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Mouse Hepatitis Virus Utilizes Two Carcinoembryonic Antigens as Alternative Receptors

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The cellular receptor for the murine coronavirus mouse hepatitis virus (MHV) has been identified as a member of the murine carcinoembryonic antigen (CEA) family (R. K. Williams, G. S. Jiang, and K. V. Holmes, Proc. Natl. Acad. Sci. USA 88:5533–5536, 1991). However, the receptor protein was not detected in some of the susceptible mouse tissues. We therefore examined whether other types of MHV receptor might exist. By polymerase chain reaction with the conserved sequences of murine CEA gene family members (mmCGM) as primers, we detected two CEA-encoding RNAs in the mouse liver. One of them (1.3 kb) encodes mmCGM1, which has previously been identified as the receptor for MHV, and the other one (0.8 kb) was shown to encode another member of mouse CGM, mmCGM2. The sequence analysis showed that mmCGM2 lacks 564 nucleotides in the middle of the gene compared with mmCGM1. These two CEA transcripts are probably derived from the same gene by an alternative splicing mechanism. Expression of either of these cDNA clones in COS-7 cells rendered these cells susceptible to MHV infection, suggesting that not only mmCGM1 but also mmCGM2 serves as a receptor for MHV. The mmCGM2 was the major CEA species in the mouse brain, which is a main target organ for the neurotropic strains of MHV. Very little mmCGM1 was detected in the mouse brain or in cells of the susceptible mouse astrocytoma cell line DBT. This result indicates that MHV may utilize different CEA molecules as the major receptor in the mouse brain and in the liver. This is a first identification of multiple receptors for a single virus. The presence of different receptors in different tissues may explain the target cell specificity of certain MHVs.

The target cell specificity of many viruses is controlled by the expression of viral receptors on specific cell types. Viral receptors have been identified for several viruses, including rhinovirus (14, 30), poliovirus (21), human immunodeficiency virus (HIV) (20), Epstein-Barr virus (37), ecotropic murine leukemia virus (16, 34), and mouse hepatitis virus (MHV) (10, 38). Each of these viruses utilizes a specific cell surface molecule as a receptor. The expression of these receptor molecules is necessary, although not always sufficient, for the susceptibility of the cells to viral infection. For example, HIV utilizes CD4 molecules on the surface of T4 cells as receptors, and transfection with CD4 rendered resistant cells susceptible to HIV infection (20). However, although T4 cells are the primary target of HIV infection, HIV also infects other cell types, such as B cells and glial cells in the central nervous system (6, 9). The mechanism of HIV entry into these cell types has not been established. One possibility is that HIV utilizes alternative receptor molecules on different cell types. Recently, the putative receptor for Sindbis virus was shown to be proteins of different sizes in different cell lines or tissues, suggesting that the virus utilizes different receptors in different cell types (33, 35). Similarly, herpes simplex virus type 1 has also been demonstrated to utilize two asymmetrically distributed molecules as receptors on polarized cells (27). However, the identity of the putative alternative receptors has not been determined for any of these viruses. We present here the first evidence that a virus may utilize an alternative receptor molecule in different tissues, thus explaining how viruses can infect cells which do not express the prototype receptor molecules.

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The murine coronavirus mouse hepatitis virus (MHV) infects liver, intestine, brain, or lymphoid tissues, depending on virus strains. For instance, the JHM strain causes either encephalitis or demyelinating diseases, MHV-2 causes hepatitis, encephalitis, or lymphadenopathy, while the A59 strain is relatively nonpathogenic (26). MHV contains a single-stranded positive-sense RNA genome of 31 kb (19, 22), which is the largest known viral RNA. The virion contains three or four structural proteins, the spike protein, the membrane protein, the nucleocapsid protein, and the optional hemagglutinin-esterase protein (18). The spike protein is responsible for virus binding to the cellular receptor and elicits neutralizing antibodies (7). Mutations in the spike protein resulted in the changes of viral host range and pathogenicity (8, 12). Passive immunization with monoclonal antibodies specific for the spike protein reduced viral neurovirulence and altered the pathogenicity of JHM from acute encephalomyelitis to subacute and/or chronic demyelination (5). Under these conditions, the viral antigen was detected in glial cells but rarely in neurons (5), suggesting that MHV infection of the neuron and glial cells requires different determining factors, possibly operating at the level of virus entry or spread.

