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A pregnancy-specific glycoprotein is expressed in the brain and serves as a receptor for mouse hepatitis virus

(carcinoembryonic antigen/coronavirus/brain expression/virus receptor)

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ABSTRACT **Mouse hepatitis virus (MHV), a murine coronavirus known to cause encephalitis and demyelination, uses murine homologues of carcinoembryonic antigens as receptors. However, the expression of these receptors is extremely low in the brain. By low-stringency screening of a mouse brain cDNA library, we have identified a member of the pregnancy-specific glycoprotein (PSG) subgroup of the carcinoembryonic antigen gene family. Unlike other PSG that are expressed in the placenta, it is expressed predominantly in the brain. Transfection of the cDNA into COS-7 cells, which lack a functional MHV receptor, conferred susceptibility to infection by some MHV strains, including A59, MHV-2, and MHV-3, but not JHM. Thus, this is a virus strain-specific receptor. The detection of multiple receptors for MHV suggests the flexibility of this virus in receptor utilization. The identification of a PSG predominantly expressed in the brain also expands the potential functions of these molecules.**

The cellular protein that functions as a viral receptor is a critical component in determining the tissue tropism of a virus. Viruses generally display a very high specificity of receptor use; however, recent studies have shown that some viruses may use alternative cell-surface molecules as receptors. For example, human immunodeficiency virus (HIV) uses CD4 as a receptor to infect T4 lymphocytes but may also use variable-region heavy-chain 3 immunoglobulin (1) molecules to infect B cells. The ability of a virus to utilize a repertoire of different receptors may allow a virus to expand its cellular and tissue spectrum.

The murine coronavirus mouse hepatitis virus (MHV) can use several different receptors (2, 3) and cause enteritis, hepatitis, encephalitis, and/or demyelination, depending on the virus strain (4). It is known that point mutations or deletions in the viral envelope spike protein, which mediates viral interaction with cellular receptors (5), and results in changes in viral pathogenicity and cellular tropism (6, 7). Conceivably, viral receptors in different tissues may vary, and different virus strains may use tissue-specific receptors at different efficiencies. Such a scenario will provide a means of regulating viral tissue tropism.

The receptors for MHV have been identified as the murine homologues of the biliary glycoprotein of the carcinoembryonic antigen (CEA) family (8, 9). Two alternatively spliced forms, $MHVR_1$ and $MHVR_2$ (formerly mmCGM1 and mmCGM2) (2, 3), and several other allele products of this gene family (10, 11) can be used as MHV receptors. However, their expression is mainly limited to the liver and intestinal brush border (12, 13). Only $MHVR_2$ is weakly expressed in the mouse brain (2). This is in contrast to the marked neurotropism of some MHV strains (14, 15). Therefore, there may exist other types of MHV receptors in the brain. Here, we report the identification of a CEA-related molecule predominantly expressed in the brain, which belongs to the pregnancy-specific glycoprotein (PSG) subgroup and functions as an MHV receptor in a virus strain-specific fashion.‖ The expression of PSG in the brain was previously unrecognized. The presence of multiple types of viral strain-specific receptors potentially allows regulation of viral tissue tropism.

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MATERIALS AND METHODS

Viruses, Cells, and Animals. MHV strains A59, JHM, MHV-2, and MHV-3 have been described (16, 17). COS-7 cells (18), derived from monkey kidney cells, were used for transfection and infection studies. Virus plaque assays were done with DBT cells (19), a murine astrocytoma cell line. Growth conditions of cells were the same as described (10). C57BL/6 mice, both pregnant and nonpregnant six-week-old mice, were purchased from The Jackson Laboratory.

cDNA Library Construction and Screening. A brain cDNA library was prepared from the poly(A)-containing RNA of a male 6-week-old C57BL/6 mouse, using Lambda ZAPII vector (Stratagene) according to the manufacturer's protocol. The amplified library contained ≈85% recombinants. This cDNA library and another library derived from the brain of an outbred newborn mouse (Stratagene) were screened at low stringency with a ^{32}P-labeled, random-primed DNA probe representing the coding sequence of receptor $MHVR_1$ (2). The hybridization was done at 42°C for 16–18 hr, and washing was sequentially performed at room temperature with 6 × standard saline citrate (SSC)/0.5% SDS, four times, 2 × SSC/0.1% SDS, two times, and 1 × SSC/0.1% SDS, two times. Isolated clones were plaque-purified three times before *in vivo* excision (Stratagene).

