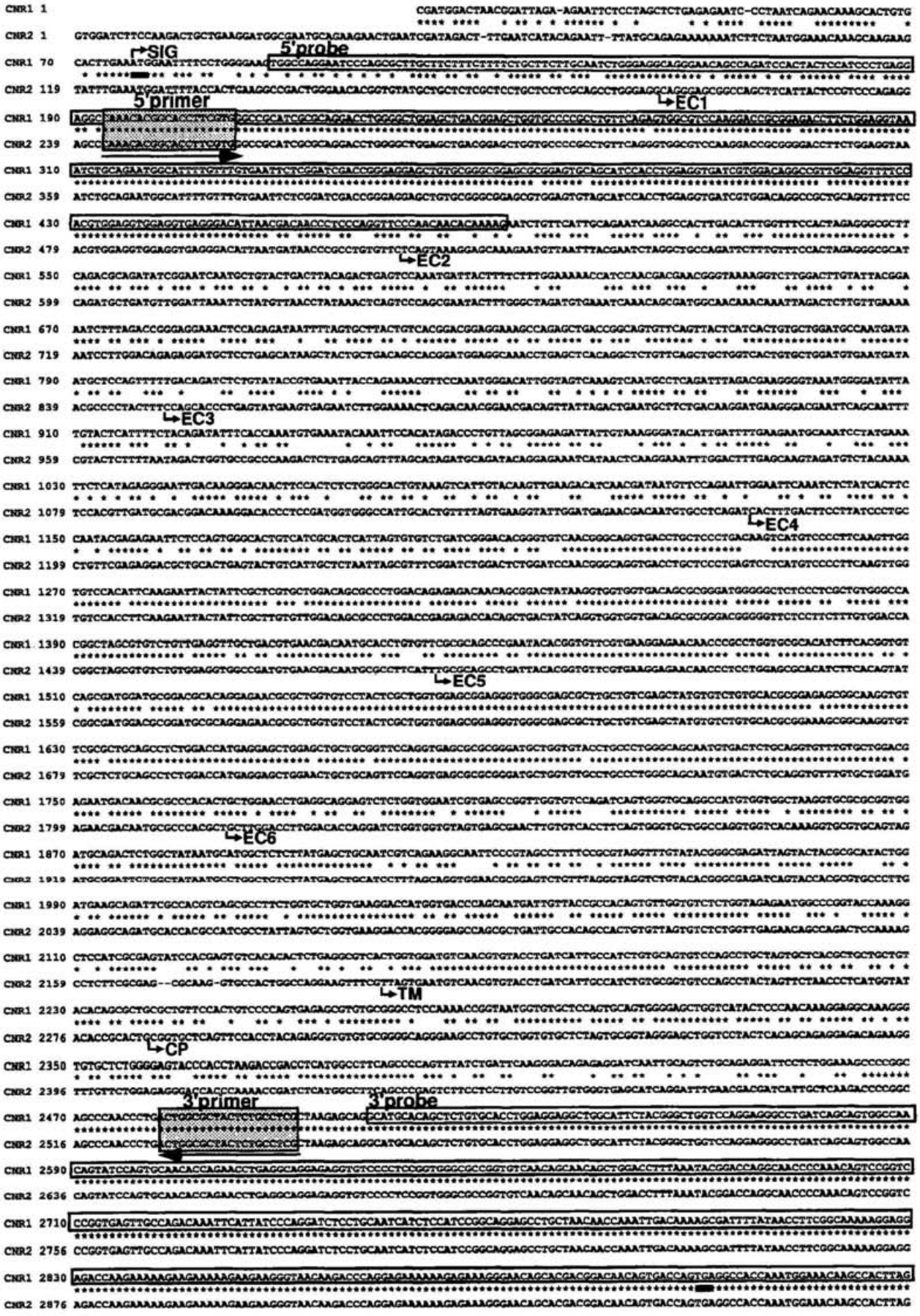
(A) Schematic representation of cDNA structures and the clones obtained by screening a C57BL/6 (B6) mouse cDNA library using the 1.8 kb cDNA fragment obtained from the two-hybrid system as probe. The clones B6-1 and B6-3 contained sequences from CNR1, and B6-2 and B6-4 contained sequences from CNR2. B6-1 and B6-4 contained nearly the full-length cDNA. Stretches of polyadenylation are found at the 3' ends of B6-1 and B6-4 cDNA clones.

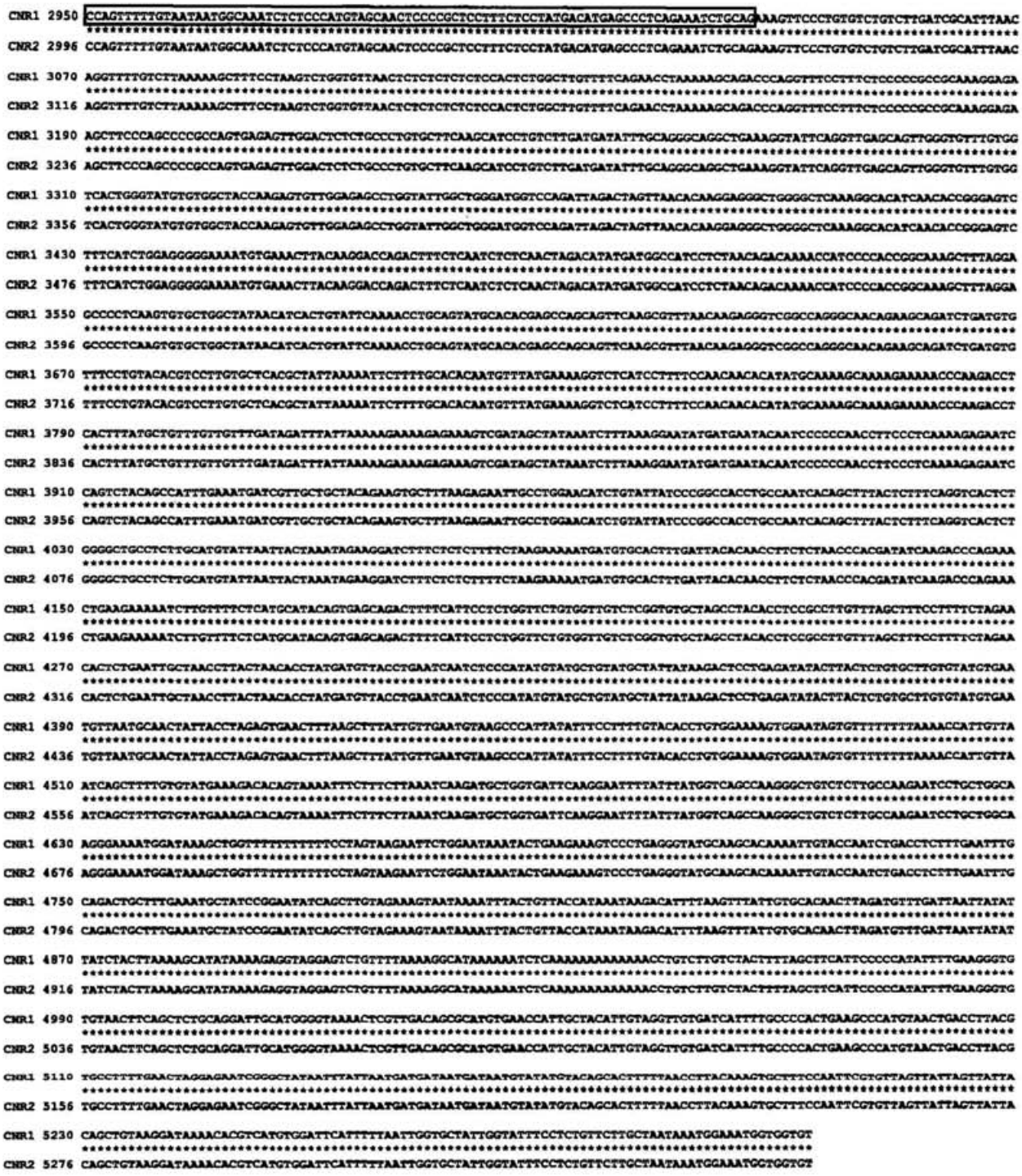
(B) and (C) Nucleotide sequence of the murine CNR1 and CNR2 genes. The deduced amino acid sequences are given underneath the nucleotide sequence using the single letter code. Numbers indicate positions from the 5' end of the cDNA and amino acid positions from the N-terminal end. The signal peptide and the putative transmembrane domain are boxed. RGD sequences are double underlined. The sites for potential N-linked glycosylation are underlined with thick lines. The polyadenylation signal is underlined by a thin line. The five cysteine residues appearing at regularly spaced intervals spanning 18, 9, 9, and 18 amino acid residues are shown as white lettering on black background. The PXXP motifs in the cytoplasmic domain are boxed with shadows. The nucleotide sequence data of CNR1 and CNR2 are available from DDBJ/EMBL/GenBank under accession number D86916 and D86917, respectively.

SIG, signal peptide; EC, extracellular domain; TM, transmembrane domain; CP, cytoplasmic domain.