The cellular receptor for MHV infection has recently been identified as a member of the murine carcinoembryonic antigen (CEA) family (10, 38). This receptor molecule, mmCGM1 (murine CEA gene family member 1), was detected in the mouse liver and the brush border membrane of the intestine (4), which are the primary target tissues of MHV infection. However, this receptor molecule was not detected in the brain (38), which is a target organ for many of the neurotropic MHV strains, including JHM (29, 36), nor was it detected in the susceptible tissue culture cell lines, such as L2 or DBT cells, the latter being an astrocytoma cell

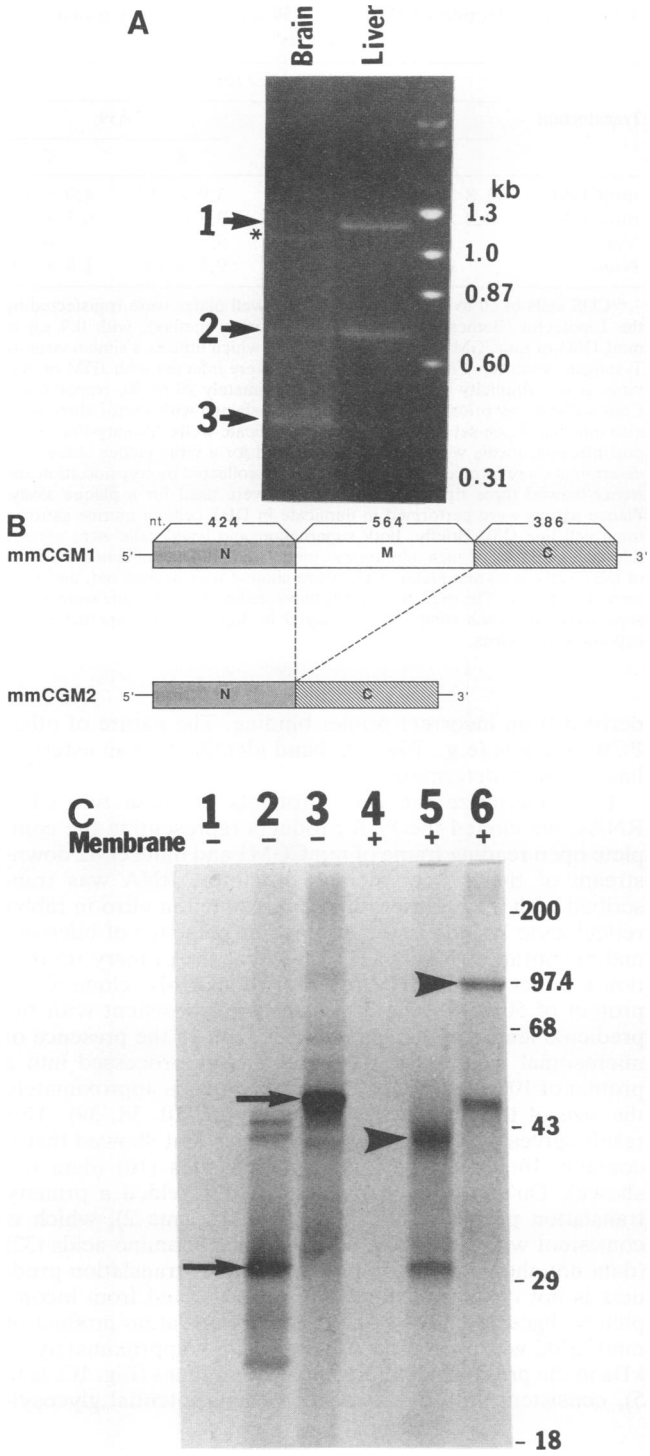

FIG. 1. Detection and characterization of MHV receptor-related CEAs. (A) RT-PCR products from poly(A)-purified RNA from C57BL/B6 mouse liver and brain, using primers specific for the conserved sequence of murine CEA (32, 38). RT was performed with primer 437 (5'-AGTTGTCAGAAGGAGCCAGATC-3'), which is complementary to the 3' end of the coding region of mmCGM1. The cDNA was amplified by PCR with a second primer, 430, which is homologous to nucleotides 151 to 195 from the first AUG of the open reading frame (5'-GTTCTTCTACTTGTTCACAATCTGC CCCTGGCGCTTGGAGCTTT-3'), and *Taq* DNA polymerase (Promega), under conditions of 94°C for 30 s, 54°C for 1.5 min, and 72°C for 3 min for 28 cycles. The PCR products were analyzed by electrophoresis on a 1% agarose gel. Major products are indicated by arrows, numbers, and the asterisk. Size markers are *Hae*III-digested φX174 DNA (Promega). (B) Schematic diagram of the sequence relationship between mmCGM1 and mmCGM2. nt., nucleotides. (C) In vitro translation of the in vitro-transcribed mmCGM1 and mmCGM2 RNAs. The complete open reading frames of mmCGM1 and mmCGM2 were obtained by RT-PCR with primer 437 and a second primer, 454 (5'-AGGCAGCAGAGACATGGAGC TGGC-3'), which is homologous to the 5' end of the leader sequence of CGM1 and CGM2 (32), including the first AUG. Products were blunt ended and cloned into the *Sma*I site of pTZ18U (U.S. Biochemical) downstream of the T7 promoter. Clones of the correct orientation were linearized with *Xba*I and transcribed in vitro into cap-containing RNA by using T7 RNA polymerase (Promega). In vitro translation was performed with [^{35}S]-methionine (Dupont, NEN Research Products) in rabbit reticulocyte lysate (Promega) in the absence (lanes 1 to 3) or presence (lanes 4 to 6) of canine pancreatic microsomal membrane (Promega) and analyzed by electrophoresis in 12.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS). Primary translation products and modified products are indicated by arrows and arrowheads. Lanes: 1 and 4, no RNA; 2 and 5, mmCGM2 RNA; 3 and 6, mmCGM1 RNA. Numbers on the right show sizes (in kilodaltons).