Transfection, Viral Infection, and Immunofluorescence. COS-7 cells were transfected with DNA via the transfection reagent DOTAP (Boehringer) and infected with MHV as per Yokomori and Lai (10). Immunofluorescence of infected cells was done 7.5 hr after infection by using monoclonal antibodies specific for N protein of strain A59 (10).

Abbreviations: PSG, pregnancy-specific glycoprotein(s); CEA, carcinoembryonic antigen(s); MHV, mouse hepatitis virus; ORF, open reading frame; FACS, fluorescence-activated cell sorting; RT-PCR, reverse transcription–PCR; bCEA, CEA-like molecule isolated from brain.

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. U34272).

Reverse Transcription–PCR (RT–PCR). Total RNA was extracted from various tissues by guanidine isothiocyanate and phenol/chloroform (20) and used for RT–PCR. The primers used for brain CEA correspond to nt 109–138 (sense) and 816–835 (antisense) of brain CEA. Primers used for murine PSG (Cea-2) correspond to nt 181–210 (sense) and 867–886 (antisense) (21). Primers used for β-actin correspond to nt 460–480 (sense primer) and 1017–1040 (antisense primer) of the murine β-actin gene (22). PCR was done at an annealing temperature of 60°C for brain CEA and PSG, or 65°C for β-actin, for 25 or 30 cycles. PCR products were transferred to Hybond-C Extra membrane (Amersham) and hybridized with a random-primed, ^{32}P-labeled brain CEA cDNA fragment (nt 1–760) as described (2).

Immunofluorescence and Fluorescence-Activated Cell Sorting (FACS) Analysis of Brain CEA Expression. Immunofluorescence and flow cytometry analyses of COS-7 cells transfected with brain CEA-encoding plasmid or PECE vector (33) alone were done with rabbit polyclonal antibodies made against brain CEA expressed in recombinant baculovirus-infected Sf9 cells. Cells were first treated with the primary antibody without prior fixation and subsequently detected by a fluorescein-conjugated goat anti-rabbit antibody. FACS analysis was performed in a Becton Dickinson FACSTAR Plus.

RESULTS

Identification of a CEA-Related cDNA Clone from Mouse Brain cDNA Libraries. To identify possible alternative receptors used by MHV in the infection of the brain, we reasoned that the alternative receptor is likely related to the known members of the CEA family. Therefore, we screened several mouse brain cDNA libraries using MHVR$_1$ (mmCGM1) cDNA as a probe. A total of 1×10^7 cDNA clones were screened, and 16 cDNA clones were obtained, 10 of which represent either partial or complete clones of a single gene, named bCEA (CEA-like molecule isolated from brain). These clones unexpectedly show the highest degree of sequence similarity to PSG (21, 23), which are members of the murine CEA family. PSG is mainly expressed in the placenta (21, 24) and was not known to be a MHV receptor. The sequence similarity is strongest from nt 217 to the 3′ end of the bCEA open reading frame (ORF) (80% similarity at the nucleotide level and 68% at the amino acid level, Fig. 1). However, PSG has an additional 255 nt upstream, which is almost completely diverged from that of bCEA. Thus, bCEA may represent a distinctive member of PSG. The sequence similarity between bCEA and the known MHV receptors, which belong to the biliary glycoprotein subgroup, is much lower. However, there are regions of strong amino acid sequence similarity (see