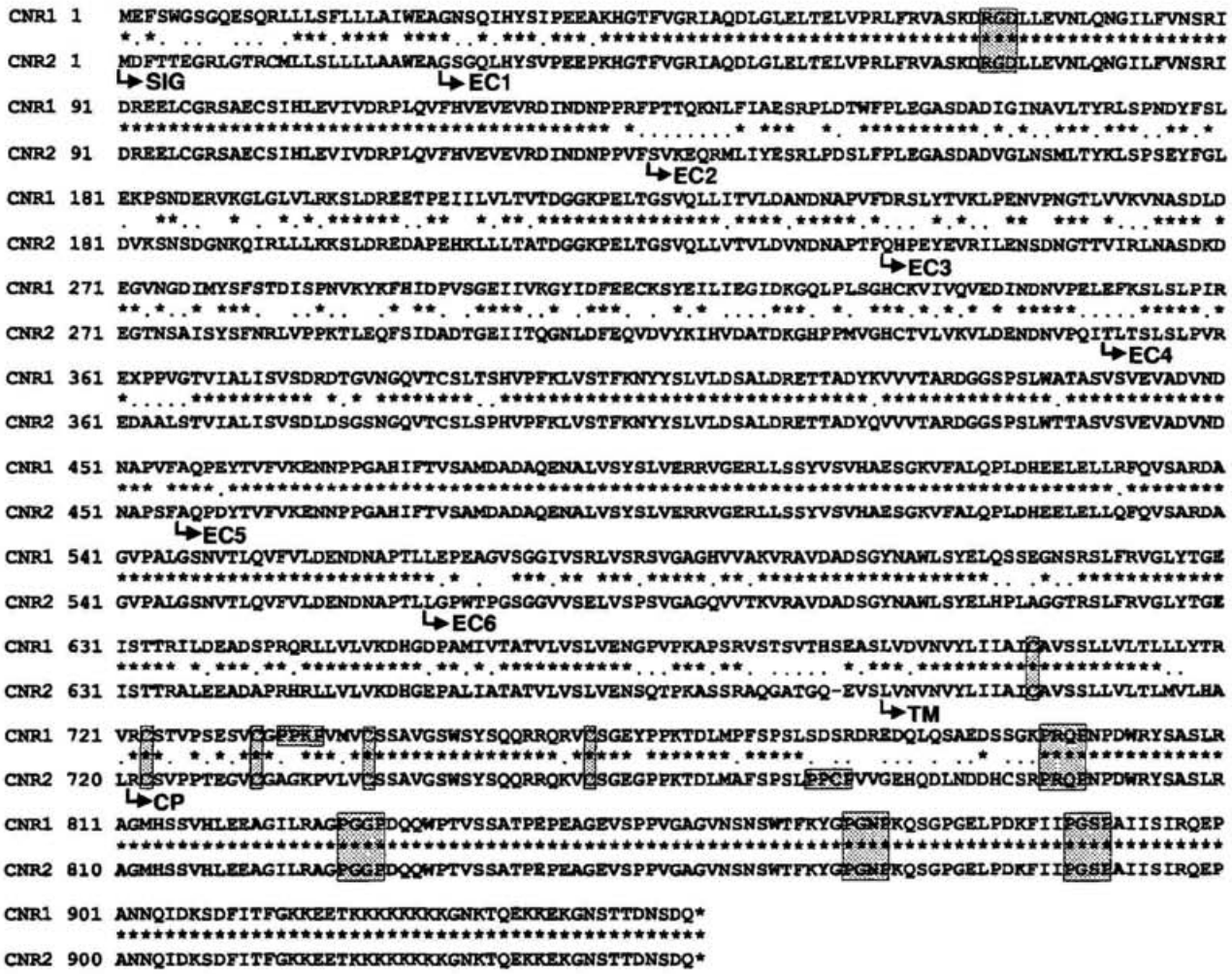
Figure 4.

A

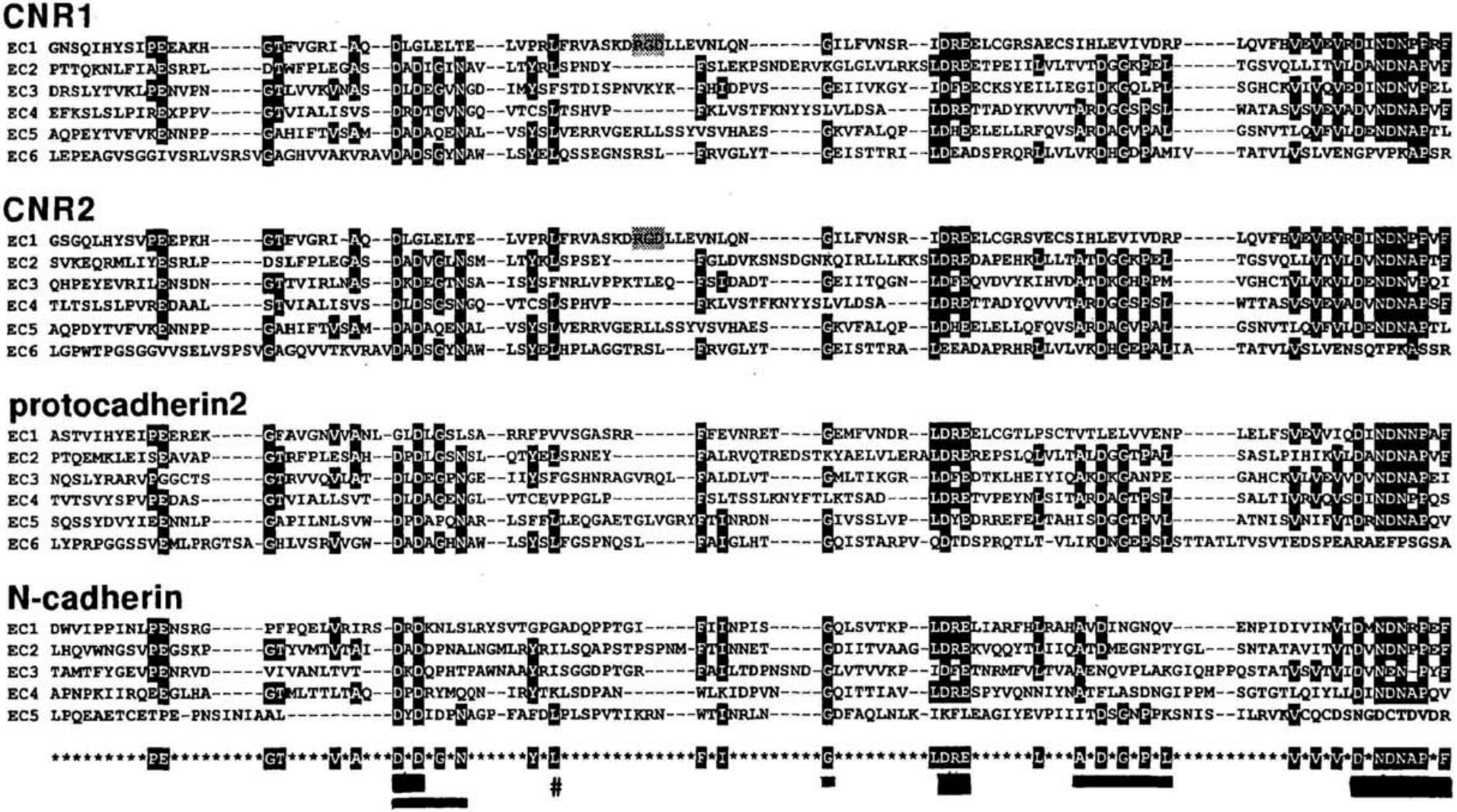

SIG
5'probe
5'primer
EC1
EC2
EC3
EC4
EC5
EC6
TM
CP
3'primer
3'probe



B



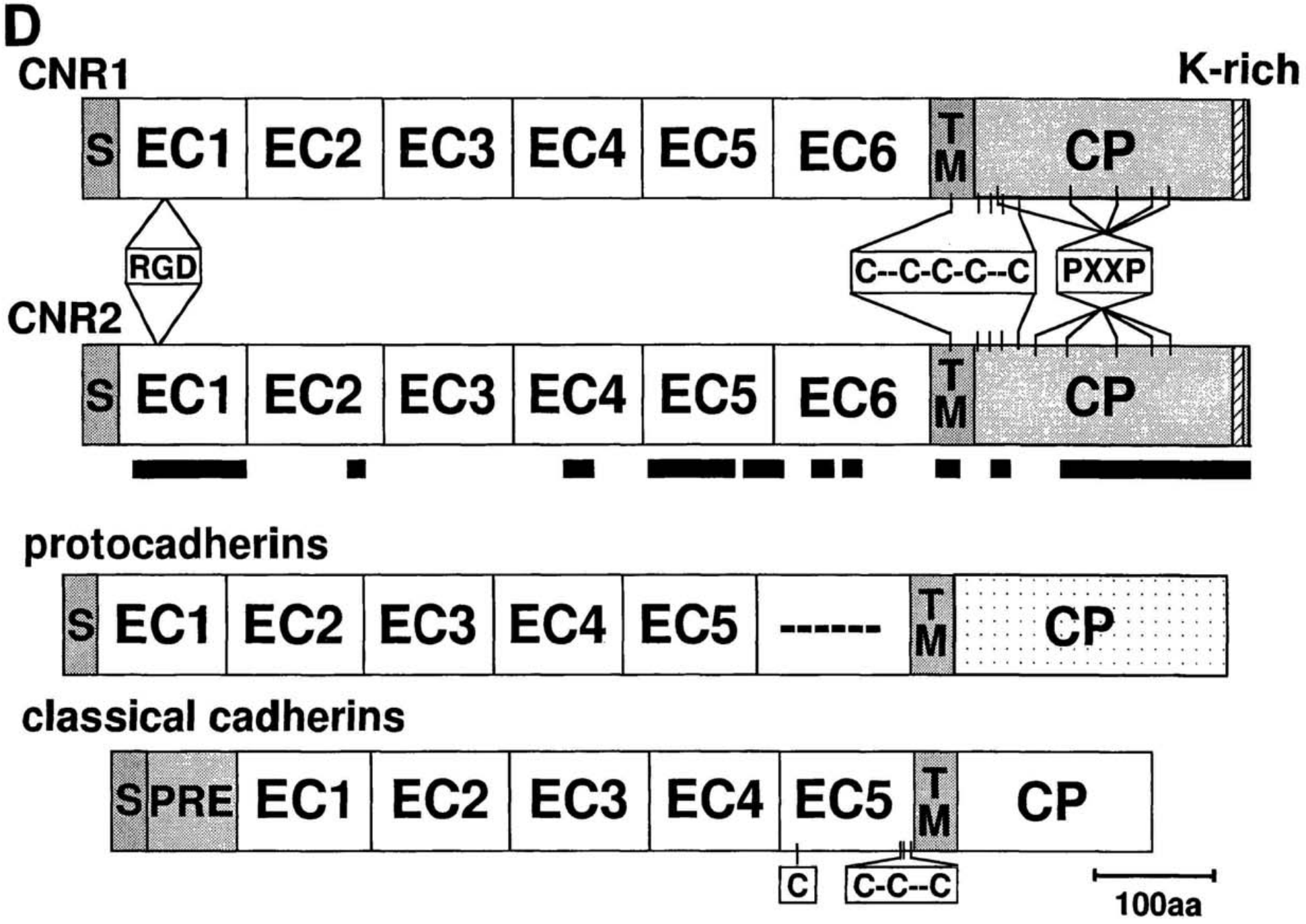
C


CNR1
EC1 GNSQIHYSIPEEAKH-----GTFVGRI-AQ--DLGLELTE---LVPRLFRVASKDRGDLLEVNLQN-------GILFVNSR--IDREELCGRSAECSIHLEVIVDRP-----LQVFHVEVEVRDINDNPPRF
EC2 PTTQKNLFIAESRPL-----DTWFPLEGAS--DADIGINAV--LTYRLSPNDY--------FSLEKPSNDERVKGLGLVLRKSLDREETPEIILVLTVTDGGKPEL------TGSVQLLITVLDANDNAPVF
EC3 DRSLYTVKLPENVPN-----GTLVVKVNAS--DLDEGVNGD--IMYSFSTDISPNVKYK--SHIDPVS-----GEIIVKGY--IDFEECKSYEILIEGIDKGQLPL------SGHCKVIVQVEDINDNVPEL
EC4 EFKSLSLPIREXPPV-----GTVIALISVS--DRDTGVNGQ--VTCSLTSHVP--------SKLVSTFKNYYSLVLDSA----LDRETTADYKVVVTARDGGSPSL------WATASVSVEVADVNDNAPVF
EC5 AQPEYTVFVKENNPP-----GAHIFTVSAM--DADAQENAL--VSYSLVERRVGERLLSSYVSVHAES-----GKVFALQP--LDHEELELLRFQVSARDAGVPAL------GSNVTLQVFVLDENDNAPTL
EC6 LEPEAGVSGGIVSRLVSRSVGAGHVVAKVRAVDADSGYNAW--LSYELQSSEGNSRSL---SRVGLYT-----GEISTTRI--LDEADSPRQRLLVLVKDHGDPAMIV----TATVLVSLVENGPVPKAPSR

