JOURNAL OF VIROLOGY, Dec. 1992, p. 6931–6938
0022-538X/92/126931-08$02.00/0

Vol. 66, No. 12

The Receptor for Mouse Hepatitis Virus in the Resistant Mouse Strain SJL Is Functional: Implications for the Requirement of a Second Factor for Viral Infection

KYOKO YOKOMORI AND MICHAEL M. C. LAI*

Howard Hughes Medical Institute and Department of Microbiology, University of Southern California School of Medicine, Los Angeles, California 90033-1054

Received 6 July 1992/Accepted 21 August 1992

The SJL mouse strain is resistant to infection by some strains of the murine coronavirus mouse hepatitis virus (MHV), such as JHM and A59. The block to virus infection has been variously attributed to defects in virus receptors or virus spread. Since the cellular receptors for MHV, mmCGM1 and mmCGM2, have recently been identified as members of the carcinoembryonic antigen family, we reexamined the possible defectiveness of the MHV receptors in SJL mouse strain. Cloning and sequencing of the cDNAs of both mmCGMs RNAs from SJL mice revealed that they were identical in size to those of the susceptible C57BL/6 (B6) mouse. There was some sequence divergence in the N terminus of the mmCGM molecules between the two mouse strains, resulting in a different number of potential glycosylation sites. This was confirmed by in vitro translation of the mmCGM RNAs, which showed that the glycosylated mmCGM2 of SJL was smaller than that of B6 mice. However, transfection of either mmCGM1 or mmCGM2 from SJL mice into MHV-resistant Cos 7 cells rendered the cells susceptible to MHV infection. The ability of the SJL mmCGM molecules to serve as MHV receptors was comparable to that of those from B6. These molecules are expressed in SJL mouse brain and liver in a similar ratio and in amounts equivalent to those in the B6 mouse. Furthermore, we demonstrated that an SJL-derived cell line was susceptible to A59 but resistant to JHM infection. We concluded that the MHV receptor molecules in the SJL mouse are functional and that the resistance of SJL mice to infection by some MHV strains most likely results from some other factor(s) required for virus entry or some other step(s) in virus replication.

Mouse hepatitis virus (MHV) is a murine coronavirus which causes a wide range of diseases in the mouse and rat, including enteritis, hepatitis, respiratory infection, and encephalomyelitis in the central nervous system (26). The tissue tropism and pathogenicity of the virus vary with the mouse strain and also with the virus strain and isolate (26). For instance, the JHM strain of MHV is highly neurotropic, whereas MHV-3 causes hepatitis in susceptible mice but also causes ventricular infection followed by chronic vasculitis in the central nervous system of semisusceptible mice (24), and the A59 strain is relatively nonpathogenic (19).

It has been reported that the resistance of mice to MHV infection is controlled by a single recessive gene (11, 12, 21), but other reports suggested that there are multiple gene determinants (1, 22). The susceptibility or resistance of animals to MHV infection also depends on the strain and age of the host animals (2) and on the virus strain, dose, and route of inoculation (3, 22). The SJL mouse is known to be resistant to infection by some strains of MHV, e.g., A59, JHM, and MHV-S (2, 3), but is susceptible to MHV-3 (29). Several pertinent observations have been made about the resistance of SJL mice to infection by some MHV strains. (i) Primary macrophage cultures were resistant to JHM infection in vitro (14). The resistance was not 100%, but only a small population of cells were infected, and subsequent virus spread was completely inhibited (14). (ii) Primary glial cells and neuronal cells derived from SJL mice were resistant to JHM and A59 infection (11, 29) but not to MHV-3, although the efficiency of virus attachment was similar for all three strains (29). (iii) In vivo, the SJL mouse was resistant to a low intranasal dose of JHM; the viral antigen was restricted to the site of inoculation and did not spread to the central nervous system (3, 4). However, high-dose inoculation led to the development of encephalitis (13). These studies suggest that the JHM strain of MHV may infect a few cells in SJL mice but cannot spread efficiently and is cleared quickly. Other reports showed that the resistance of SJL mice to JHM infection was age dependent; newborn mice of strain SJL were highly susceptible to JHM infection and easily developed fatal central nervous system infection (23).

The mechanism of the resistance of SJL mice to MHV infection is not clear. SJL macrophages are intrinsically resistant to MHV infection in vitro (14, 23). In addition, SJL mice may also produce some factors that modulate infection by certain MHV strains (21, 23). Recently, it was shown that A59 virus failed to bind to the receptor molecule from the SJL mouse liver in an in vitro virus-binding assay, the virus overlay protein blot assay (5, 28), suggesting that the SJL mouse virus receptor is nonfunctional. It was further reported that the MHV receptor carried a deletion in SJL mice, since these receptor molecules appeared to migrate faster than those of BALB/c mice on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (28). On the contrary, the resistance of primary glial cells derived from SJL mice was shown to involve not virus binding but rather virus dissemination (29), and it was suggested that the resistance lies in a defective proteolytic activity in SJL mice which is required for A59 and JHM infection but not for MHV-3 (29). It is not clear why these results were so discrepant.

The cellular receptor for MHV infection has been identified as a member of the murine homolog of the carcinoembryonic antigen (CEA) family, mouse CEA gene family

* Corresponding author.

member 1 (mmCGM1) (6, 27). We have recently shown that another mouse CEA, mmCGM2 (25), which is probably generated by alternative splicing of the same CEA gene, can also be utilized as an MHV receptor (31). mmCGM1 contains an additional 564-nucleotide sequence in the middle of the open reading frame (ORF) compared with mmCGM2. Interestingly, mmCGM2 but very little mmCGM1 is expressed in the mouse brain, suggesting that mmCGM2 is the major MHV receptor used in the mouse brain (31).

To provide a better understanding of the molecular basis of SJL resistance to infection by certain MHV strains, we examined whether the SJL mouse expresses a functional receptor for MHV infection. In this report, we show that the SJL mouse has fully functional receptors, which, upon transfection, permitted MHV to infect a resistant cell line, Cos 7. However, an SJL-derived cell line, PSJLSV, was partially resistant to MHV A59 and fully resistant to MHV JHM infection, suggesting that additional factors are required for MHV infection. Possible factors involved in the susceptibility and resistance to MHV infection will be discussed.