line (15). Nevertheless, MHV infection in mouse brain could be prevented by monoclonal antibody to the purified MHV receptor (28), which is a protein of roughly 110 kDa (10, 38, 39). Thus, it was inferred that this receptor molecule must be present in the brain, albeit in very low, undetectable quantities (38). We considered an alternative possibility that MHV utilizes a different receptor molecule, which could be related to the prototype receptor mmCGM1, in different tissues. To test this possibility, we initially examined whether different mmCGM1-related molecules might be present in different mouse tissues or cell lines which are susceptible to MHV infection. We used two synthetic oligonucleotides representing the conserved sequences of the murine CEA family (32, 38) as primers and poly(A)-selected RNA from the liver or brain of the C57BL/B6 mouse (a strain susceptible to MHV) as templates to perform reverse transcription-polymerase chain reaction (RT-PCR) analysis. Figure 1A shows that two PCR products (bands 1 and 2) could be detected from the liver RNA. Cloning and sequencing of these two PCR products revealed that band 1 represented the MHV receptor cDNA identified previously (10, 38) and was equivalent to mmCGM1 (2, 10, 32). The sequence of band 2 is identical to that of band 1, except that band 2 has a 564-nucleotide deletion in the middle of the gene (Fig. 1B). The band 2 sequence corresponds to that of a second member of the murine CEA family, mmCGM2 (32) (data not shown). The first domain of these two CEAs was similar to the exons and splicing sites of the rat CEA genes (17, 24). Thus, these two PCR products most likely represent alternatively spliced mRNAs of the murine CEA gene. Although alternative splicing of murine CEA genes has not been reported previously, alternative splicing of the human CEA RNAs has been observed (1). Interestingly, the brain RNA yielded only the PCR product corresponding to mmCGM2 but not the prototype MHV receptor mmCGM1 (Fig. 1A). This was consistent with the reported failure to detect the MHV receptor in the mouse brain (38). The brain RNA also generated several additional PCR products, which were different in size from that of either mmCGM1 or mmCGM2. Sequencing of some of these PCR products (e.g., Fig. 1A, band 3) showed that they were nonspecific PCR products