CNR2
EC1 GSGQLHYSVPEEPKH-----GTFVGRI-AQ--DLGLELTE---LVPRLFRVASKDRGDLLEVNLQN-------GILFVNSR--IDREELCGRSVECSIHLEVIVDRP-----LQVFHVEVEVRDINDNPPVF
EC2 SVKEQRMLIYESRLP-----DSLFPLEGAS--DADVGLNSM--LTYKLSPSEY--------FGLDVKSNSDGNKQIRLLLKKSLDREDAPEHRLLLTATDGGKPEL------TGSVQLLVTVLDVNDNAPTF
EC3 QHPEYEVRILENSDN-----GTTVIRLNAS--DKDEGTNSA--ISYSFNRLVPPKTLEQ--FSIDADT-----GEIITQGN--LDFEQVDVYKIHVDATDKGHPPM------VGHCTVLVKVLDENDNVPQI
EC4 TLTSLSLPVREDAAL-----STVIALISVS--DLDSGSNGQ--VTCSLSPHVP--------SKLVSTFKNYYSLVLDSA----LDRETTADYQVVVTARDGGSPSL------WTTASVSVEVADVNDNAPSF
EC5 AQPDYTVFVKENNPP-----GAHIFTVSAM--DADAQENAL--VSYSLVERRVGERLLSSYVSVHAES-----GKVFALQP--LDHEELELLQFQVSARDAGVPAL------GSNVTLQVFVLDENDNAPTL
EC6 LGPWTPGSGGVVSELVSPSVGAGQVVTKVRAVDADSGYNAW--LSYELHPLAGGTRSL---SRVGLYT-----GEISTTRA--LEEADAPRHRLLVLVKDHGEPALIA----TATVLVSLVENSQTPKASSR

protocadherin2
EC1 ASTVIHYEIPEEREK-----GFAVGNVVANL-GLDLGSLSA--RRFPVVSGASRR------FFEVNRET----GEMFVNDR--LDREELCGTLPSCTVTLELVVENP-----LELFSVEVVIQDINDNNPAF
EC2 PTQEMKLEISEAVAP-----GTRFPLESAH--DPDLGSNSL--QTYELSRNEY--------FALRVQTREDSTKYAELVLERALDREREPSLQLVLTALDGGTPAL------SASLPIHIKVLDANDNAPVF
EC3 NQSLYRARVPGGCTS-----GTRVVQVLAT--DLDEGPNGE--IIYSFGSHNRAGVRQL--FALDLVT-----GMLTIKGR--LDFEDTKLHEIYIQAKDKGANPE------GAHCKVLVEVVDVNDNAPEI
EC4 TVTSVYSPVPEDAS-----GTVIALLSVT--DLDAGENGL--VTCEVPPGLP--------FSLTSSLKNYFTLKTSAD----LDRETVPEYNLSITARDAGTPSL------SALTIVRVQVSDINDNPPQS
EC5 SQSSYDVYIEENNLP-----GAPILNLSVW--DPDAPQNAR--LSFELLEQGAETGLVGRYFTINRDN-----GIVSSLVP--LDYEDRRREFELTAHISDGGTPVL------ATNISVNIFVTDRNDNAPQV
EC6 LYPRPGGSSVEMLPRGTSA-GHLVSRVVGW--DADAGHNAW--LSYSLFGSPNQSL-----FAIGLHT-----GQISTARPV-QDTDSPRQTLT-VLIKDNGEPSLSTTATLTVSVTEDSPEARAEFPSGSA

N-cadherin
EC1 DWVIPPINLPENSRG-----PFPQELVRIRS-DRDKNLSLRYSVTGPGADQPPTGI-----FIINPIS-----GQLSVTKP--LDRELIARFHLRAHAVDINGNQV------ENPIDIVINVIDMNDNRPEF
EC2 LHQVWNGSVPEGSKP-----GTYVMTVTAI--DADDPNALNGMLRYRILSQAPSTPSPNM-FTINNET-----GDIITVAAG-LDREKVQQYTLIIQATDMEGNPTYGL---SNTATAVITVTDVNDNPPEF
EC3 TAMTFYGEVPENRVD-----VIVANLTVT---DKDQPHTPAWNAAYRISGGDPTGR-----FAILTDPNSND-GLVTVVKP--IDFETNRMFVLTVAAENQVPLAKGIQHPPQSTATVSVTVIDVNEN-PYF
EC4 APNPKIIRQEEGLHA-----GTMLTTLTAQ--DPDRYMQQN--IRYTKLSDPAN-------WLKIDPVN----GQITTIAV--LDRESPYVQNNIYNATFLASDNGIPPM--SGTGTLQIYLLDINDNAPQV
EC5 LPQEAETCETPE-PNSINIAAL----------DYDIDPNAGP-FAFDLPLSPVTIKRN---WTINRLN-----GDFAQLNLK-IKFLEAGIYEVPIIITDSGNPPKSNIS--ILRVKVCQCDSNGDCTDVDR


D
CNR1
K-rich
S
EC1
EC2
EC3
EC4
EC5
EC6
TM
CP
RGD
C--C-C-C--C
PXXP
CNR2
S
EC1
EC2
EC3
EC4
EC5
EC6
TM
CP
protocadherins
S
EC1
EC2
EC3
EC4
EC5

TM
CP
classical cadherins
S
PRE
EC1
EC2
EC3
EC4
EC5
TM
CP
C
C-C--C
100aa

Figure 4. Alignment and Structural Comparison of CNR1 and CNR2 to Other Cadherins.

(A) Alignment of the nucleotide sequence of CNR1 and CNR2 cDNAs. Identical nucleotides between CNR1 and CNR2 are shown by asterisks. The ATG initiation codon and TGA stop codon are represented with thick bars. The regions spanning the 5' and 3' probes for Southern blot analysis in figure 5A are boxed, and the sequences of the 5' and 3' primers for RT-PCR in figure 5B are boxed with shadows.

(B) Alignment of the deduced amino acid sequences of CNR1 and CNR2. The RGD motif, the PXXP motif, and cysteine repeats appearing at regularly spaced intervals spanning 18, 9, 9, and 18 amino acid residues are boxed with shadows.
SIG, signal peptide; EC, extracellular domain; TM, transmembrane domain; CP, cytoplasmic domain.

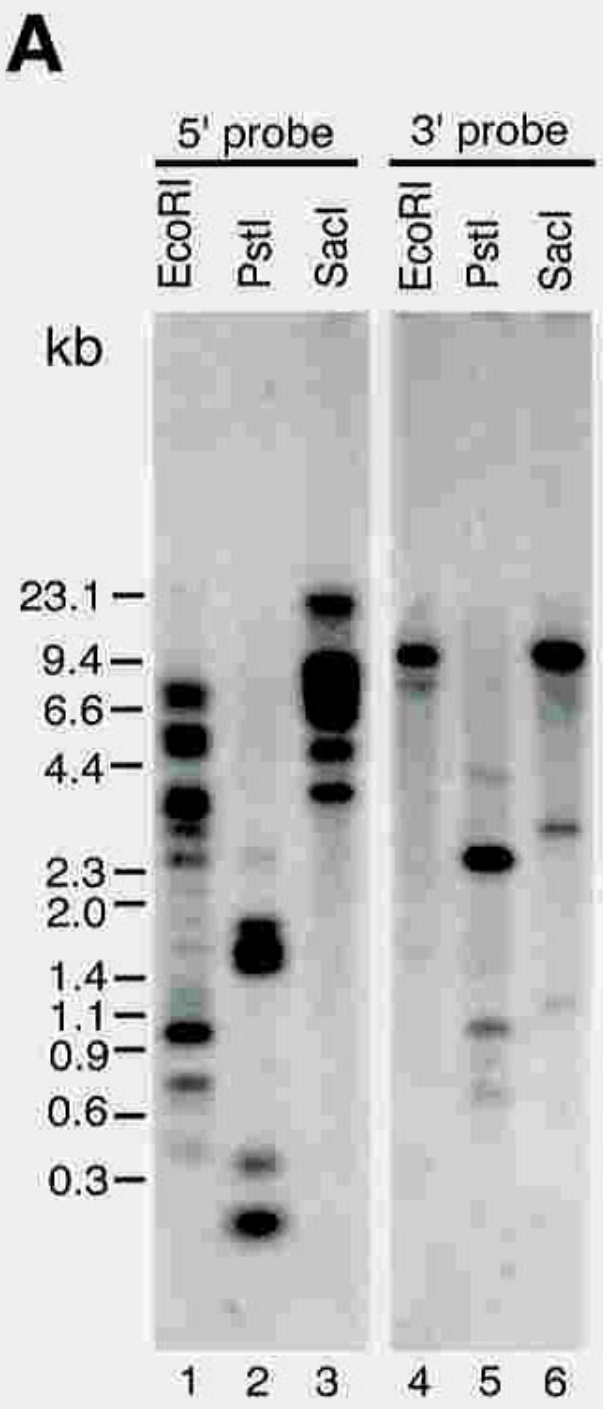
(C) Alignment of internal cadherin repeats in the extracellular domains of CNR1, CNR2, protocadherin2 and N-cadherin. The white letters on black background represent the well-conserved residues. Consensus repeats are shown at the bottom of the sequences. The negatively-charged amino acids containing the DXD, DRE, and DXNDNAPXF sequence motifs, which are the major conserved motifs among typical cadherin families, are underlined in thick lines under the consensus sequence. The DXDXGXN, AXDXGXPXL motifs and glycine residue in the middle of repeat except for the EC2 and EC4 regions, which are the conserved features among the CNR1, CNR2 and protocadherin family, are underlined in thin lines under consensus sequence. The single leucine residue, which is the predominantly conserved feature between CNR1 and CNR2, is indicated by #. The RGD motifs in the EC1 region of CNR1 and CNR2 are boxed with shades.