MATERIALS AND METHODS

Viruses, cells, and animals. MHV strains A59 and JHM(2) were used throughout this study. JHM(2) is a clonal isolate of the original JHM strain (16) and expresses a large amount of the hemagglutinin-esterase protein (20, 32). The virus plaque assay was done in DBT cells, a murine astrocytoma cell line (10). Cos 7 cells (9) were used for transfection and subsequent infection assays; these cells were resistant to both A59 and JHM infection but were able to support viral replication when transfected with viral genomic RNA (unpublished observation). A simian virus 40 (SV40)-transformed SJL cell line derived from the pancreas of an adult SJL mouse, PSJLSV, was kindly provided by Barbara B. Knowles, Wistar Institute, Philadelphia, Pa.

SJL and C57BL/6 (B6) mice, 6 weeks old, were obtained from Jackson Laboratories, Bar Harbor, Maine.

RNA extraction from tissues and cell lines. Total cellular RNA was extracted from SJL mouse liver and brain with 4 M guanidine isothiocyanate by a published procedure (15). Briefly, the mouse tissues were homogenized in a guanidine isothiocyanate solution, and total cellular RNA was extracted with phenol and chloroform and precipitated with isopropanol. Cytoplasmic RNA was extracted from culture cell lines by proteinase K digestion after removal of nuclei by Nonidet P-40 treatment as described previously (17). Polyadenylated [poly(A)$^+$] RNA was selected by passing total RNA through an oligo(dT) column by the procedure recommended by the manufacturer (Collaborative Research Inc., Bedford, Mass.).

RT-PCR and cDNA cloning. Reverse transcription (RT) of poly(A)$^+$ RNA from mouse liver and brain was done with avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) and primer 437 (5′-AGTTGTCAGAAGGAGC CAGATC-3′), which is complementary to the 3′ end of the coding region of both mmCGM1 and mmCGM2. To detect the mmCGM1 and mmCGM2 transcripts among total RNA, cDNA was amplified by polymerase chain reaction (PCR) with a second primer, 430 (5′-GTTCTTCTACTTGTTC ACAATCTGCCCCTGGCGCTTGGAGCTTT-3′), which is homologous to nucleotides 151 to 195 from the first AUG of the ORF. To obtain a cDNA clone containing a full-length ORF, primer 543 (5′-AGCAGAGACATGGAG CTGGCCTCAGCACATCTCCACAAAG-3′), which is homologous to the extreme 5′ end of both the mmCGM1 and mmCGM2 ORFs, including the first AUG, was used as the second primer. PCR was performed with *Taq* DNA polymerase (Boehringer Mannheim) for 30 cycles at 94°C for 30 s, 54°C for 1.5 min, and 72°C for 3 min. The PCR products were separated by electrophoresis in 1.2% low-melting-point agarose (SeaPlaque; FMC Bioproducts). The full-length PCR products were extracted from the gel, blunt ended, and cloned into the *Sma*I site of vector pTZ18U (U.S. Biochemicals), which contains the T7 promoter at one side.

DNA sequencing. The cDNA clones were sequenced by the dideoxyribonucleotide chain termination method as recommended by the manufacturer (Sequenase kit; U.S. Biochemicals), with universal, reverse, or specific primers corresponding to various regions of the mmCGM1 and mmCGM2 ORFs. At least two clones of every region were sequenced.

In vitro transcription and translation and SDS-PAGE analysis. After sequencing, cDNA clones of the correct orientation were linearized with *Xba*I and transcribed in vitro into cap-containing RNA by using T7 polymerase (Promega) as described previously (30). The RNA was used for in vitro translation in a rabbit reticulocyte lysate (Promega). The translation product was analyzed by SDS-PAGE in 12.5% polyacrylamide gels as described previously (30).

Southern blot analysis of RT-PCR products. One microgram of poly(A)$^+$ RNA from SJL mouse liver or brain was used for RT-PCR with primers complementary and homologous to the 3′ and 5′ ends of the mmCGM coding regions, respectively, as described above. The PCR products were analyzed by electrophoresis in 1.2% agarose gels. Gels were treated briefly with 0.25 M HCl, alkaline denatured, and transferred to a Highbond C Extra membrane (Amersham). The membrane was 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 and hybridized overnight in the same solution containing ^{32}P-labeled mmCGM2 cDNA probe prepared by random priming (random priming kit; Boehringer Mannheim) and 17% dextran sulfate. After hybridization, the membrane was washed with a solution containing 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS four times for 5 min each at room temperature and then with a solution containing 0.1× SSC and 0.1% SDS for 2 h at 55°C.

Transfection and infection assay. mmCGM1 and mmCGM2 cDNAs were cloned into the mammalian expression vector pECE (7), which contains an SV40 T-antigen promoter. Cos 7 cells at 60 to 80% confluence in 24-well plates were transfected with DNA (0.7 μg per well) by lipofection method with either Lipofectin (BRL) or DOTAP (Boehringer Mannheim) as recommended by the manufacturers. Forty hours later, cells were infected with JHM or A59 virus at a multiplicity of infection of approximately 20 to 50. Cells transfected with vector pECE and cells that had not been given any treatment were also infected. After 1 h of virus adsorption, the cells were washed twice with Dulbecco's modified Eagle's medium (DMEM), and 1 ml of DMEM containing 1% fetal calf serum was added to each plate. At 24 h postinfection, supernatant and cells were collected for the virus plaque assay. Cells were disrupted by trypsinization and freeze-thawing three times. Cell debris was removed by brief centrifugation, and the supernatant was used in the plaque assay for titering cell-associated virus. The transfec-

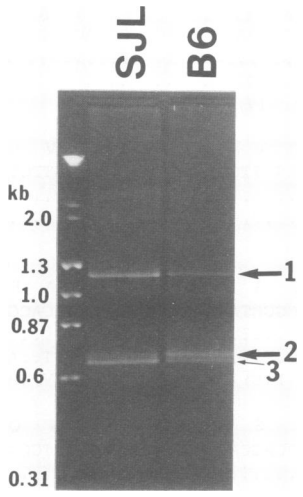

FIG. 1. Detection of mmCGM1 and mmCGM2 PCR products from SJL and B6 mouse livers. Primers 430 and 437 were used for RT-PCR. Size markers are *Hin*dIII-digested lambda DNA and *Hae*III-digested φX174 DNA (Promega). DNA was stained with ethidium bromide. Band 1, mmCGM1; band 2, mmCGM2; band 3, PCR artifact.

tion-infection experiments were repeated at least three times. Standard virus plaque assays were performed in duplicate in DBT cells. The precise procedure has been described elsewhere (31).