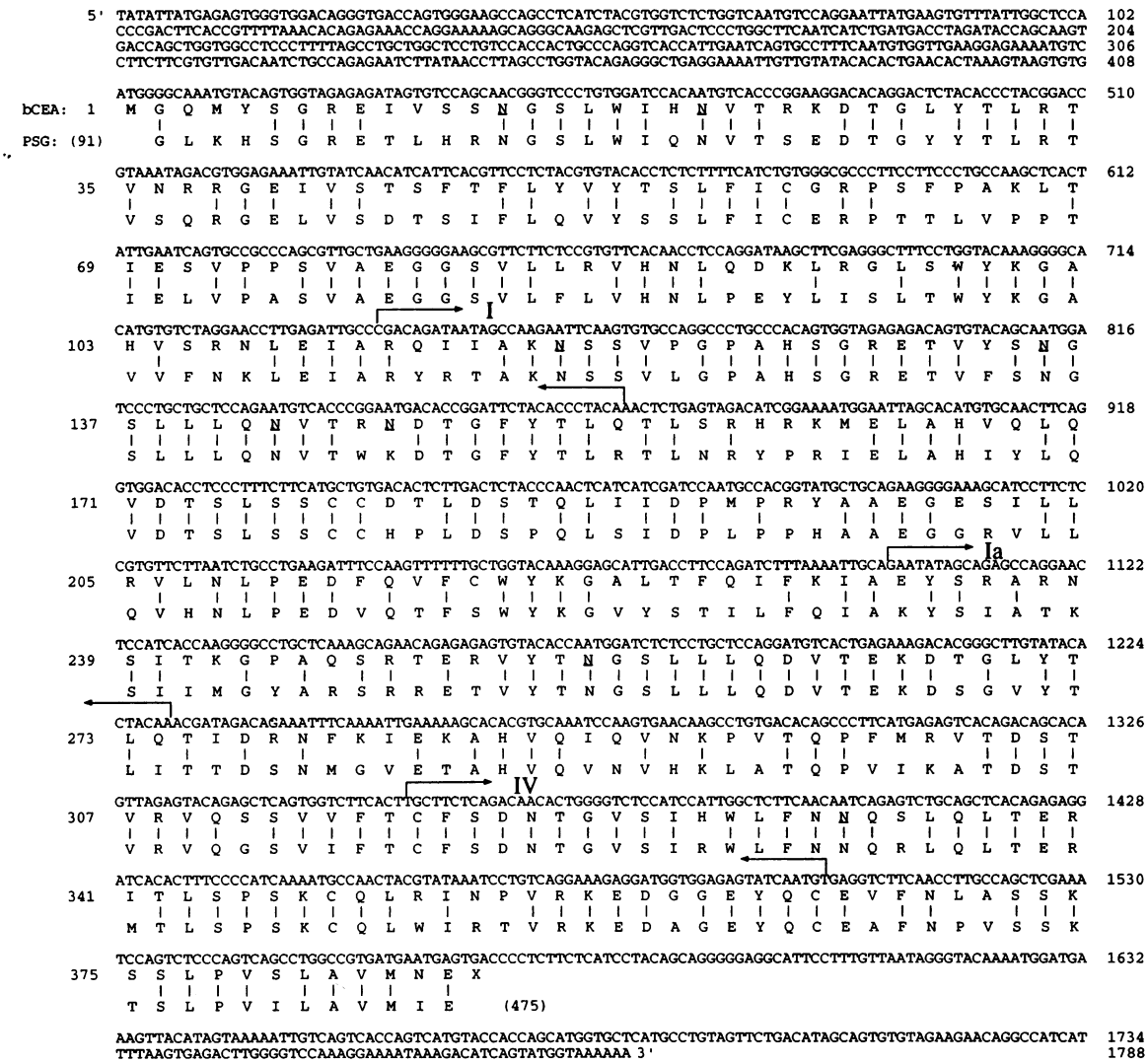

FIG. 1. Nucleotide and predicted amino acid sequences of bCEA cDNA. The amino acid sequence of a murine PSG, Cea-2 (amino acid residues 91–475) (21), is aligned for comparison. Potential N-linked glycosylation sites are underlined; the three immunoglobulin-like loops of bCEA are demarcated by arrows.