(D) Schematic structural comparison of CNR1 and CNR2 with protocadherins and classical

cadherins. The RGD motif, the five cysteine residues appearing at regularly spaced intervals spanning 18, 9, 9, and 18 amino acid residues, and the five PXXP motifs in the cytoplasmic domains of CNR1 and CNR2 are shown. The lysine-rich sequence in the C-terminal region of the cytoplasmic domain are shown in hatched boxes. The long sequence of more than 15 amino acid residues conserved between CNR1 and CNR2 is underlined with a thick bar at the bottom of the structure of CNR2.

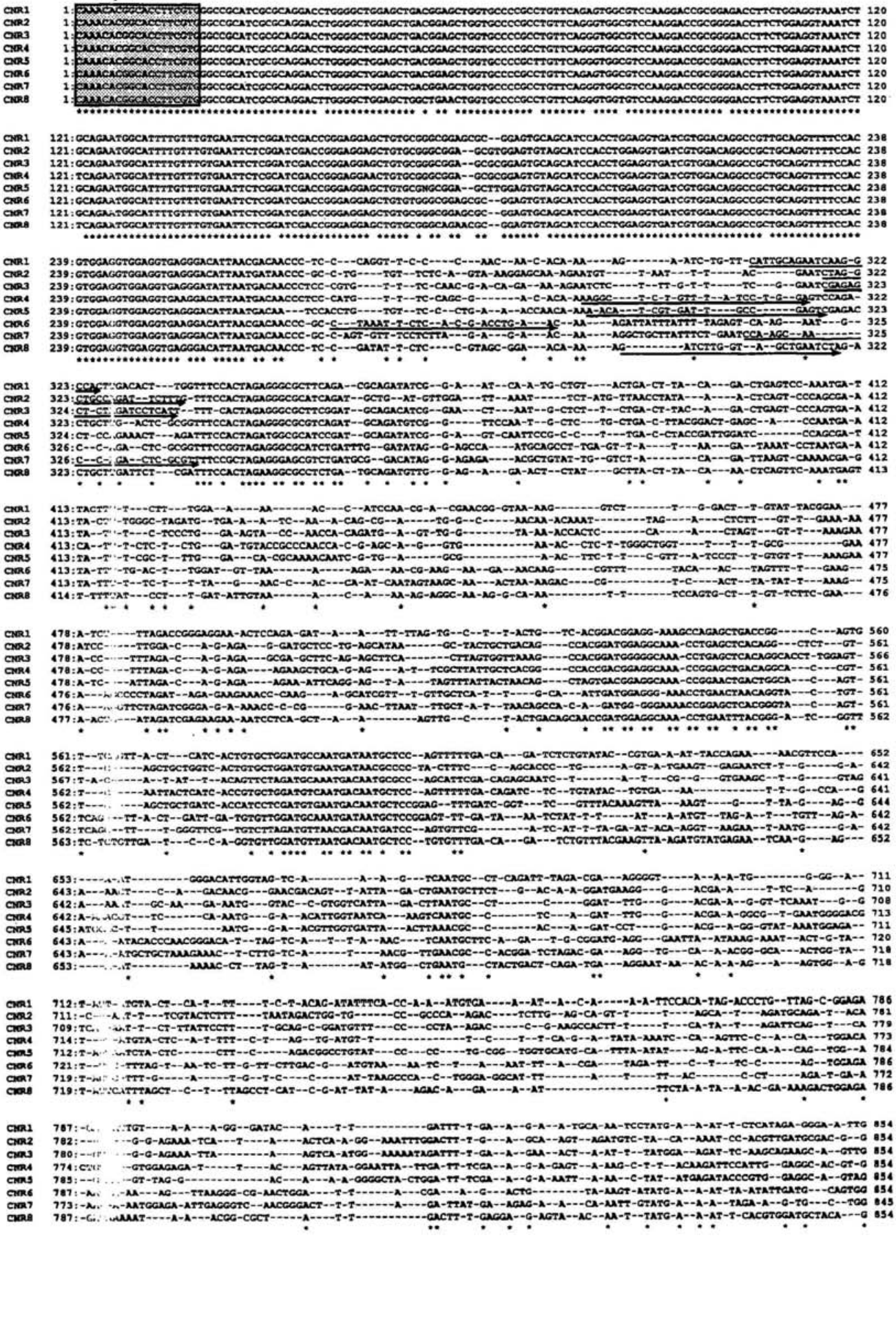
S, signal peptide; EC, extracellular domain; TM, transmembrane domain; CP, cytoplasmic domain.

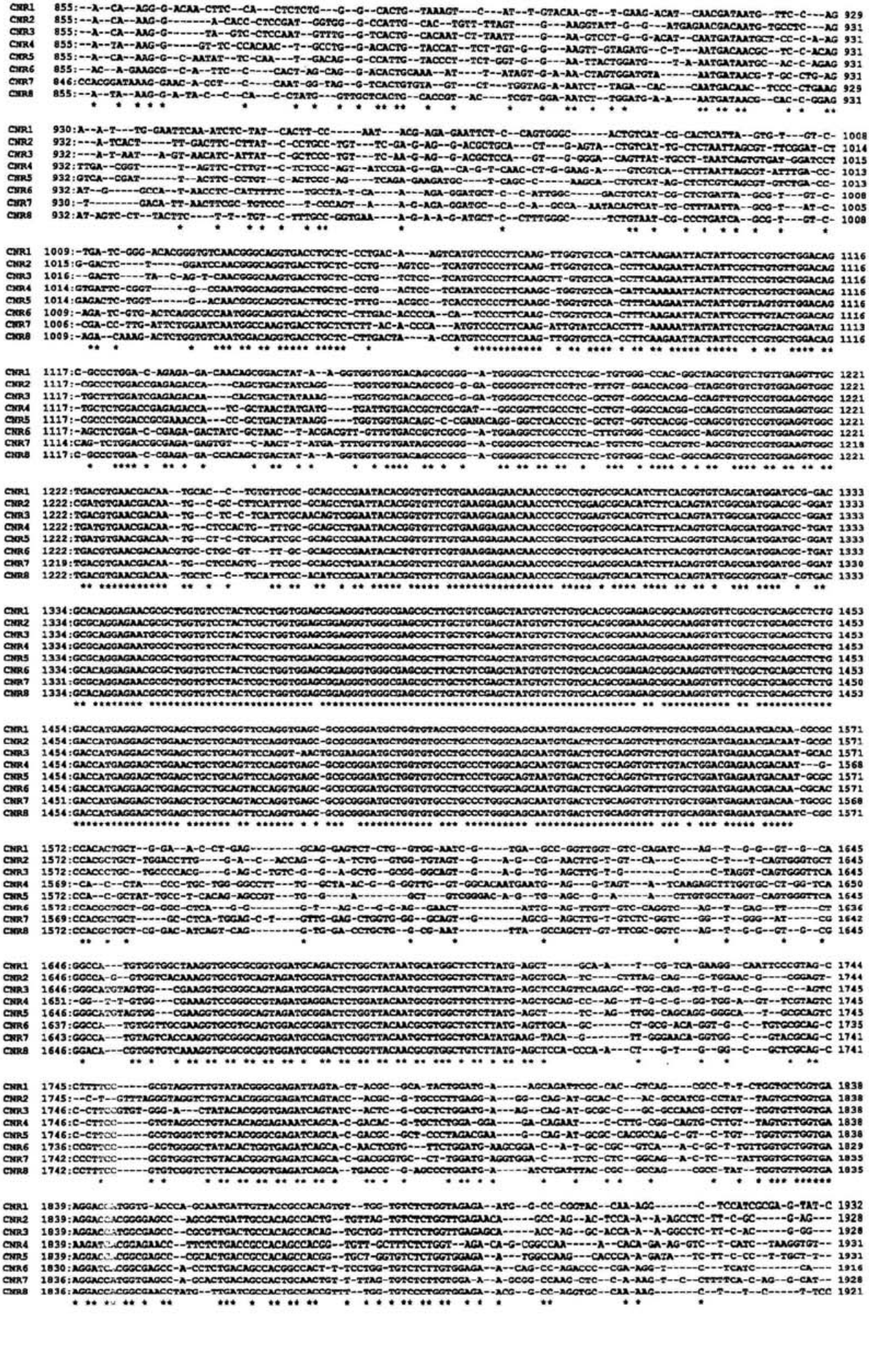
Figure 5.


A
5' probe
3' probe
EcoRI
PstI
SacI
EcoRI
PstI
SacI
kb
23.1
9.4
6.6
4.4
2.3
2.0
1.4
1.1
0.9
0.6
0.3
1
2
3
4
5
6