Nucleotide sequence accession numbers. The GenBank accession numbers for C57BL/6 mmCGM2 and SJL mmCGM1 are M96934 and M96935, respectively.

RESULTS

Characterization of mmCGM1 and mmCGM2 from SJL mice. To compare the expression patterns of transcripts of the MHV receptors mmCGM1 and mmCGM2 in the susceptible mouse strain B6 and the resistant strain SJL, poly(A)$^+$ liver RNA was used for RT-PCR, initially to obtain partial cDNA clones of mmCGM1 and mmCGM2, with primers specific for the 3′ end and for nucleotides 151 to 195 downstream from the 5′ end of the mmCGM ORF, respectively. Under this condition, two major products (bands 1 and 2), which correspond to mmCGM1 and mmCGM2 (see below), respectively, were obtained (Fig. 1). The patterns of PCR products were identical between B6 and SJL, and their sizes were indistinguishable. A few additional PCR products, most notably band 3, were also detected. Sequence analysis of band 3 showed that it was a nonspecific product caused by mispriming during PCR (31; data not shown). Other minor PCR products have not been characterized. Previous studies have shown that under the conditions used, the relative amounts of RT-PCR products closely parallel the amounts of RNA (31). This result thus indicates that the SJL mouse expresses amounts of mmCGM1 and mmCGM2 RNAs comparable to those in the B6 mouse, and these molecules are identical in size between these two strains of mice. These results together suggest that the mmCGM gene of the SJL mouse does not have a deletion.

Sequence comparison of mmCGM1 and mmCGM2 between SJL and B6 mice. To further characterize mmCGM1 and mmCGM2 of SJL mice, full-length cDNA clones of mmCGM1 and mmCGM2 were obtained by RT-PCR with two primers specific for the extreme 3′ and 5′ ends of the ORFs, respectively, which are common to both mmCGM1 and mmCGM2 (6, 25, 31). Two major products of 1.3 and 0.8 kb, which correspond to full-length mmCGM1 and mmCGM2, respectively, were cloned into vector pTZ18U, which contains the T7 polymerase promoter.

The cDNA clones of both SJL and B6 mmCGMs were sequenced, and the sequences were compared with the published sequence for mmCGM1 from the BALB/c mouse (6). The sequence of B6 mmCGM1 is nearly identical to that of BALB/c mmCGM1 (6), whereas SJL mmCGM1 shows a high degree of sequence divergence from that of B6 at both the nucleotide and amino acid levels (Fig. 2). In the N-terminal portion, which is common to mmCGM1 and mmCGM2, there are 30 amino acid differences between SJL and B6. In the middle domain, which is present only in mmCGM1, and the C-terminal region, which is the same in mmCGM1 and mmCGM2, sequences are conserved among all three mouse strains, except for 2 amino acids in the middle and 3 amino acids in the C-terminal region. Furthermore, the number of potential N-linked glycosylation sites is different between B6 and SJL mice (Fig. 2, indicated by underlines). There are 16 potential glycosylation sites in BALB/c and B6 mmCGM1: 3 sites in the N-terminal, 11 in the middle, and 2 in the C-terminal region. In the SJL mouse, two of the glycosylation sites in the N-terminal region were lost because of mutations (indicated by cross marks); however, a new glycosylation site (shown by an asterisk) was generated. Glycosylation sites are summarized in Fig. 3. Thus, there are only two potential glycosylation sites in the N-terminal region of SJL mmCGM1 and mmCGM2. These analyses indicate that there is no structural deletion in the mmCGM gene of the SJL mouse, in contrast to the previous suggestion (28).

Comparison of in vitro translation products of mmCGM1 and mmCGM2 from SJL and B6 mice. To characterize the gene products of the mmCGM genes in the SJL mouse, in vitro-transcribed RNAs from both B6 and SJL full-length mmCGM1 and mmCGM2 ORF clones were used for in vitro translation in a rabbit reticulocyte lysate in the presence or absence of canine pancreatic microsomal membrane. The primary product of B6 mmCGM2 was approximately 30 kDa in size (Fig. 4, lane 3, asterisk), which was processed to a protein of approximately 43 kDa in the presence of microsomal membrane (Fig. 4, lane 4, arrow). SJL mmCGM2 also yielded a primary translation product of 30 kDa (Fig. 4, lane 5); this product appeared to migrate slightly faster than the B6 counterpart. Since they have an identical number of amino acids (Fig. 2), the reason for the slight discrepancy in their electrophoretic behaviors is not clear; most likely it was caused by charge differences in their amino acid sequences. Two minor products of approximately 43 kDa were also detected, the origin of which is not clear. Similar products have occasionally been observed with B6 mmCGM2 (31). In the presence of microsomal membrane, the translation product of SJL mmCGM2 was processed into a protein of approximately 40 kDa (Fig. 4, lane 6, arrow), which was smaller than that of B6 mmCGM2 (Fig. 4, lane 4). This result indicates that SJL mmCGM2 is smaller than B6 mmCGM2 despite the identical sizes of their ORFs. This size difference (3 kDa) could be accounted for by the difference of one glycosylation site between these two molecules, since the number of glycosylation sites in SJL mmCGM2 is four, in contrast to five for that of B6 (Fig. 3). On the other hand, the size difference in mmCGM1 between B6 and SJL mice was not apparent; B6 mmCGM1 (lanes 7 and 8) and SJL