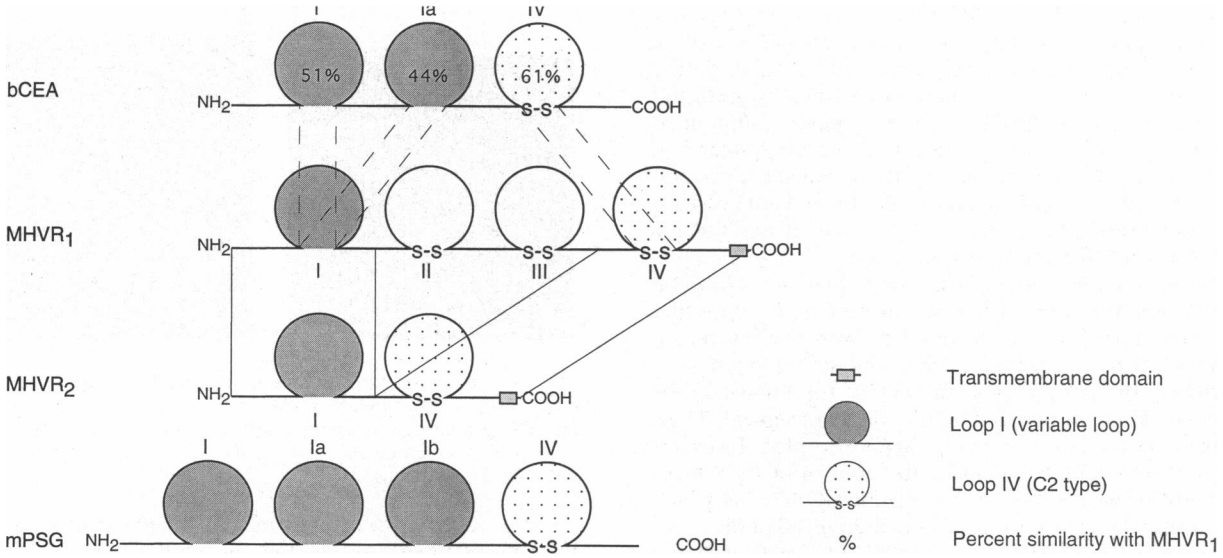

FIG. 2. Schematic diagram of the immunoglobulin-like loops in bCEA, $MHVR_1$, $MHVR_2$, and murine PSG.

below). Significantly, no cDNA clones coding for $MHVR_1$ or $MHVR_2$ were detected; this result is consistent with the previous finding that these MHV receptors are expressed at very low levels in the brain (2).

Characterization of the Structure and Surface Expression of bCEA. The longest bCEA clone, bCEA-1 (1.8 kb in length), has a large ORF, with a coding capacity for 386 amino acids (43 kDa) (Fig. 1). The predicted product contains eight potential N-linked glycosylation sites and may form three immunoglobulin-like loops. The structure and sequence of these domains are most similar to those of PSG, except that the latter contains an additional immunoglobulin-like loop, reflecting its longer upstream sequence (Fig. 2). The two N-terminal loops, designated loop I and loop Ia, are variable-type loops lacking a disulfide bond (25). These two loops share 60% amino acid sequence similarity between them; they also share 51% and 44% sequence similarity, respectively, with the N-terminal loop (loop I) of the known MHV receptor, $MHVR_1$ (8). The third immunoglobulin-like loop, designated loop IV, is a C_2-type loop (25), which is closed by a disulfide bond. Similar to PSG, bCEA does not have a strongly hydrophobic region but does contain a long stretch of uncharged sequence near the C terminus (data not shown).

To characterize the gene product of bCEA, *in vitro* transcription and translation of the bCEA-1 clone were done with and without microsomal membranes (Fig. 3). A primary translation product of 40 kDa was generated without microsomal membranes (Fig. 3*B*, lane b). In addition, a smaller protein of 36 kDa was also detected, which was likely the result of premature termination or alternative initiation, as the generation of this product was variable. With microsomal membranes, the size of the primary translation product increased to 55 kDa, suggesting posttranslational modifications, most likely glycosylation. This result suggests that the bCEA-1 product could be inserted into the endoplasmic reticulum and potentially expressed on the cell surface. Surface expression of bCEA was shown by immunofluorescence staining of COS-7 cells transiently transfected with bCEA-1 clone under the control of a simian virus 40 early promoter (PECE vector) (33), using a polyclonal antibody generated against the purified recombinant bCEA (Fig. 4). It was also confirmed by FACS analysis of the transfected cells using the same antibody (data not shown).