B

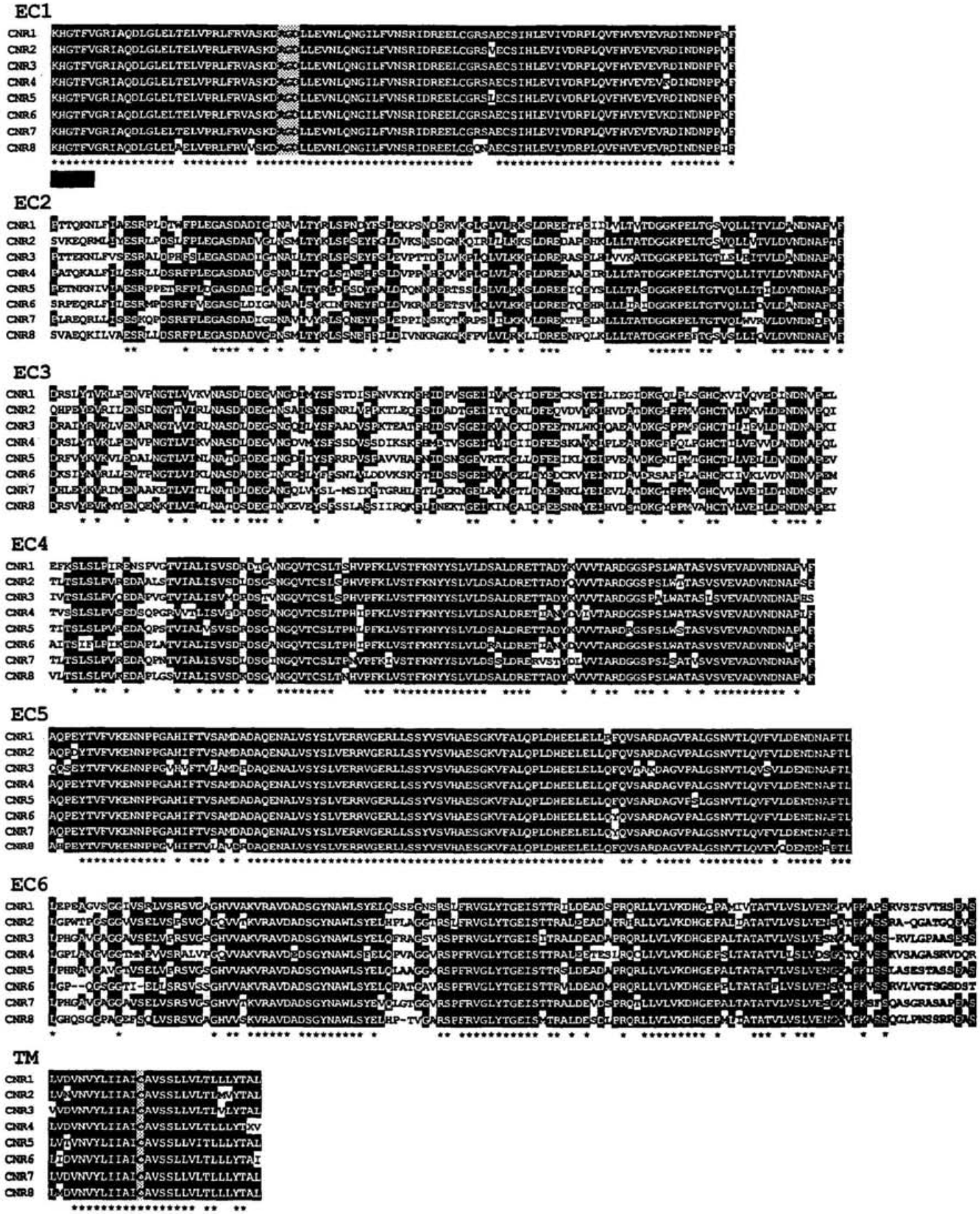
5'primer



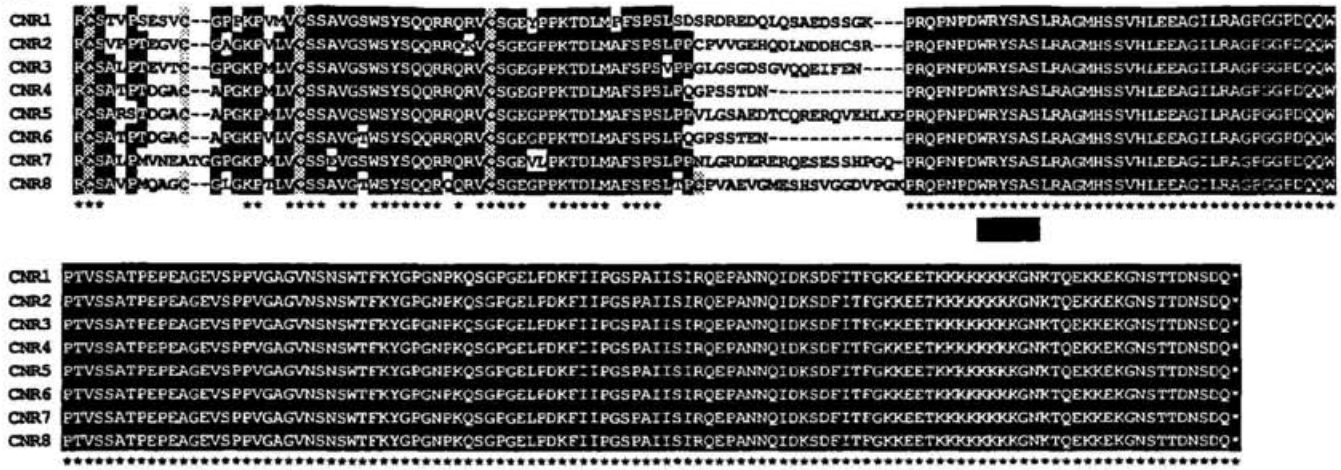



3'primer

C


EC1
EC2
EC3
EC4
EC5
EC6
TM
CNR1
CNR2
CNR3
CNR4
CNR5
CNR6
CNR7
CNR8

CP



D

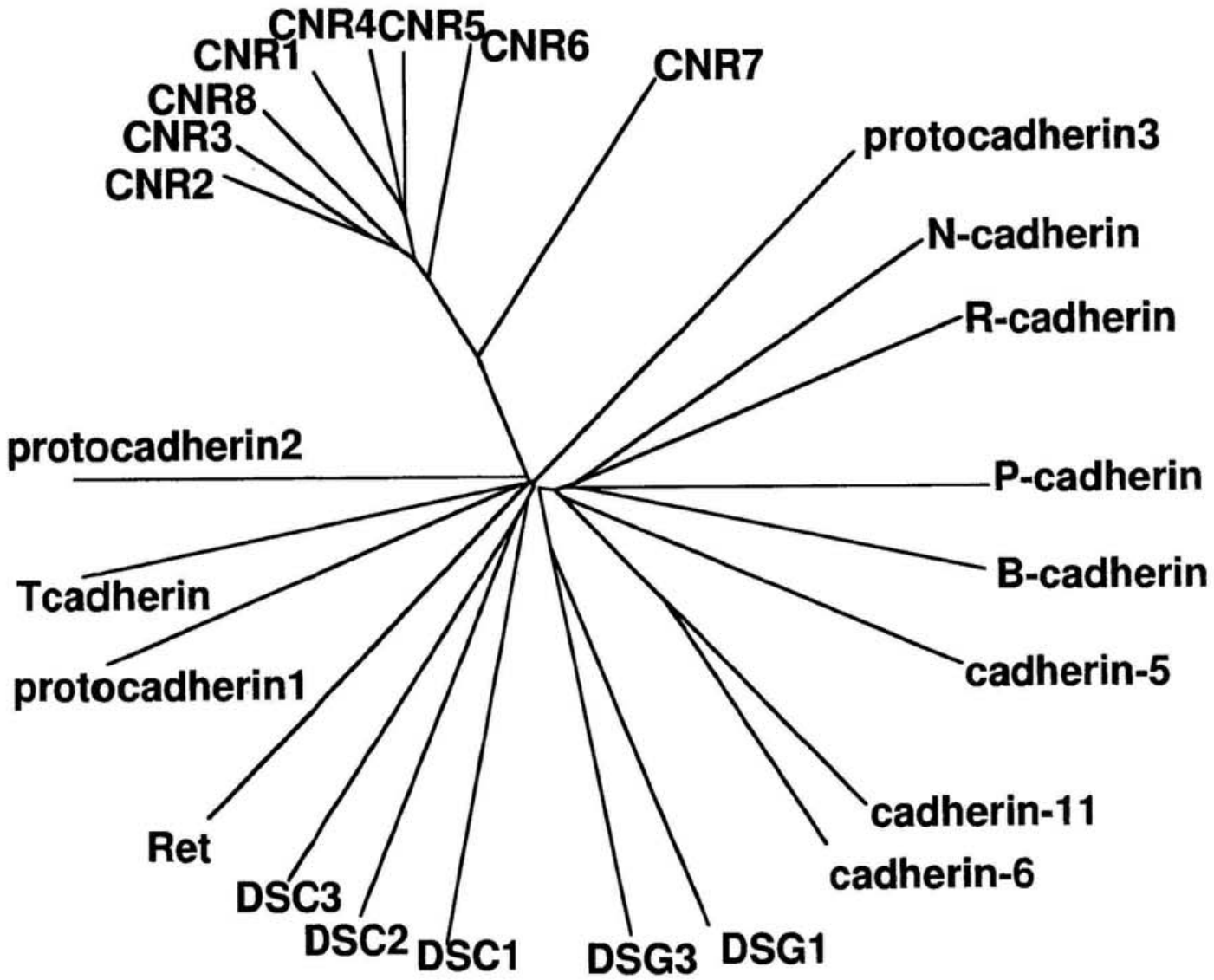

CNR4
CNR5
CNR6
CNR1
CNR7
CNR8
CNR3
CNR2
protocadherin3
N-cadherin
R-cadherin
protocadherin2
P-cadherin
B-cadherin
Tcadherin
cadherin-5
protocadherin1
cadherin-11
Ret
cadherin-6
DSC3
DSC2
DSC1
DSG3
DSG1

Figure 5. CNRs Constitute a Multi-gene Family.

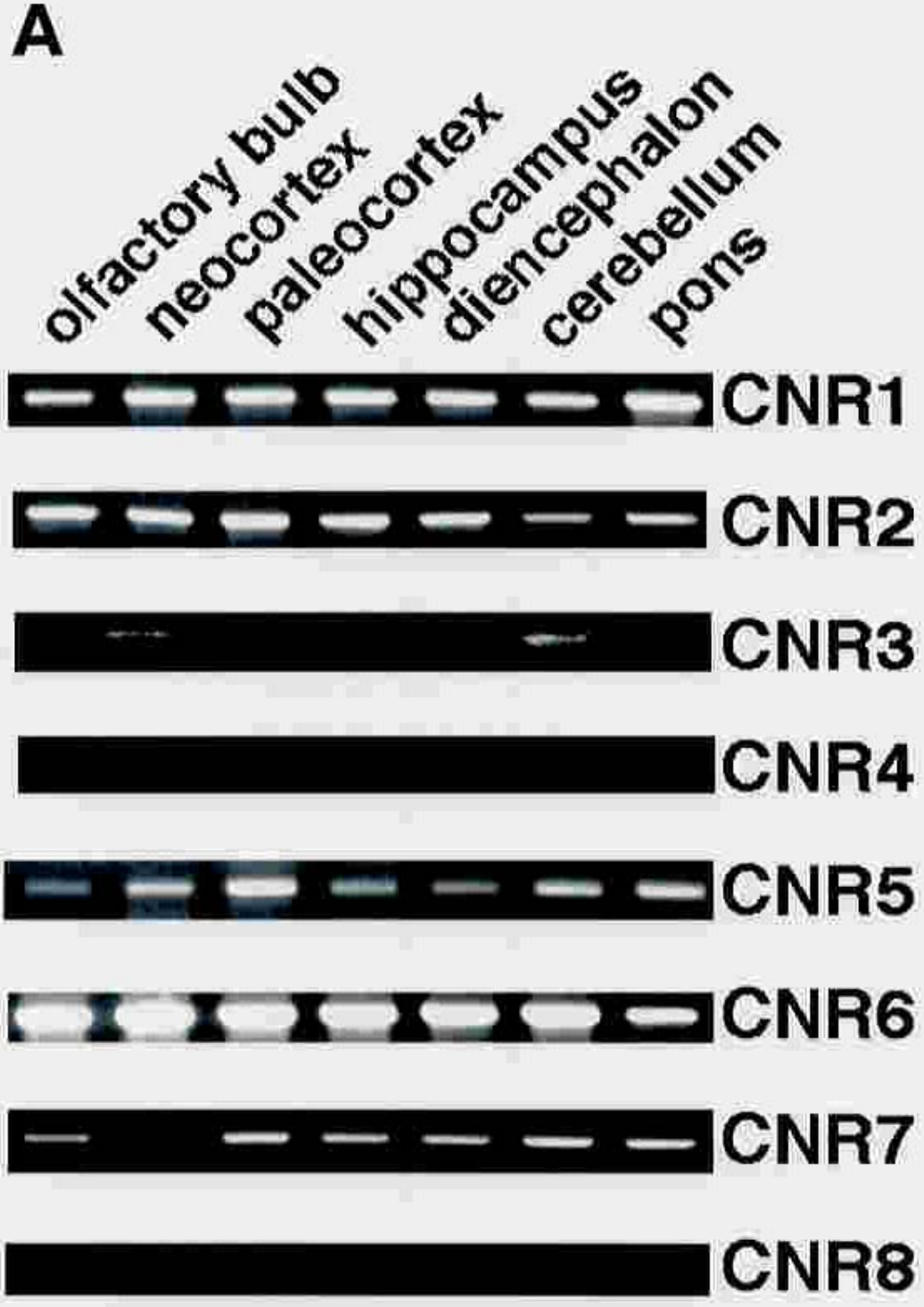
(A) Genomic Southern blot analysis using 5' (left) and 3' (right) conserved sequences between CNR1 and CNR2 as probes. The sequences of the probes are shown in Figure 4A. Ten ug of genomic DNA from C57BL/6 mouse liver digested with *Eco*RI (lane 1 and 4), *Pst*I (lane 2 and 5), and *Sac*I (lane 3 and 6) was loaded in each lane. DNA size markers are λ/*Hind*III and λ/*Hae*III.