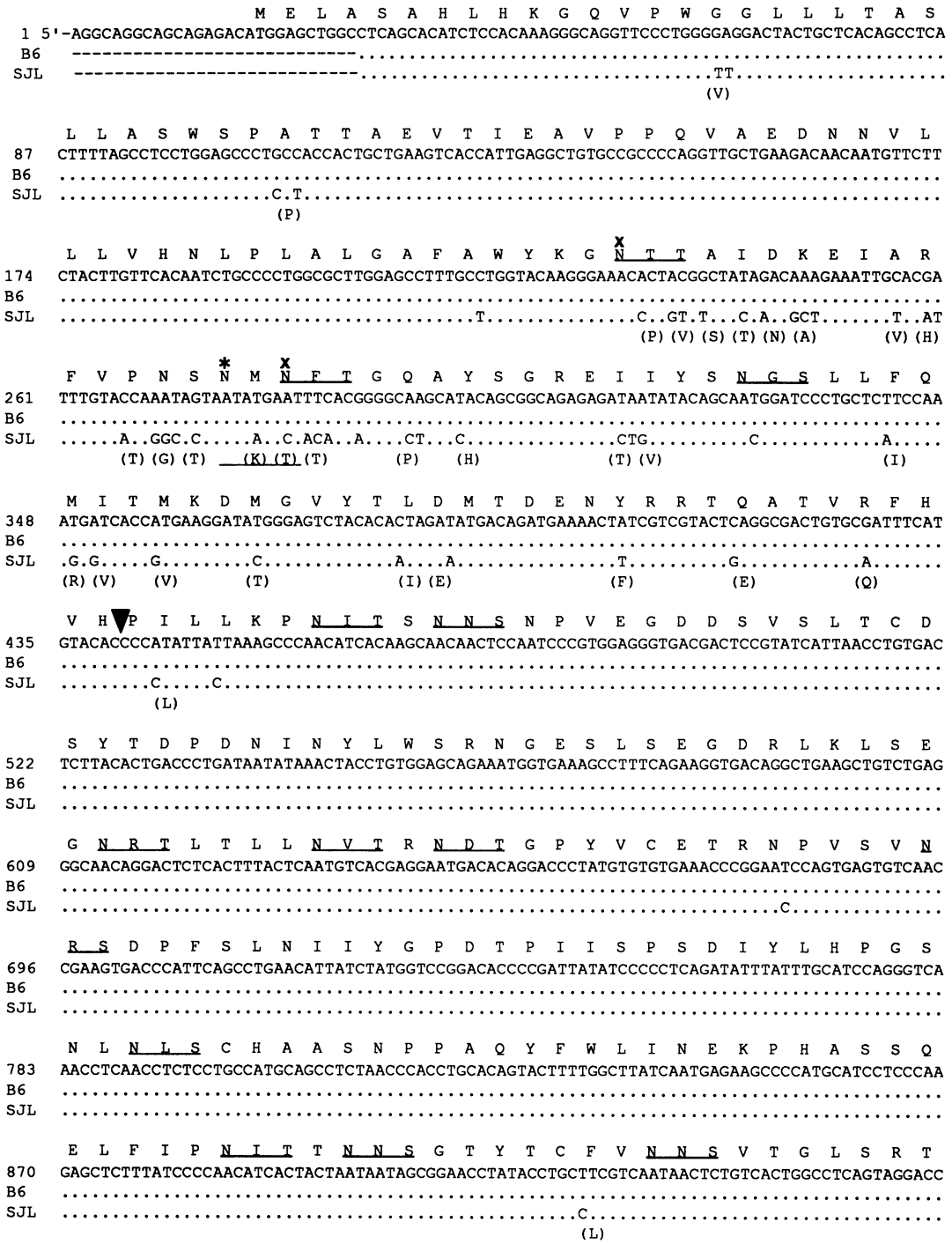

FIG. 2. Sequences of mmCGM1 and mmCGM2 in B6 and SJL mice. Sequences are compared with the published BALB/c sequence (5) (top line). Dots indicate identical nucleotides. Potential N-linked glycosylation sites are underlined. Splicing sites are indicated by arrowheads (mmCGM2 does not contain the middle region between the two arrowheads). Amino acid changes are indicated underneath in parentheses. Glycosylation sites lost due to amino acid changes are indicated by ×, and generation of a new site is indicated by an asterisk. Dashed lines show the primers used for PCR.

mmCGM1 (lanes 9 and 10) yielded products of similar sizes both in the absence (45 kDa; Fig. 4, lanes 7 and 9, asterisks) and in the presence (approximately 95 kDa; Fig. 4, lanes 8 and 10, arrows) of membrane, probably because of the limitation of resolution in the high-molecular-weight range.

Functional analysis of mmCGM1 and mmCGM2 of the SJL mouse. We reported previously that both mmCGM1 and mmCGM2 of the B6 mouse were functional as MHV receptors when they were expressed in Cos 7 cells, which are otherwise resistant to MHV infection (31). Because of the

```
      T  V  K  N  I  T  V  L  E  P  V  T  Q  P  F  L  Q  V  T  N  T  T  V  K  E  L  D  S  V
957   ACAGTCAAGAACATTACAGTCCTTGAGCCAGTGACTCAGCCCTTCCTCCAAGTCACCAACACCACAGTCAAAGAACTAGACTCTGTG  1043
B6    .....................................................................................
SJL   ..........................................C..........................................C
                                               (S)

      T  L  T  C  L  S  N  D  I  G  A  N  I  Q  W  L  F  N  S  Q  S  L  Q  L  T  E  R  M  T
1044  ACCCTGACCTGCTTGTCGAATGACATTGGAGCCAACATCCAGTGGCTCTTCAATAGCCAGAGTCTTCAGCTCACAGAGAGAATGACA  1130
B6    .....................................................................................
SJL   .....................................................................................

      L  S  Q  N  N  S  I  L  R  I  D  P  I  K  R  E  D  A  G  E  Y  Q  C  E  I  S  N  P  V
1131  CTCTCCCAGAACAACAGCATCCTCAGAATAGACCCTATTAAGAGGGAAGATGCCGGCGAGTATCAGTGTGAAATCTCGAATCCAGTC  1217
B6    .....................................................................................
SJL   .....................................................................................

      S  V  R  R  S  N  S  I  K  L  D  I  I  F  D  P  T  Q  G  G  L  S  D  G  A  I  A  G  I
1218  AGCGTCAGGAGGAGCAACTCAATCAAGCTGGACATAATATTTGACCCAACACAAGGAGGCCTCTCAGATGGCGCCATTGCTGGCATC  1304
B6    ..........................................................................CG.........
SJL   .......A..................................................................CG.........
            (K)                                                                   (R)

      V  I  G  V  V  A  G  V  A  L  I  A  G  L  A  Y  F  L  Y  S  R  K  S  G  G  S  G  S  F
1305  GTGATTGGAGTTGTGGCTGGGGTGGCTCTAATAGCAGGGCTGGCATATTTCCTCTATTCCAGGAAGTCTGGCGGATCTGGCTCCTTC  1391
B6    ..........................................................................----------
SJL   ..........................................................................----------
      Z
1392  TGACAACTCTCCTAACAA-3'
      --------
```