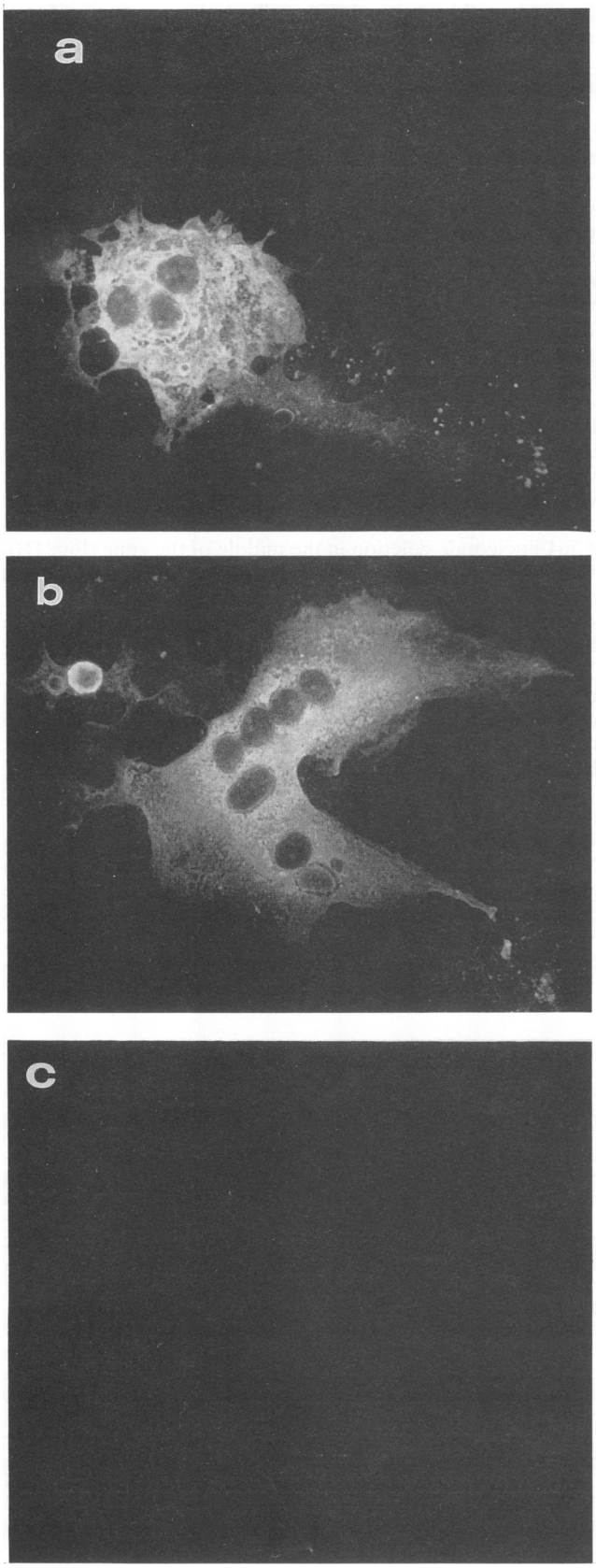

TABLE 1. Infection of JHM and A59 viruses in CEA-transfected COS cells[a]

Transfectant	Virus titer (PFU/ml)			
	JHM		A59	
	S	C	S	C
mmCGM1	1.8×10^3	4.9×10^3	3.9×10^3	4.9×10^3
mmCGM2	4.2×10^2	2.3×10^3	3.8×10^2	6.5×10^2
Vector	0	1.6×10^1	5.5×10^1	0
None	0	0	9.5×10^1	1.8×10^1

[a] COS cells of 60 to 80% confluence in 24-well plates were transfected by the Lipofectin (Bethesda Research Laboratories) method, with 0.7 μg of mmCGM1 or mmCGM2 in pECE vector (11), which utilizes a simian virus 40 T-antigen promoter. Forty hours later, cells were infected with JHM or A59 virus at a multiplicity of infection of approximately 20 or 50, respectively. Cells without any prior treatment or cells transfected with vector alone were also infected. Each set was performed in duplicate wells. Twenty-four hours postinfection, media were harvested and used for a virus plaque assay. For determination of intracellular virus, cells were collected by trypsinization and freeze-thawed three times, and the lysates were used for a plaque assay. Plaque assays were performed in duplicate in DBT cells, a murine astrocytoma cell line (15). Briefly, both supernatant and lysed cells were serially diluted, and 0.2 ml of each dilution was inoculated onto a confluent monolayer of DBT cells in 60-mm plates. Cells were stained with neutral red, and PFU were determined. The duplicated wells harvested at the same time were tested separately, and each sample was assayed in duplicate. S, supernatant; C, cell-associated virus.

derived from incorrect primer binding. The nature of other PCR products (e.g., Fig. 1A, band identified by an asterisk) has not been determined.