(B) Alignment of the nucleotide sequences of eight CNR cDNA identified by RT-PCR. Asterisks show the common nucleotides among the eight CNRs. The sequences of 5' and 3' primers are boxed with shadows. The sequences of specific primers for Figure 6A are underlined.

(C) Alignment of the deduced amino acid sequences of eight CNRs by each domain. Asterisks show the common amino acid residues among the eight CNRs. Amino acid residues common to at least five of the eight sequences are shown as white lettering on black background. The sequences corresponding to 5' and 3' primers are underlined with thick bars. The RGD motif and cysteine repeats are shadowed.

(D) Phylogenetic tree of the cadherin super family deduced from amino acid sequence similarity.

Figure 6.


A
olfactory bulb
neocortex
paleocortex
hippocampus
diencephalon
cerebellum
pons
CNR1
CNR2
CNR3
CNR4
CNR5
CNR6
CNR7
CNR8

B

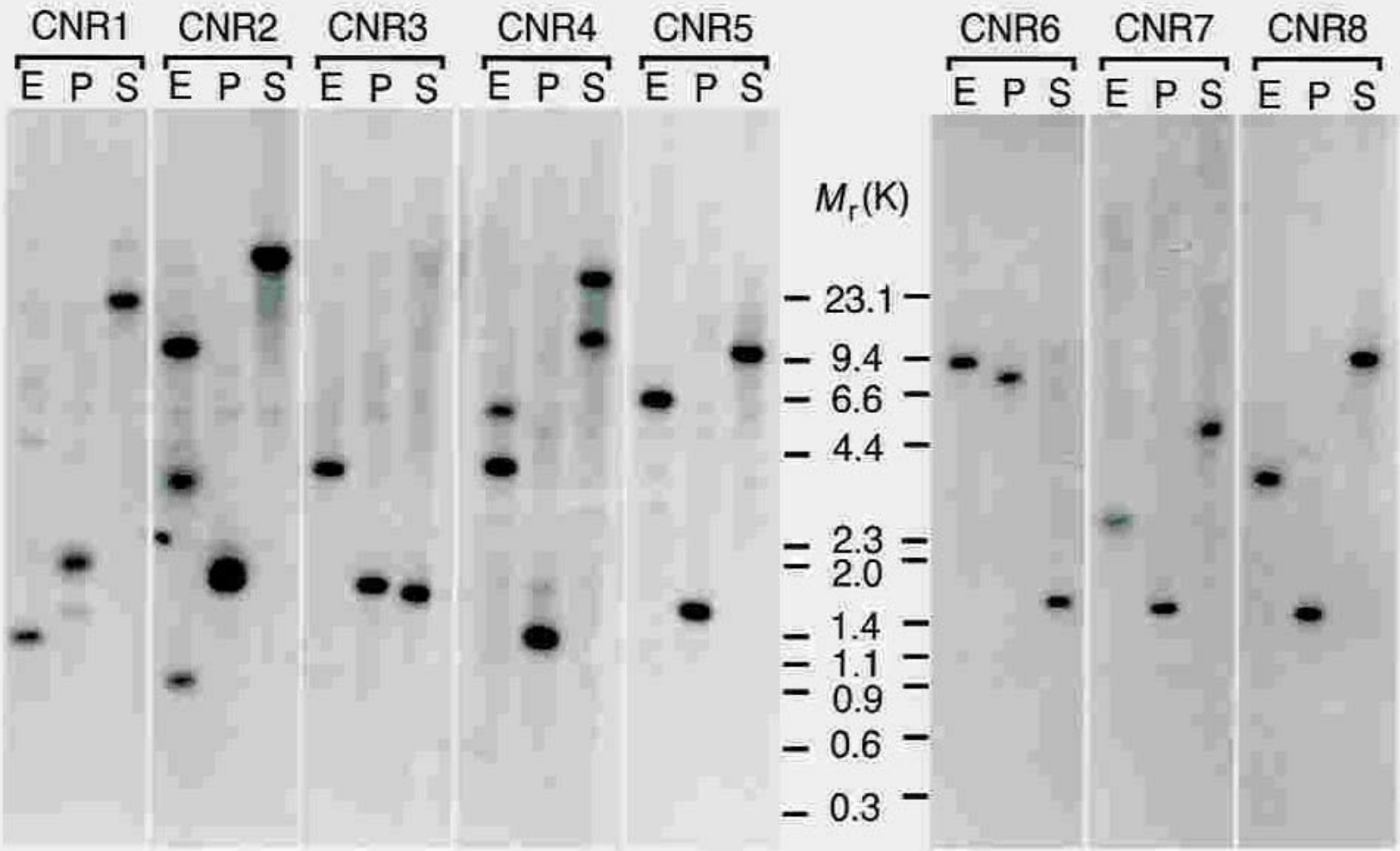
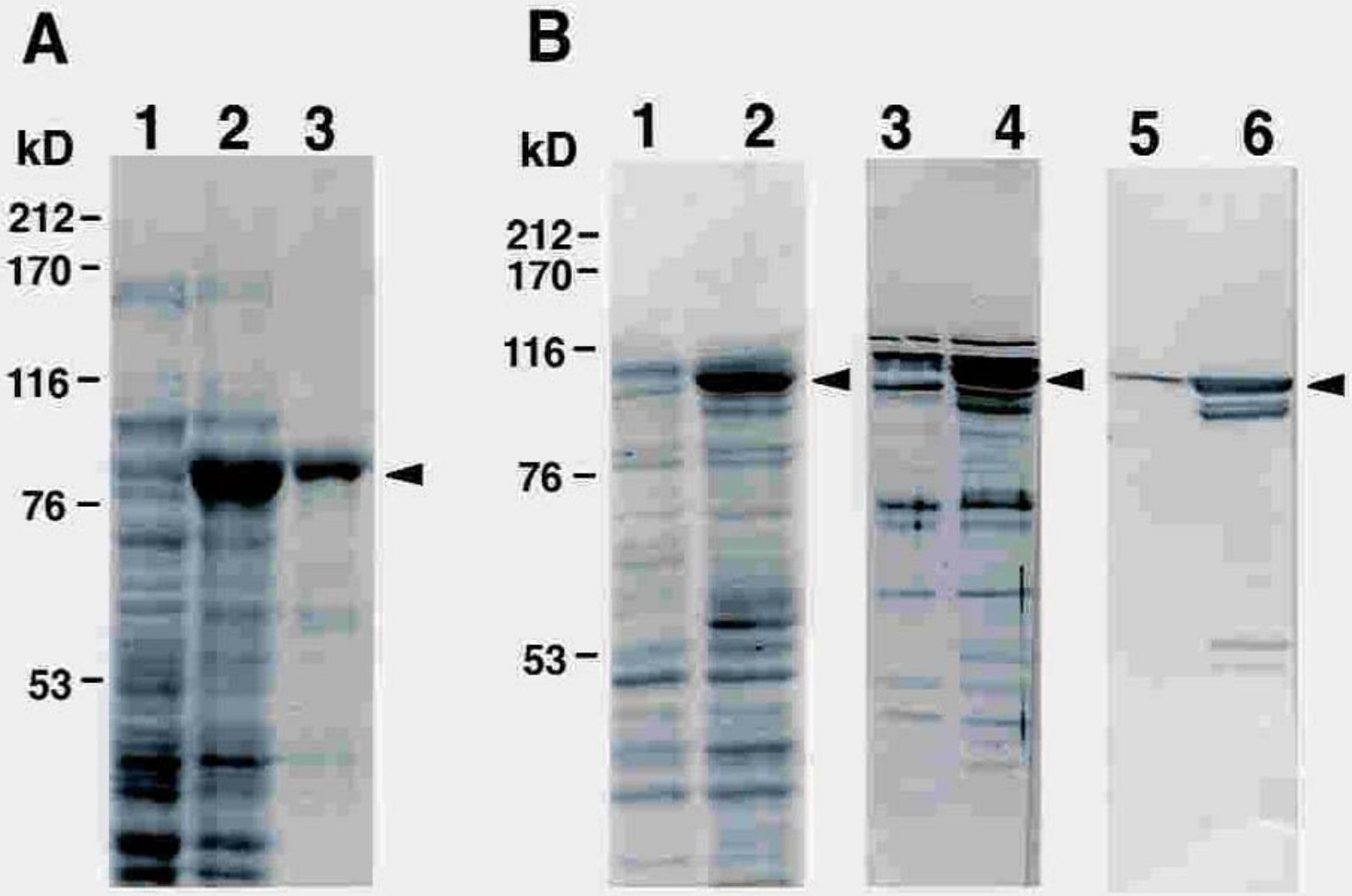

CNR1
CNR2
CNR3
CNR4
CNR5
CNR6
CNR7
CNR8
E P S
M_r(K)
23.1
9.4
6.6
4.4
2.3
2.0
1.4
1.1
0.9
0.6
0.3

Figure 6. Identification of Eight Distinct CNRs in the Mouse Genome, and Expression of Their Respective mRNAs in the Brain.