FIG. 2—*Continued.*

differences in amino acid sequence and glycosylation sites of the mmCGM proteins between SJL and B6 mice and the finding that SJL receptors failed to bind MHV in an in vitro virus-binding assay, the virus overlay protein blot assay (5, 28), we wondered whether the SJL mmCGM molecules could serve as functional receptors for MHV. We examined this possibility by transfecting SJL mmCGM1 and mmCGM2 into Cos 7 cells and testing their susceptibility to both A59 and JHM virus infection. The results are summarized in Table 1.

Surprisingly, both SJL mmCGM1 and mmCGM2 could serve as functional receptors for MHV. In JHM infection, cells transfected with either SJL-derived mmCGM1 or mmCGM2 yielded virus titers in the range of 200 to 3,000 PFU/ml in both the supernatant and the cell lysate. These virus yields were roughly at the same level as those of B6 mmCGM1- and mmCGM2-transfected cells. In contrast, in the vector-transfected and untreated cells, either no virus was detected or the virus titer was lower than 10 PFU/ml, which was probably derived from the virus inoculum remaining attached to the cells. With A59 infection, in both B6 and SJL mmCGM1-transfected cells, the virus titer was in the range of 10^3 PFU/ml, which was 2 $\log_{10}$ higher than the background. The A59 virus titers from both B6 and SJL mmCGM2-transfected cells were approximately 10-fold lower than those of mmCGM1-transfected cells; nevertheless, they were significantly higher than the background. The JHM virus yields from the mmCGM1- and mmCGM2-trans-

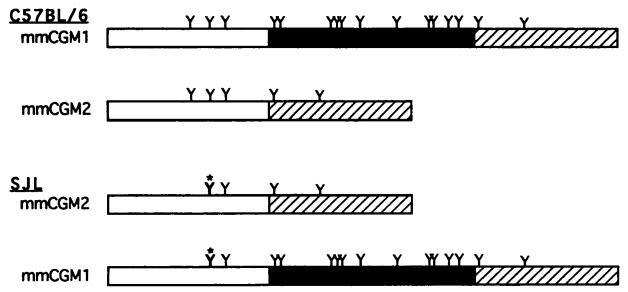

FIG. 3. Diagram of mmCGM1 and mmCGM2. Potential glycosylation sites are indicated by Y; the novel glycosylation site present only in SJL molecules is indicated by an asterisk.

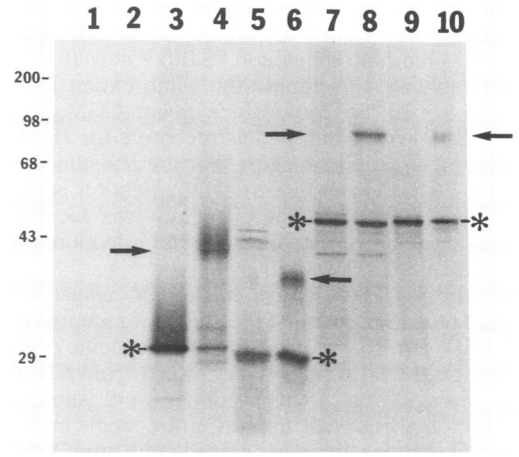

FIG. 4. In vitro translation products of full-length ORFs of mmCGM1 and mmCGM2 of SJL and B6 mice. Cap-containing RNA was transcribed with T7 RNA polymerase, used for in vitro translation with [^{35}S]methionine in the absence (primary products, indicated by asterisks) and presence (processed products, indicated by arrows) of canine pancreatic microsomal membranes, and analyzed by SDS-PAGE on 12.5% polyacrylamide gels. Odd-numbered lanes, without membranes; even-numbered lanes, with membranes. Lanes: 1 and 2, no RNA; 3 and 4, B6 mmCGM2; 5 and 6, SJL mmCGM2; 7 and 8, B6 mmCGM1; 9 and 10, SJL mmCGM1. Sizes are shown in kilodaltons.

TABLE 1. Receptor functions of mmCGM molecules from SJL and B6 mice in Cos 7 cells[a]

Transfectant	Virus titer (PFU/ml)			
	JHM		A59	
	S	C	S	C
mmCGM1				
B6	1.1×10^3	2.0×10^3	4.4×10^3	7.0×10^3
SJL	2.1×10^2	9.0×10^2	2.0×10^3	3.7×10^3
mmCGM2				
B6	3.1×10^2	1.4×10^3	3.1×10^2	7.5×10^2
SJL	3.8×10^2	3.2×10^3	2.1×10^2	1.9×10^2
Vector (pECE)	0	0	2.0×10^1	0
None	0	7.5×10^0	3.3×10^1	1.5×10^1

[a] Cos 7 cells were transfected with various DNAs and infected with either JHM or A59 at 40 h posttransfection. Viruses were harvested from the supernatant (S) or cell lysates (C) 24 h after infection. The virus was plaque assayed on DBT cells.