To characterize the gene products of these two CEA RNAs, we cloned the PCR products representing the complete open reading frame of mmCGM1 and mmCGM2 downstream of the T7 polymerase promoter. RNA was transcribed with T7 polymerase and translated in vitro in rabbit reticulocyte lysates in the absence or presence of microsomal membranes. Figure 1C shows that the primary translation product of the MHV receptor (mmCGM1) clone was a protein of 50 kDa (lane 3), which was consistent with the predicted length of 458 amino acids (10). In the presence of microsomal membrane, this product was processed into a protein of 100 kDa (Fig. 1C, lane 6), which is approximately the size of the identified MHV receptor (10, 38, 39). This result agrees with the sequence analysis that showed that it contains 16 potential N-glycosylation sites (10) (data not shown). On the other hand, mmCGM2 yielded a primary translation product of 30 kDa (Fig. 1C, lane 2), which is consistent with the predicted length of 270 amino acids (32) (data not shown). The origin of the minor translation products is not clear; possibly, they were derived from incompletely digested cDNA. The primary translation product of mmCGM2 was processed into a protein of approximately 50 kDa in the presence of microsomal membranes (Fig. 1C, lane 5), consistent with the presence of five potential glycosyl-

FIG. 2. Immunofluorescent staining of viral antigen in COS cells. Transfection with mmCGM1 or mmCGM2 and infection with JHM virus were as described in Table 1, footnote *a*, except that cells were seeded on coverslips. Twenty hours postinfection, cells were fixed with acetone at −20°C and incubated with a mixture of monoclonal antibodies specific for the spike protein and the nucleocapsid protein of JHM (41). Rhodamine-conjugated goat anti-mouse immunoglobulin G antibody (HyClone, Logan, Utah) was used to stain cells. (A) CGM1 transfected. (B) CGM2 transfected. (C) Vector pECE transfected.