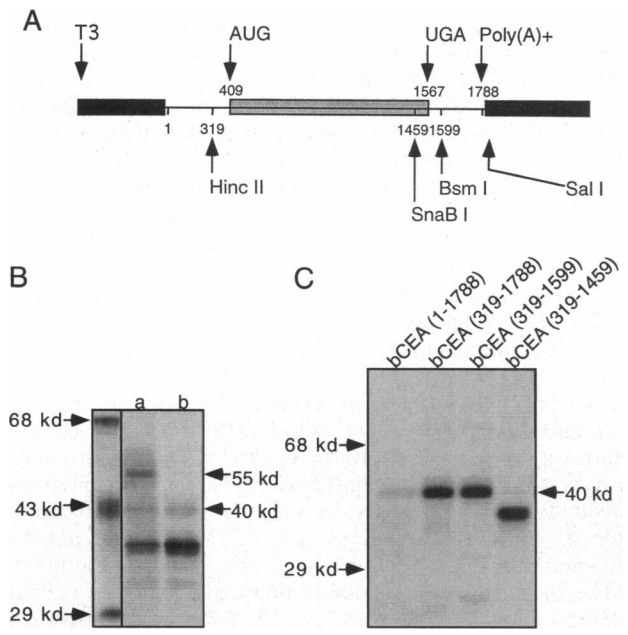

FIG. 3. *In vitro* transcription–translation of bCEA cDNA. (*A*) Schematic diagram of structure of bCEA cDNA used. Shaded area represents ORF, and black areas indicate vector sequences. (*B*) *In vitro* translation of full-length bCEA RNA. Lane: a, with microsomal membranes (Promega); b, without microsomal membranes. (*C*) *In vitro* translation of truncated bCEA RNAs. kd, kDa.

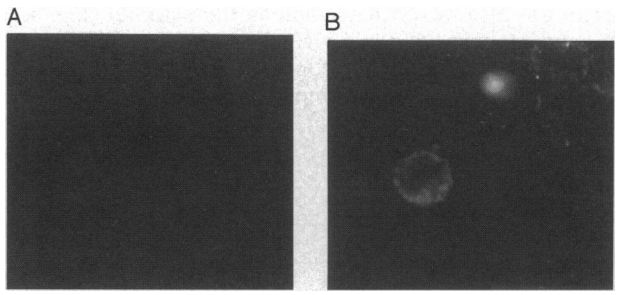

FIG. 4. Detection of surface expression of bCEA by immunofluorescence. COS-7 cells transfected with bCEA-1 under simian virus 40 early promoter were stained with a rabbit polyclonal antibody directed against bCEA and subsequently stained with goat anti-rabbit antibody. (*A*) Vector-transfected cells. (*B*) bCEA-transfected cells.

The ORF of bCEA-1 was further confirmed by *in vitro* transcription and translation of various truncated clones. Truncation of the 3′-terminal sequence had no effect on the size of the protein product until it reached within the predicted termination codon, establishing the termination codon at nt 1567–1569 (Fig. 3 *A* and *C*). Truncations of the 5′-terminal sequence up to 90 nt upstream of the predicted initiation codon enhanced the translation efficiency but did not decrease the size of the translated protein. Thus, the predicted ORF is likely the actual ORF used for translation.

Viral Receptor Function of bCEA Gene Product. The gene product of bCEA contains two immunoglobulin-like loops that have considerable similarity to loop I of known MHV receptors (Fig. 2). This domain is the virus-binding domain (26). Furthermore, the consensus sequences of the known MHV receptors are also conserved in bCEA (data not shown). Thus, bCEA could potentially serve as an MHV receptor. To examine this possibility, bCEA-1 under the control of the simian virus 40 early promoter was transfected into COS-7 cells, which lack a functional receptor for MHV but contain all of the other factors necessary for MHV infection (27, 28). The transfected COS-7 cells were examined for susceptibility to infection by different MHV strains. The known MHV receptors, $MHVR_1$ and $MHVR_2$ (2, 3), were used for comparison.

The results (Table 1) showed that bCEA-1-transfected cells were susceptible to A59, MHV-2, and MHV-3 infection, all of which yielded virus titers at least one logarithm higher than those from cells transfected with the PECE vector. Furthermore, A59 and MHV-2 infection of bCEA-1-transfected cells and $MHVR_1$- or $MHVR_2$-transfected cells yielded very similar virus titers, although MHV-3 yielded a slightly lower titer in bCEA-1-transfected cells. In contrast, strain JHM yielded no detectable virus from cells transfected with bCEA-1. Virus infection of the bCEA-1-transfected cells was confirmed by immunofluorescence of the infected cells using monoclonal antibodies specific for the nucleocapsid protein of MHV (Fig. 5). These results indicate that bCEA can serve as a receptor for strains A59, MHV-2, and MHV-3, but not for strain JHM; thus, in contrast to other known MHV receptors, the receptor function of bCEA is virus strain-specific. This is the only known PSG to serve as a MHV receptor.