(A) RT-PCR analysis using specific primers for each CNR. The primer sequences are shown in Figure 5B.

(B) Genomic Southern blot analyses with non-cross-hybridizing probes of CNR cDNAs. Ten ug of genomic DNA from C57BL/6 mouse liver digested with *Eco*RI (E), *Pst*I (P), and *Sac*I (S) was applied to each lane. The probes used are from extracellular domains 2 and 3, which comprise the least-conserved region among the CNRs. Note that each CNR does not exhibit the same pattern of bands.

Figure 7.



C

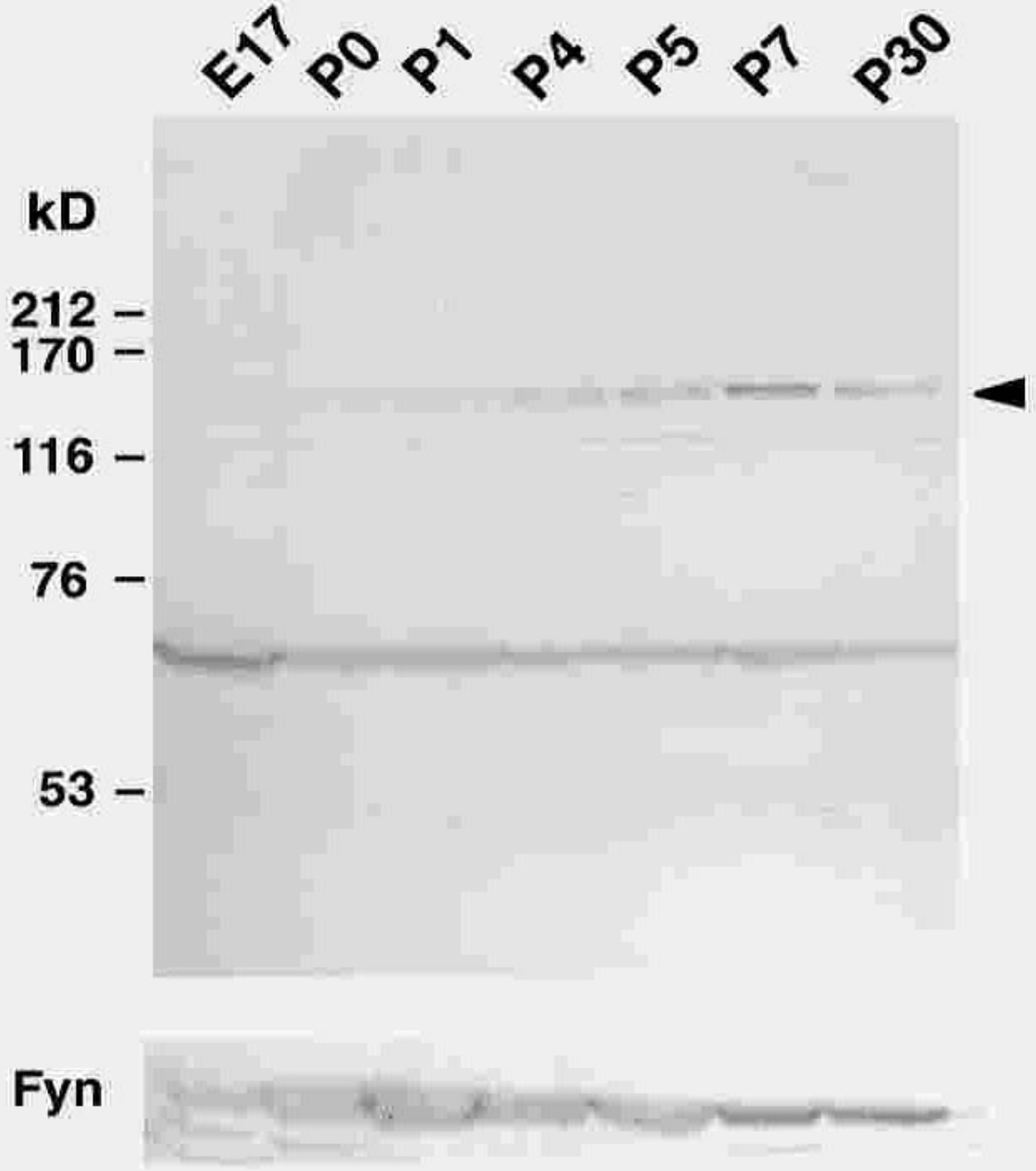


Figure 7. Establishment of CNR1 antibodies.

(A) SDS-PAGE analysis of extracts from trxA-CNR1EC-producing *E. coli* before induction (lane 1), after induction (lane 2) and purified fusion protein (lane 3) are stained with CBB. The purified fusion protein was injected into rabbit and mouse.

(B) Left panel shows SDS-PAGE analysis of extracts from GST-CNR1EC-producing *E. coli* before induction (lane 1) and after induction (lane 2). The gel was stained with CBB. Center panel shows Western blot analysis of the *E. coli* extracts before (lane 3) and after induction (lane 4) incubated with rabbit anti-serum. Right panel shows Western blot analysis of extracts from GST-CNR1EC-producing *E. coli* before (lane 5) and after induction (lane 6) incubated with 6-1B mouse monoclonal antibody.

(C) Developmental profile of CNR1 protein expression. 30 ug of P2 fractions at embryonic day 17 (E17), postnatal (P0) day 0, 1, 4, 5, 7, and 30 were loaded for Western blot analysis. The membranes were incubated with anti-CNR1 monoclonal antibody 6-1B (top panel) and the anti-Fyn monoclonal antibody γC3 (bottom panel).

Figure 8.

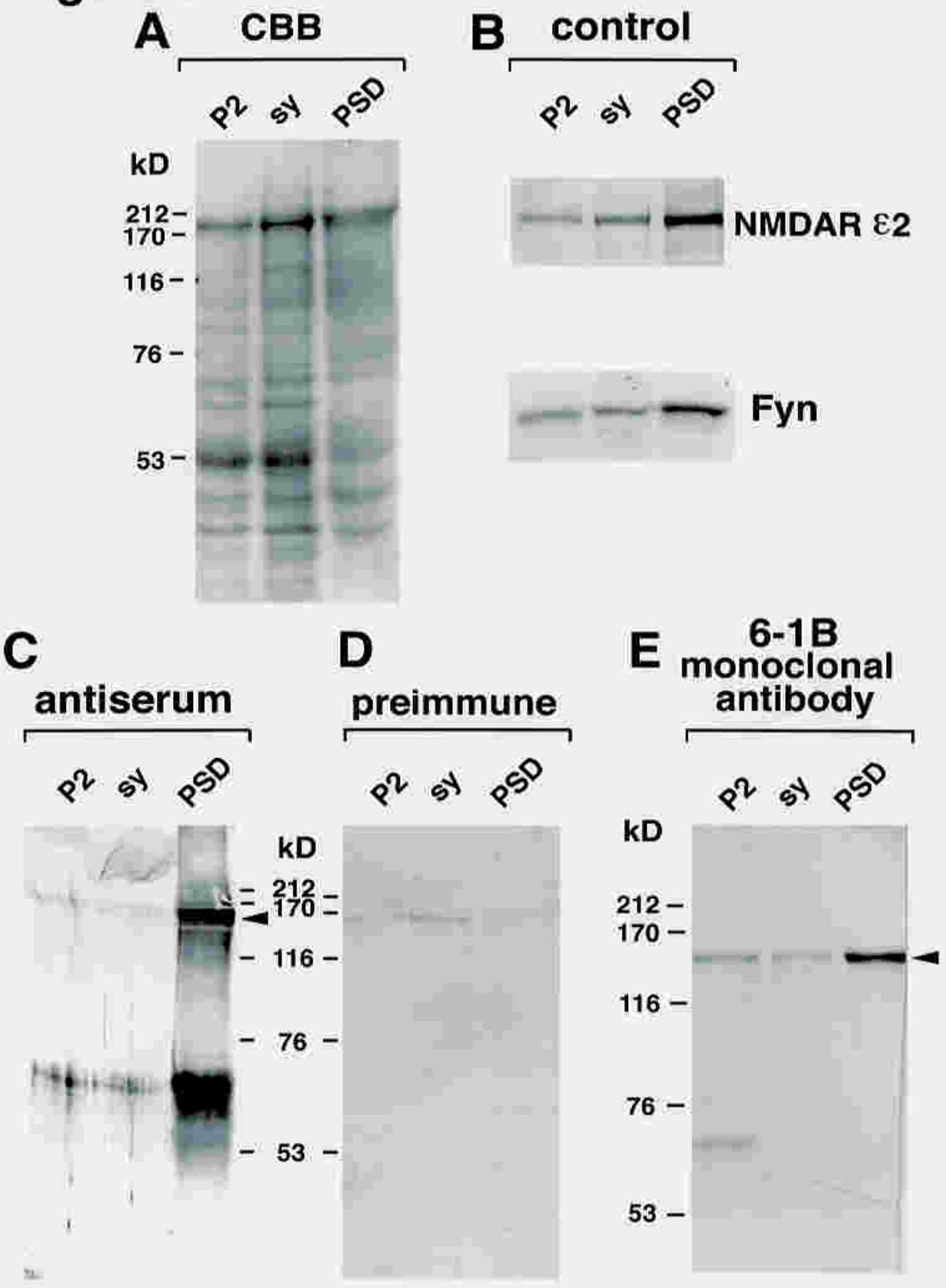

A
CBB
P2
sy
PSD
kD
212
170
116
76
53
B
control
P2
sy
PSD
NMDAR ε2
Fyn
C
antiserum
P2
sy
PSD
kD
212
170
116
76
53
D
preimmune
P2
sy
PSD
E
6-1B
monoclonal
antibody
P2
sy
PSD
kD
212
170
116
76
53

Figure 8. CNR1 is Concentrated in the Postsynaptic Density Fraction.

P2 fraction (P2), synaptosomal fraction (sy), and postsynaptic density fraction (PSD) were prepared as described in Materials and Methods. 10 ug of the samples was loaded in each lane.

(A) SDS-PAGE analysis of the samples stained with CBB.

(B) The NMDA receptor ε2 and Fyn were quantitated as fraction preparation controls.

(C) CNR1 antiserum reacted with 160 kDa-protein. The protein was concentrated in the PSD fraction.

(D) Pre-immune antiserum was incubated for control.