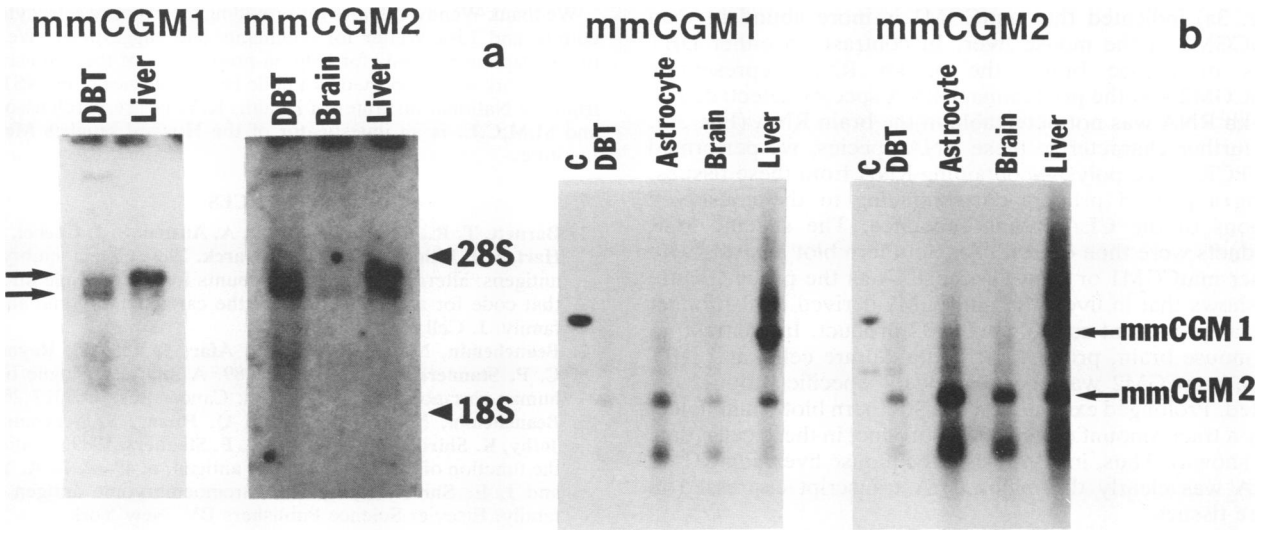

FIG. 3. Analysis of mmCGM1 and mmCGM2 expression in different tissues and cell lines. (A) Northern blot analysis. Poly(A)-purified RNA from B6 mouse liver or brain (1.2 μg of RNA each) or from DBT cells (15 μg of RNA) was denatured with glyoxal and separated by electrophoresis on a 1% agarose (SeaKem) gel. The RNA was transferred to Highbond C extra membrane (Amersham). The membranes were prehybridized at 42°C for 4 h in a buffer containing 35% formamide, 0.6 M NaCl, 0.06 M sodium citrate, 100 mM sodium phosphate, 50 mM Tris-HCl (pH 7.1), 8× Denhardt's reagent without bovine serum albumin, 0.1% SDS, 0.5 mg of tRNA per ml, and 1.0 mg of salmon sperm DNA per ml. The membrane was then hybridized overnight with the same solution containing ^{32}P-labeled mmCGM1 or mmCGM2 cDNA and 17% dextran sulfate. The membranes were washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS four times for 5 min each at room temperature. The probes used are indicated on the top. The arrows indicate the two RNA transcripts detected (approximately 4.3 and 3.7 kb, respectively). (B) Southern blot analysis of RT-PCR products. One microgram each of poly(A)-purified RNA from C57BL/B6 mouse liver or brain, primary astrocyte cell culture from B6 mouse, or DBT cells was used for RT-PCR. RT-PCR was performed as described in the legend to Fig. 1, using two primers corresponding to part of the mmCGM1 and mmCGM2 coding regions as in Fig. 1A. Gels were alkaline denatured and bidirectionally transferred to two membrane filters. Hybridization and washing conditions were the same as in panel a, except that an additional washing with 0.1× SSC–0.1% SDS for 2 h at 60°C was performed. The thick arrows denote the specific PCR products. The two small arrows indicate the full-length mmCGM1 and mmCGM2 cDNA clones (lane C) used as markers. The probes used are indicated on the top.

ation sites (data not shown). Interestingly, the processed protein of mmCGM2 was similar in size to a minor protein which was occasionally detected in MHV binding assays (39) and by the monoclonal antibody specific for the purified MHV receptor (38).

Since mmCGM2 was the predominant CEA family member detected in the B6 mouse brain, we examined whether this molecule could possibly serve as the receptor for MHV. Both the full-length mmCGM1 and mmCGM2 cDNAs were cloned into a mammalian expression vector pECE (11) under the simian virus 40 early promoter. The DNA was transfected into COS-7 cells, which are resistant to MHV infection. We have previously shown that this resistance is due to the deficiency of viral receptors or other components of the early events of viral infection, since these cells support viral replication when transfected with MHV RNA (unpublished observation). The mmCGM1- or mmCGM2-transfected cells were then infected with either the A59 or JHM strain of MHV at 40 h posttransfection, and the virus was harvested 24 h after infection. Table 1 shows that COS-7 cells transfected with mmCGM1 DNA yielded an average virus titer of 3×10^3 PFU/ml, which was approximately 100- to 1,000-fold higher than the virus yield from the untransfected COS-7 cells or cells transfected with the vector only. Similarly, mmCGM2-transfected cells yielded a virus titer of 4×10^2 PFU/ml, which was 10- to 100-fold higher than the control culture, suggesting that mmCGM2 can also serve as the receptor for MHV. To ensure that the virus yields represented the virus replication inside the cells, we also examined the titer of intracellular or cell-associated virus particles. The intracellular virus particles from both mmCGM1- and mmCGM2-transfected cells consistently showed as much as a fivefold-higher titer than that of the supernatant. Furthermore, MHV infection in mmCGM1- or mmCGM2-transfected COS-7 cells showed cell fusion typical of MHV infection in a variety of mouse cell lines, and MHV viral antigens could be detected in the cytoplasm of the cell syncytium (Fig. 2). This result clearly indicates that both mmCGM1 and mmCGM2 can serve as the receptor for MHV. A59 appeared to utilize mmCGM1 approximately 10 times more efficiently than mmCGM2 (Table 1). This was a consistent observation in three separate transfection experiments (data not shown). In contrast, JHM utilized mmCGM1 only slightly better than mmCGM2.