Tissue Expression Pattern of bCEA. Because PSG is known to be highly expressed in the placenta (21, 24) and other CEA family members, in general, are known to be expressed in the liver and gastrointestinal tracts but not in the brain (29), isolation of a PSG-like clone from brain cDNA libraries was a surprise. Therefore, we determined the expression pattern of bCEA in different mouse tissues. Poly(A)-containing RNA from different organs of adult C57BL/6 mice (placenta was taken from 14-day pregnant mice) was subjected to RT–PCR using bCEA-specific primers and analyzed by Southern blot analysis. The results showed that a single species of bCEA-specific PCR product was detected in the brain; this same species was also weakly detectable in the placenta (Fig. 6*A*), lung, and spleen on long exposure (data not shown). This result indicates that bCEA is a predominantly brain-specific PSG molecule. As a control, the amounts of β-actin-specific PCR products were similar in all tissues examined (data not shown). When primers representing the murine PSG Cea-2 (21) were used for PCR amplification, multiple products were detected in the placenta (Fig. 6*B*). The major PSG product in the placenta, which probably represents Cea-2 (21), was also detected at a very low level in the brain (data not shown). Therefore, there is likely an additional PSG-like molecule expressed in the brain.

Table 1. Virus titers from COS-7 cells after transfection with various cDNAs

DNA transfected	Viral titer, pfu/ml			
	A59	MHV-2	MHV-3	JHM
Control (PECE vector)	6.9×10^1	1.7×10^3	0	0
MHVR-1	3.3×10^3	7.0×10^4	1.0×10^5	1.1×10^3
MHVR-2	1.6×10^3	3.6×10^4	5.0×10^4	3.1×10^2
bCEA	1.2×10^3	1.3×10^4	4.4×10^3	0

Each value represents viral titer in plaque-forming units per ml of collected supernatant.

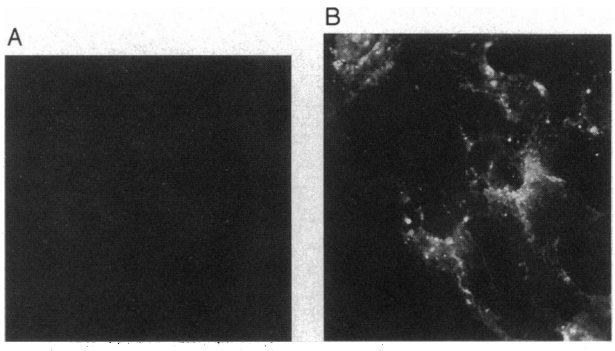

FIG. 5. Immunofluorescence staining of MHV antigens in COS-7 cells transfected with bCEA or PECE vector and infected with strain A59. The primary antibody used was a monoclonal antibody against the N protein of strain A59. (*A*) PECE vector-transfected cells. (*B*) bCEA-transfected cells.

The results of PCR-Southern experiments were confirmed by Northern-blot analysis of RNA from several tissues using a bCEA probe. Two RNA bands of ≈1.8 and 2.4 kb were visible in the brain samples. No bands were detectable in the RNA from the kidney, liver, or intestine (Fig. 6*C*). In contrast, the same amounts of β-actin RNA were expressed in all tissues. Therefore, bCEA is expressed predominantly in the brain.

DISCUSSION

MHV has been shown to utilize various alleles of the biliary glycoprotein subgroup of CEA family as receptors for infection (2, 3, 10, 11). Here, we have identified another type of MHV receptor, which belongs to the PSG subgroup of the CEA family, thus further expanding the repertoire of MHV receptors. The identification of multiple cellular receptors for MHV demonstrates marked flexibility of virus–host interaction and provides a mechanism for MHV to expand its tissue tropism. This finding may be a paradigm for other viruses as well, as recent studies have demonstrated the flexibility of virus–receptor interaction for an increasing number of viruses.