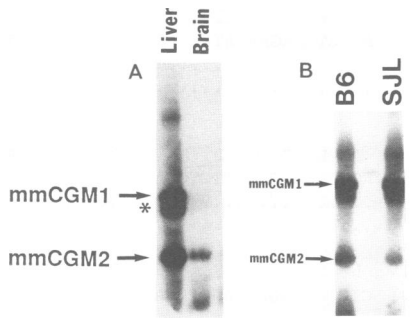

FIG. 5. PCR-Southern analysis of mmCGM products of liver and brain from an SJL mouse. (A) Poly(A)$^+$ RNA from the liver and brain of an SJL mouse was used for RT-PCR to amplify mmCGM1 and mmCGM2 transcripts. The PCR products were detected by Southern blot with an mmCGM-specific probe. The product indicated by an asterisk was not studied further. (B) One microgram of poly(A)$^+$ RNA from the livers of an SJL and a B6 mouse was used for RT-PCR, and mmCGM1 and mmCGM2 products were detected as described for panel A. The mmCGM1 and mmCGM2 products are indicated by arrows.

fected cells were equivalent. These results indicate that both mmCGM1 and mmCGM2 from SJL mice are functional receptors for both the A59 and JHM strains of MHV. This finding is in contrast to the conclusion deduced from the in vitro virus-binding analysis (5, 28). mmCGM1 was probably a better receptor than mmCGM2 for A59. By contrast, mmCGM1 and mmCGM2 served as equally effective receptors for JHM.

Susceptibility of an SJL-derived cell line to MHV infection. Since the MHV receptors in the SJL mouse are functional, we tested the cell line PSJLSV, which was derived from an adult SJL mouse and transformed with SV40 T antigen (14a), with regard to its ability to support MHV infection. The results showed that A59 replicated in this cell line, suggesting that the resistance of the SJL mouse strain to MHV infection is not absolute. However, with the same amount (multiplicity of infection of 10) of input A59 virus, PSJLSV cells yielded only 6×10^3 PFU/ml, versus 7.5×10^6 PFU/ml in the BALB/c-derived astrocytoma cell line (DBT cells). In contrast, JHM did not replicate in PSJLSV cells (0 PFU/ml), whereas it replicated to almost the same extent as A59 in DBT cells (5.5×10^6 PFU/ml). Since the mmCGM molecules from the SJL mouse functioned as receptors for JHM in the Cos 7 cells, these results suggest that the resistance of SJL mice or the PSJLSV cell line to JHM infection is due to a defect in some factor(s) other than the virus receptor and that the requirement for establishing viral infection differs in different strains of MHV.

Expression of MHV receptors in the SJL mouse and cultured cells. To further examine the status of receptor expression in the SJL mouse, we used mmCGM-specific primers to obtain PCR products from poly(A)$^+$ RNA from SJL mouse liver and brain. These PCR products were subjected to Southern blot analysis with an mmCGM-specific probe (Fig. 5A). The SJL mouse liver expressed both mmCGM1 and mmCGM2 in roughly equal amounts. In contrast, the SJL mouse brain expressed mainly mmCGM2 and the overall amounts of mmCGM in the brain were slightly lower than in the liver. A trace amount of mmCGM1 was also detected in brain samples after prolonged exposure (data not shown). A PCR product which was slightly smaller than mmCGM1 was also detected with the mmCGM probe in Southern blot (Fig. 5A). As indicated above (Fig. 1), the nature of this product remains elusive, since we have so far been unable to clone this product. Nevertheless, this tissue-specific expression pattern in SJL mice is similar to that in B6 mice (31). Furthermore, the amounts of both mmCGM1 and mmCGM2 PCR products were roughly equivalent between B6 and SJL mice (Fig. 5B). Although the quantitation of PCR products is not necessarily linear in relation to the amount of RNA, preliminary data and the previous study (31) showed a correlation between Northern (RNA blot) analysis and PCR-Southern analysis. Thus, we conclude that the receptor molecules in SJL mice are properly expressed. Furthermore, transfection of B6 mmCGM1 and mmCGM2 into PSJLSV cells did not render the cells susceptible to JHM infection (data not shown). These data suggest that the presence of MHV receptors does not necessarily correlate with the level of MHV replication in these cells. Thus, a second factor likely regulates MHV infection.

DISCUSSION

Two CEA gene family members, mmCGM1 and mmCGM2, have been shown to serve as MHV receptors (6, 31). The A59- and JHM-resistant SJL mouse expresses both proteins at the same levels as the susceptible mouse strain BALB/c (28); however, these proteins appeared to be smaller in SJL than in BALB/c mice (28). Furthermore, the SJL receptor molecules failed to bind to a monoclonal antibody (MAb), CC1, which recognizes the corresponding BALB/c receptor molecules and blocks A59 infection in BALB/c mice (6, 28). In addition, A59 did not bind to the mmCGM molecules from the liver or intestinal brush border membrane of the SJL mouse in an in vitro virus-binding assay (5). Thus, it was suggested that the resistance of SJL mice to MHV infection is due to defective virus receptors (5, 28). Other reports, however, showed that the resistance of SJL mice to JHM infection is due to some other factor, e.g., a defect in virus spread (29). Moreover, newborn SJL mice are highly susceptible to JHM infection (23); thus, it seems unlikely that there is a structural deletion in the MHV receptor in SJL mice.

In this study, we cloned the mmCGM1 and mmCGM2 molecules from adult SJL mice and showed that they were functional MHV receptors. Sequence analysis of these clones revealed that there are multiple amino acid differ-

ences, especially in the N-terminal domain, between the MHV receptors of the susceptible BALB/c and B6 and resistant SJL mouse strains, and the number of the potential N-linked glycosylation sites is different (Fig. 2 and 3). In vitro translation studies further indicated that the differences in the extent of glycosylation between the B6 and SJL receptors led to the difference in the sizes of their receptor proteins. These differences likely also accounted for the failure of MAb CC1 to recognize SJL receptors (5) and the failure of MHV to bind to the receptors in vitro (5, 28). Surprisingly, despite the sequence divergence, the transfected SJL receptors were functional and allowed both A59 and JHM infection of the otherwise resistant monkey kidney cell line Cos 7 cells (Table 1). Thus, the SJL receptor molecules have lost the MAb CC1-binding activity in vitro but not the in vivo virus-binding capability. MAb CC1 probably recognizes a site that is close to but not the same as the virus-binding site, and the interference of MAb CC1 with virus binding (6, 28) was most likely caused by steric hindrance. Since the N-terminal portion of the mmCGM molecules is the most divergent part, MAb CC1 most likely recognizes this region. Our data also suggest that the virus overlay protein blot assay results (5) do not correspond to virus binding in vivo and do not necessarily reflect the presence or absence of a functional virus receptor in vivo. It has recently been shown that in vitro virus binding requires proper glycosylation of the receptor molecules (18). The alteration of glycosylation in SJL mmCGM molecules may have prevented virus binding in vitro (5, 28); however, these alterations apparently did not affect their receptor functions, again suggesting that virus binding has different requirements in vitro and in vivo.