We then examined further the distribution of the different CEA family members in different mouse tissues and tissue culture cell lines (Fig. 3a). Northern (RNA) blot analysis with either mmCGM1 or mmCGM2 as the probe detected two RNA species of approximately 4.3 and 3.7 kb in the B6 mouse liver. These two RNAs most likely represented mmCGM1 and mmCGM2 RNAs, respectively (2, 32). The size difference between these two RNAs corresponded to the difference in the size of the open reading frame between mmCGM1 and mmCGM2. The mmCGM2 probe was approximately three times more sensitive than the mmCGM1 probe in the detection of the 3.7-kb RNA as determined by measuring the hybridization to a fixed amount of their cDNA clones (data not shown). Hybridization with either probe

(Fig. 3a) indicated that mmCGM1 is more abundant than mmCGM2 in the mouse liver. In contrast, in either DBT cells or mouse brain, the 3.7-kb RNA representing mmCGM2 was the predominant RNA species detected. The 4.3-kb RNA was not detectable in the brain RNA (Fig. 3a). To further characterize these RNA species, we performed RT-PCR of the poly(A)-containing RNA from these tissues, using a pair of primers corresponding to the conserved regions of the CEA-coding sequence. The specific PCR products were then detected by Southern blot analysis with either mmCGM1 or mmCGM2 cDNA as the probe. Figure 3b shows that in liver, the mmCGM1-derived PCR product was as prominent as the mmCGM2 product. In contrast, in B6 mouse brain, primary astrocyte culture cells, and DBT cells, mmCGM2 was the major CEA-specific product detected. Prolonged exposure of the Southern blot could detect only a trace amount of mmCGM1 product in these cells (data not shown). Thus, in contrast to the mouse liver, mmCGM2 RNA was clearly the major CEA transcript expressed in these tissues.

The results presented here showed that MHV could utilize at least two different CEA molecules as the receptor. This is the first report showing the utilization of alternative receptors in different cell types by a virus. Although a small amount of mmCGM1 may be present in brain or DBT cells, it is most likely that mmCGM2 is the major receptor used by MHV for central nervous system infection or tissue culture infection since it is the most abundant CEA protein possessing the functional receptor activity. Preliminary results suggested that different strains of MHV utilize these two different receptors at different efficiency, e.g., A59 virus utilizes mmCGM2, which is the major MHV receptor in the brain, less efficiently and, correspondingly, is less neurotropic (25). This finding may explain the basis of viral neurotropism. However, this hypothesis needs to be confirmed with stable transformants expressing higher levels of the receptor molecules. Also, viral tropism may be more complex than the mere expression of viral receptors. For example, mmCGM2 is expressed to a high level in kidney (32), which is not the site of replication for MHV. Similarly, poliovirus does not infect kidney despite the high level of expression of the poliovirus receptor (13). Resistance of certain mouse strains to MHV infection and viral tissue tropism could also be affected by steps subsequent to virus binding to the receptor (40). Furthermore, we cannot rule out the possibility that there are additional tissue-specific CEA molecules, which can also serve as a receptor for MHV infection in different tissues. Since the expression of CEA is developmentally regulated (3), there is a possibility that different CEA molecules are utilized as MHV receptors at different developmental stages. Such a possibility has also been discussed previously (23, 39). An intriguing example is that SJL mice are susceptible to MHV infection as newborns, but become resistant as adults (31). Whether different CEA molecules are differentially expressed in this strain of mouse is not clear. Since mmCGM1 and mmCGM2 represent gene products of alternatively spliced mRNAs, the susceptibility of different cell types to MHV infection at different developmental stages (23) may also be regulated by alternative splicing of CEA mRNAs. It is likely that the different CEA species have different biological functions, e.g., Ca^{2+}-dependent adhesion molecule, ATPase, or immunomodulator (32). This report thus provides a paradigm for other viral infections such as HIV or Sindbis virus, in that alternative receptors may explain the susceptibility of different cell types to viral infections.

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