(E) CNR1 monoclonal antibody 6-1B reacted with 160 kDa-protein. The protein was concentrated in the PSD fraction.

Figure 9.

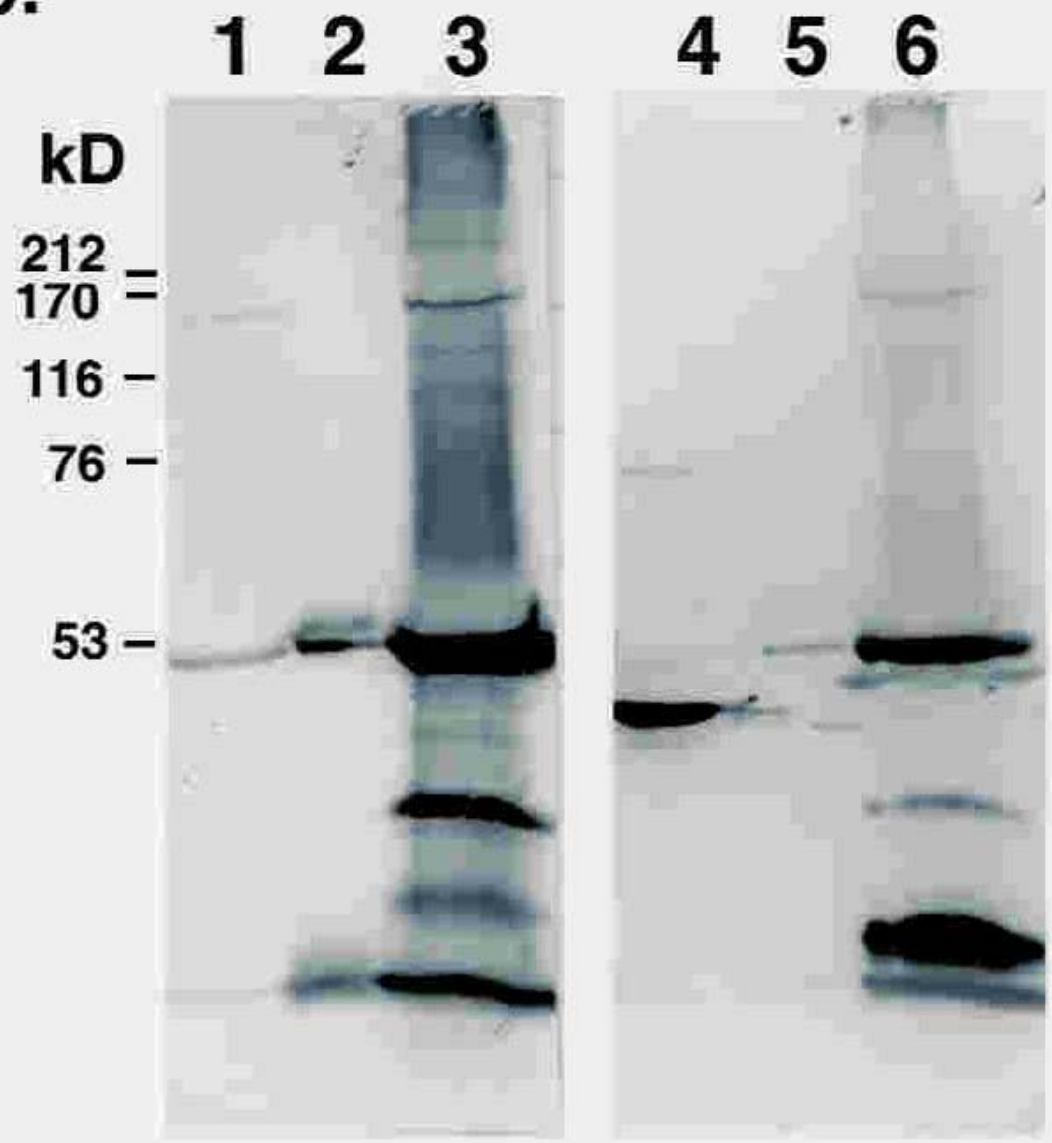
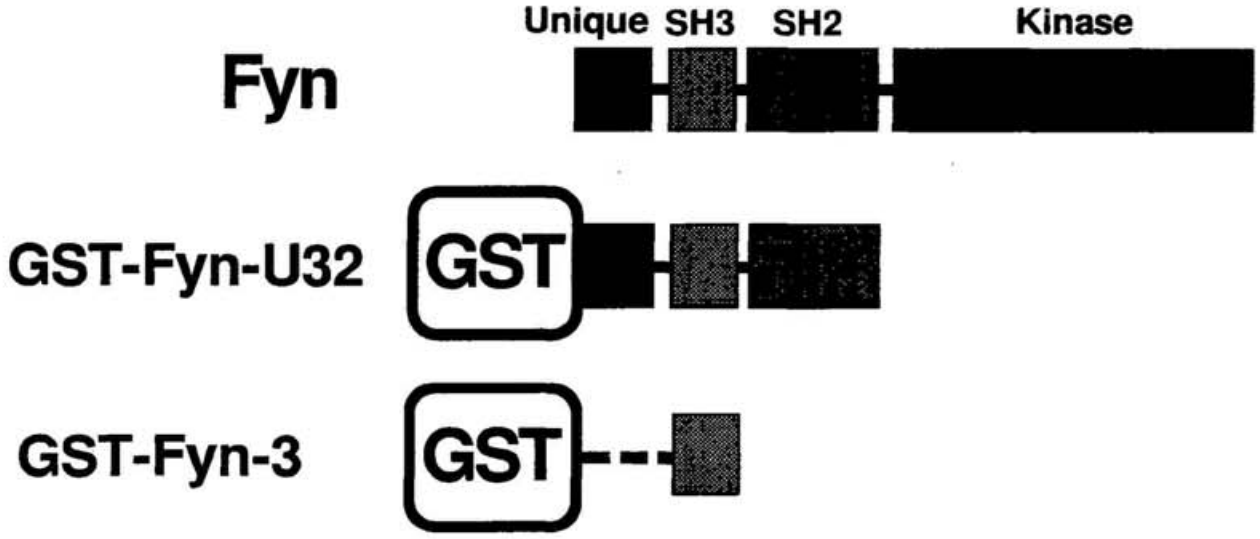


Figure 9. Co-immunoprecipitation of CNR1 with Fyn.

Mouse brain homogenate in RIPA buffer was incubated with protein G beads coated with anti-Fyn monoclonal antibody γC3. Immunoprecipitates were analyzed by Western blot analysis using CNR1 anti-serum (left) and anti-CNR1 monoclonal antibody 6-1B (right). Lane 1 and lane 4, whole brain homogenate in RIPA buffer; lane 2 and lane 5, protein G beads reacted with brain homogenate; lane 3 and lane 6, immunoprecipitate with γC3 monoclonal antibody-coated protein G beads. 160 kD-protein bands were detected in both antibodies.

Figure 10.

A



B

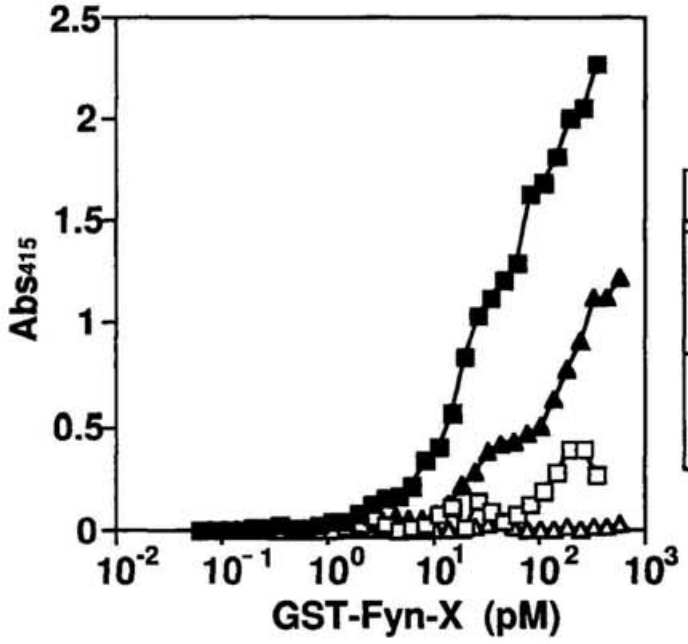


plate coating	incubation
GST-CNR-CP	GST-Fyn-U32
	GST-Fyn-3
GST	GST-Fyn-U32
	GST-Fyn-3

Figure 10. The Conserved Cytoplasmic Region in All CNRs Bind the Fyn Regulatory Region.

(A) Schematic representation of Fyn and GST-fusion proteins. The DNA fragment of unique-SH3-SH2 and SH3 regions were cloned into pGEX-2T expression vector to yield GST-Fyn-U32 fusion protein and GST-Fyn-3 fusion protein, respectively. Fusion proteins were tested for their ability to bind to the conserved cytoplasmic region of CNR family by ELISA.

(B) The conserved sequence of CNR1 and CNR2 was cloned into pGEX-2T expression vector to yield GST-CNR-CP fusion protein. This protein was coated at 2.0 ug/well in duplicate rows. Only GST protein was also coated at 2.0 ug/well in another pair of duplicate rows. The GST-Fyn-U32 and the GST-Fyn-3 proteins were incubated after being serially diluted (3:4) starting at 600 pM and 770 pM, respectively. The graph shows the dose-response binding curve of GST-Fyn-fusion proteins to the conserved-CNR cytoplasmic region.

Abs415 = absorbance at 415 nm; X = GST-Fyn-U32, GST-Fyn-3.

Table 1. **No. of clones by RT-PCR**

Stage \ Type	CNR1	CNR2	CNR3	CNR4	CNR5	CNR6	CNR7	CNR8
P0	1	11	0	0	0	0	0	28
P30	7	5	5	6	5	3	1	0

Acknowledgments

I would express my sincere appreciation to Dr. Takeshi Yagi and Professor Kazuhiro Ikenaka for their continuous encouragements and helpful advice throughout this study.

I wish to thank all the members of our laboratory, especially Miss Sachiko Makino for her expert technical assistance and Dr. Nobuyuki Kai for his helpful cooperation on several points in my study. I also thank Dr. Masahiro Yasuda for teaching me hybridoma techniques and for many helpful suggestions during the course of this study. I am also sincerely grateful to Dr. Rie Yasuda, Dr. Masahiko Taniguchi, Mr. Makoto Sanbou, Mrs. Chiaki Sanbou, and Mr. Hiroyuki Ishii for their helpful criticism.

I also appreciate my parents from the bottom of my heart for supporting me in everything without compensation. Finally, I would express my special thanks to my fiancee, Miki for her encouragements throughout my study.