The results obtained in this study did not contradict the previous studies on macrophage infection (14), glial cell infection (29), animal infection by intranasal inoculation (3, 4), and some other studies in animals (1, 2, 13) which showed that replication and the establishment of disease by JHM were deficient in SJL mice and SJL-derived cells, although a small population of cells can be infected at the beginning. Those studies and our study suggest that the resistance of SJL mice to MHV infection does not reside in a defect in the receptor molecule itself but rather in the deficiency of some other factor(s), which either cooperates with the receptor or is required for subsequent steps of virus infection.

Although our data showed that SJL mice express fully functional MHV receptors and an SJL-derived cell line is susceptible to at least A59 infection, the resistance of SJL mice to A59 or JHM infection could still be due to possible processing defects of the MHV receptor. It is possible that the receptor molecules are inappropriately modified in SJL mice. Since we have shown that mmCGM1 and mmCGM2 serve as functional MHV receptors in Cos 7 cells, the genetic defects of SJL mice that prevent MHV infection could lie in the mechanism for modification of the receptor or in a second factor associated with the receptor. The second factor could be a high-affinity molecule or protease or some other enzyme, which changes the conformation of the viral spike protein or other structural proteins to stabilize or trigger the internalization of the virus-receptor complex or virus uncoating. It can be assumed that BALB/c and B6 mice and Cos 7 cells have this factor but SJL mice do not express it, although SJL cells in culture can express this factor. The second factor also could act at any step after virus internalization and uncoating; in this case, virus binds to the receptor but one of the subsequent steps is inhibited. That additional factors are required to establish viral infection is supported by our recent finding that certain B6-derived cell lines are resistant to JHM infection and even the transfection of an otherwise fully functional receptor into these cells did not render them susceptible (unpublished observation).

The preliminary studies suggested that this factor acted at a very early stage of infection. In this regard, it is interesting that a rat cell line has been shown to be defective in the internalization of JHM virus (8). Our finding is in contrast to the previous observation that JHM infection in SJL-derived glial cells is blocked at the virus dissemination step (29). However, in the published data (29), JHM replication within the first replication cycle in SJL-derived cells was also inhibited, suggesting that viral replication was restricted early in infection. It is conceivable that different cultured cells may have different defects that block viral infection. The nature of the defective second factor in the SJL-derived cell line and others is currently being studied. It is tempting to suggest that the multiple genes involved in determining the genetic resistance of SJL mice and other mouse strains to MHV infection may control this second factor.

It is interesting that, although the SJL receptors were used equally effectively by A59 and JHM when the receptors were transfected into Cos cells, JHM does not grow in the SJL-derived cell line PSJLSV, whereas A59 does. Thus, the putative second factor should be able to discriminate A59 from JHM infection. Furthermore, since the receptor molecules are expressed even in the MHV-resistant cells, the second factor must be delicately regulated. Such high discrimination and sensitive regulation make this factor an ideal candidate for the regulation of viral tropism and pathogenicity. The identity of this second factor in different strains of mice is currently being investigated.

ACKNOWLEDGMENTS

We thank Daphne Shimoda for help in preparation of the manuscript.

This work was supported by grant NS18146 from the National Institutes of Health. K.Y. is a Research Associate and M.M.C.L. is an Investigator of the Howard Hughes Medical Institute.

REFERENCES

1. **Bang, F. S.** 1981. The use of a genetically incompatible combination of host and virus (MHV) for the study of mechanisms of host resistance. Adv. Exp. Med. Biol. **142**:359–373.
2. **Barthold, S. W.** 1987. Host age and genotypic effects on enterotropic mouse hepatitis virus infection. Lab. Anim. Sci. **37**:36–40.
3. **Barthold, S. W., D. S. Beck, and A. L. Smith.** 1986. Mouse hepatitis virus nasoencephalopathy is dependent upon virus strain and host genotype. Arch. Virol. **91**:247–256.
4. **Barthold, S. W., and A. L. Smith.** 1987. Response of genetically susceptible and resistant mice to intranasal inoculation with mouse hepatitis virus JHM. Virus Res. **7**:225–239.
5. **Boyle, J. F., D. G. Weismiller, and K. V. Holmes.** 1987. Genetic resistance to mouse hepatitis virus correlates with the absence of virus-binding activity on target tissues. J. Virol. **61**:185–189.
6. **Dveksler, G. S., M. N. Pensiero, C. B. Cardellichio, R. K. Williams, G.-S. Jiang, K. V. Holmes, and C. W. Dieffenbach.** 1991. Cloning of the mouse hepatitis virus (MHV) receptor: expression in human and hamster cell lines confers susceptibility to MHV. J. Virol. **65**:6881–6891.
7. **Ellis, L., E. Clauser, D. O. Morgan, M. Edery, R. A. Roth, and W. J. Rutter.** 1986. Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. Cell **45**:721–732.
8. **Flintoff, W. F., and S. Van Dinter.** 1989. Several rat cell lines share a common defect in their inability to internalize murine coronaviruses efficiently. J. Gen. Virol. **70**:1713–1724.
9. **Gluzman, Y.** 1981. SV40-transformed simian cells support the