The ability of bCEA to serve as a receptor for only certain MHV strains, but not others, is unique among the known MHV receptors. The differential use by various MHV strains of different receptors in different tissues may explain the variation in pathogenicity of different MHV strains. However, bCEA does not seem to be the major determinant for MHV tissue tropism because bCEA serves as the receptor for strains A59 and MHV-3, but not strain JHM, and yet JHM is neurotropic (14), whereas some A59 and MHV-3 strains infect restricted areas of the brain (15, 30). This finding raises the possibility of the presence of yet another MHV receptor. Indeed, Northern-blot analysis (Fig. 6*C*) suggests the possible presence of another CEA-related RNA species in the brain.

The brain-specific expression of bCEA, which is a PSG-related molecule, is unusual. No CEA family members had previously been found to be expressed specifically in the brain. Our current data showed that, although many PSG molecules are expressed in the placenta, probably only two of them are expressed in the brain. Most interestingly, bCEA is expressed much more abundantly in the brain than in the placenta. This brain-specific expression of a PSG-like molecule may suggest

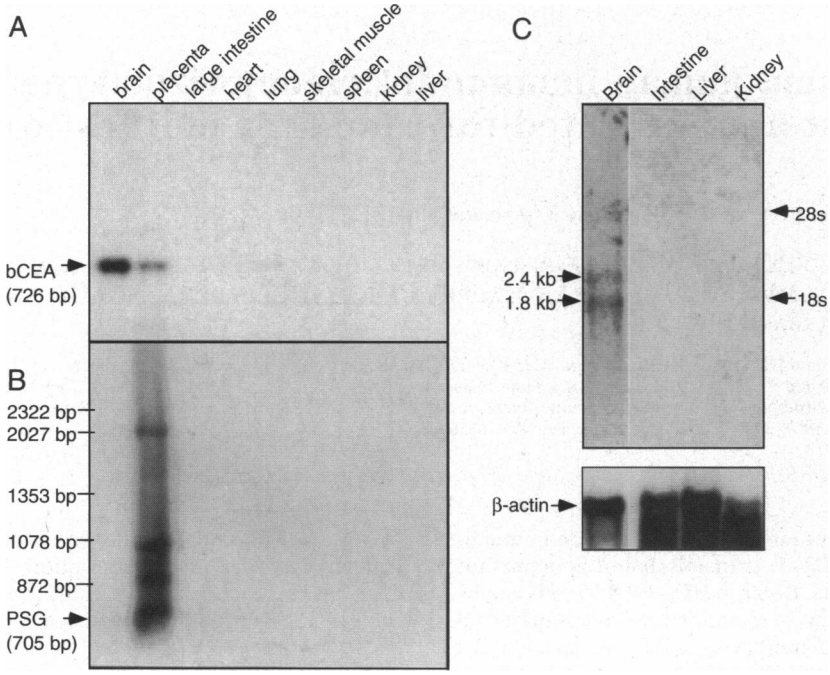

FIG. 6. Tissue-specific expression of bCEA. (*A*) PCR–Southern analysis of bCEA. RT–PCR products from various tissues were obtained with bCEA-specific primers and then probed with bCEA-specific cDNA. (*B*) RT–PCR products were obtained with PSG (Cea-2)-specific primers (21) and probed with a cDNA containing sequences common to Cea-2 and bCEA. (*C*) Northern blot with a probe containing sequences common to bCEA and PSG (*Upper*) and β-actin probe (*Lower*).

the involvement of PSG-like molecules in the development and function of the brain. Although bCEA lacks an apparent transmembrane domain, it is expressed on the cell surface as detected by immunofluorescence and FACS analysis of cells transfected with bCEA. *In vitro* translation studies also showed that bCEA can be posttranslationally modified in the presence of microsomal membranes despite the absence of a signal sequence motif. Some CEA molecules, including human CEA, have been shown to be linked to membranes by a posttranslational glycosylphosphatidylinositol linkage (31, 32). bCEA may anchor to the membrane by a similar linkage or by forming a complex with an integral or glycosylphosphatidylinositol-linked membrane protein. The nature of its membrane anchor remains to be determined.

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