replication of early SV40 mutants. Cell **23**:175–182.
10. **Hirano, N., K. Fujiwara, S. Hino, and M. Matsumoto.** 1974. Replication and plaque formation of mouse hepatitis virus (MHV-2) in mouse cell line DBT culture. Arch. Gesamte Virusforsch. **44**:298–302.
11. **Knobler, R. L., M. V. Haspel, and M. B. A. Oldstone.** 1981. Mouse hepatitis virus type 4 (JHM strain)-induced fatal central nervous system disease. I. Genetic control and the murine neuron as the susceptible site of disease. J. Exp. Med. **153**:832–843.
12. **Knobler, R. L., B. A. Taylor, M. K. Wooddell, W. G. Beamer, and M. B. A. Oldstone.** 1984. Host genetic control of mouse hepatitis virus type-4 (JHM strain) replication. II. The gene locus for susceptibility is linked to the Svp-2 locus on mouse chromosome 7. Exp. Clin. Immunogenet. **1**:217–222.
13. **Knobler, R. L., L. A. Tunison, P. W. Lampert, and M. B. A. Oldstone.** 1982. Selected mutants of mouse hepatitis virus type 4 (JHM strain) induce different CNS diseases. Pathology of disease induced by wild type and mutants ts8 and ts15 in BALB/c and SJL/J mice. Am. J. Pathol. **109**:157–168.
14. **Knobler, R. L., L. A. Tunison, and M. B. A. Oldstone.** 1984. Host genetic control of mouse hepatitis virus type 4 (JHM strain) replication. I. restriction of virus amplification and spread in macrophages from resistant mice. J. Gen. Virol. **65**:1543–1548.

14a. **Knowles, B. B.** Personal communication.

15. **Macnaughton, T. B., E. J. Gowans, A. R. Jilbert, and C. J. Burrell.** 1990. Hepatitis delta virus RNA, protein synthesis and associated cytotoxicity in a stably transfected cell line. Virology **177**:692–698.
16. **Makino, S., and M. M. C. Lai.** 1989. Evolution of the 5'-end of genomic RNA of murine coronaviruses during passages *in vitro*. Virology **169**:227–232.
17. **Makino, S., F. Taguchi, N. Hirano, and K. Fujiwara.** 1984. Analysis of genomic and intracellular viral RNAs of small plaque mutants of mouse hepatitis virus, JHM strain. Virology **139**:138–151.
18. **Pensiero, M. N., G. S. Dveksler, C. B. Cardellichio, G.-S. Jiang, P. E. Elia, C. W. Dieffenbach, and K. V. Holmes.** 1992. Binding of the coronavirus mouse hepatitis virus A59 to its receptor expressed from a recombinant vaccinia virus depends on post-translational processing of the receptor glycoprotein. J. Virol. **66**:4028–4039.
19. **Robb, J., and C. W. Bond.** 1979. Pathogenic murine coronaviruses. I. Characterization of biological behavior in vitro and virus-specific intracellular RNA of strongly neurotropic JHMV and weakly neurotropic A59 viruses. Virology **94**:352–370.
20. **Shieh, C.-K., H. J. Lee, K. Yokomori, N. La Monica, S. Makino, and M. M. C. Lai.** 1989. Identification of a new transcriptional initiation site and the corresponding functional gene 2b in the murine coronavirus RNA genome. J. Virol. **63**:3729–3736.
21. **Smith, A. L., D. F. Winograd, and M. S. de Souza.** 1991. In vitro splenic T cell responses of diverse mouse genotypes after oronasal exposure to mouse hepatitis virus, strain JHM. Lab. Anim. Sci. **41**:106–111.
22. **Stohlman, S. A., and J. A. Frelinger.** 1978. Resistance to fatal central nervous system disease by mouse hepatitis virus, strain JHM. I. Genetic analysis. Immunogenetics **6**:277–281.
23. **Stohlman, S. A., J. A. Frelinger, and L. P. Weiner.** 1980. Resistance to fatal central nervous system disease by mouse hepatitis virus, strain JHM. II. Adherent cell-mediated protection. J. Immunol. **124**:1733–1739.
24. **Tardieu, M., A. Goffinet, G. Harmart-van Rijckevorsel, and G. Lyon.** 1982. Ependymitis, leukoencephalitis, hydrocephalus, and thrombotic vasculitis following chronic infection by mouse hepatitis virus 3 (MHV 3). Acta Neuropathol. (Berlin) **58**:168–176.
25. **Turbide, C., M. Rojas, C. P. Stanners, and N. Beauchemin.** 1991. A mouse carcinoembryonic antigen gene family member is a calcium-dependent cell adhesion molecule. J. Biol. Chem. **266**:309–315.
26. **Wege, H., S. Siddell, and V. ter Meulen.** 1982. The biology and pathogenesis of coronaviruses. Curr. Top. Microbiol. Immunol. **99**:165–200.
27. **Williams, R. K., G.-S. Jiang, and K. V. Holmes.** 1991. Receptor for mouse hepatitis virus is a member of the carcinoembryonic antigen family of glycoproteins. Proc. Natl. Acad. Sci. USA **88**:5533–5536.
28. **Williams, R. K., G.-S. Jiang, S. W. Snyder, M. F. Frana, and K. V. Holmes.** 1990. Purification of the 110-kilodalton glycoprotein receptor for mouse hepatitis virus (MHV)-A59 from mouse liver and identification of a nonfunctional, homologous protein in MHV-resistant SJL/J mice. J. Virol. **64**:3817–3823.
29. **Wilson, G. A. R., and S. Dales.** 1988. In vivo and in vitro models of demyelinating disease: efficiency of virus spread and formation of infectious centers among glial cells is genetically determined by murine host. J. Virol. **62**:3371–3377.
30. **Yokomori, K., L. R. Banner, and M. M. C. Lai.** 1991. Heterogeneity of gene expression of the hemagglutinin-esterase (HE) protein of murine coronaviruses. Virology **183**:647–657.
31. **Yokomori, K., and M. M. C. Lai.** 1992. Mouse hepatitis virus utilizes two carcinoembryonic antigens as alternative receptors. J. Virol. **66**:6194–6199.
32. **Yokomori, K., N. La Monica, S. Makino, C.-K. Shieh, and M. M. C. Lai.** 1989. Biosynthesis, structure, and biological activities of envelope protein gp65 of murine coronavirus. Virology **173**:683–691.