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## Mouse Hepatitis Virus Strain JHM Infects a Human Hepatocellular Carcinoma Cell Line

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Mouse hepatitis virus (MHV) strain JHM is a coronavirus that causes encephalitis and demyelination in susceptible rodents. The known receptors for MHV are all members of the carcinoembryonic antigen family. Although human forms of the MHV receptor can function as MHV receptors in some assays, no human cell line has been identified that can support wild-type MHV infection. Here we describe the infection of a human hepatocellular carcinoma cell line, HuH-7, with MHV. HuH-7 cells were susceptible to strains JHM-DL and JHM-DS, yielding virus titers nearly identical to those seen in mouse DBT cells. In contrast, HuH-7 cells were only marginally susceptible or completely resistant to infection by other MHV strains, including A59. JHM produced a strong cytopathic effect in HuH-7 cells with the formation of round plaques. Studies of various recombinant viruses between JHM and A59 strains suggested that the ability of JHM to infect HuH-7 cells was determined by multiple viral genetic elements. Blocking the viral spike (S) protein with a neutralizing antibody or a soluble form of the MHV receptor inhibited infection of HuH-7 cells, suggesting that infection is mediated through the S protein. Transfection with the prototype mouse receptor, biliary glycoprotein, rendered HuH-7 cells susceptible to infection by other MHV strains as well, suggesting that JHM uses a receptor distinct from the classical MHV receptor to infect HuH-7 cells. Possible implications for human disease are discussed. © 1999 Academic Press

### INTRODUCTION

The host range of a virus is often determined by the degree to which the sequence of its receptor is conserved among different animal species. A virus which uses a highly conserved protein or a sugar moiety as a receptor (e.g., vesicular stomatitis virus) will often display a wide host range as a result. Although most viruses are believed to use only one specific receptor, many examples of a single virus using alternate receptors or coreceptors have been documented in recent years (Geraghty *et al.*, 1998; Moore *et al.*, 1997; Selinka *et al.*, 1998; Smith and Vanderplasschen, 1998; Thoulouze *et al.*, 1998). Human immunodeficiency virus (HIV), for example, can use several different chemokine receptors to gain entry into CD4-positive cells (Moore *et al.*, 1997). Different isolates of HIV display tropism for either macrophages or T cells, depending on the coreceptor recognized. Similarly, mouse hepatitis virus (MHV), a coronavirus, has been shown to be capable of using any one of a number of carcinoembryonic antigen (CEA)-related proteins as a receptor (Chen *et al.*, 1995, 1997; Dveksler *et al.*, 1993; Yokomori and Lai, 1992, 1994).

While some MHV strains (including the hepatotropic strain A59) can utilize any one of many CEA-related molecules, other strains (including the neurotropic strain

JHM) appear to be much more restricted in their receptor utilization (Chen *et al.*, 1995, 1997). Of the known CEA-related proteins, JHM can apparently use only the prototype MHV receptor mouse biliary glycoprotein (mBGP). mBGP is expressed primarily in the liver and intestine and interacts with the MHV spike (S) protein to allow virus entry (Dveksler *et al.*, 1991; Kubo *et al.*, 1994; Williams *et al.*, 1990). Yet, paradoxically, JHM causes very little hepatitis or enteritis in normal mice. Instead, it infects glial cells and neurons, causing a neurological disease that resembles the human disease multiple sclerosis (Fleury *et al.*, 1980; Goto *et al.*, 1977; Herndon *et al.*, 1975; Powell and Lampert, 1975). This apparent paradox has led some to speculate that JHM may have the ability to use some undiscovered receptor or coreceptor to infect cells within the central nervous system (Chen *et al.*, 1995).

Members of the coronavirus family are responsible for a wide variety of illnesses in a number of different animal species. However, the natural host range of each coronavirus is quite limited, typically consisting of a single species (Compton *et al.*, 1993). For instance, the two human coronavirus strains 229E and OC43 are not known to have any animal reservoir. Likewise, MHV infects only mice, although some strains (including JHM) are able to infect rats (Compton *et al.*, 1993; Nagashima *et al.*, 1978). However, replication of JHM has been detected in the brains of experimentally infected owl monkeys after either peripheral or intracerebral inoculation

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(Cabirac *et al.*, 1994; Murray *et al.*, 1992b, 1997), indicating that the host range of JHM may be broader than originally believed. In addition, long-term passage of susceptible mouse 17 Cl-1 cells persistently infected with MHV strain A59 has yielded a mutant virus strain capable of infecting a broad range of cell lines, including some human cell lines, demonstrating a theoretical potential for cross-species infection and an altered receptor specificity (Schickli *et al.*, 1997).

Some human cell lines, such as HeLa cells, are susceptible to wild-type MHV infection if transfected with exogenous mBGP or other appropriate MHV receptors (Dveksler *et al.*, 1991). Conversely, the human CEA-related proteins hBGP and hCEA can be used as MHV receptors by strains A59 and MHV-2 when transfected into receptor-deficient cell lines (Chen *et al.*, 1997). Nevertheless, to date no wild-type MHV strain has been reported to successfully infect any human cell line. For instance, the hepatocellular carcinoma cell line HepG2 is resistant to MHV infection despite high levels of hBGP expression (Baric *et al.*, 1999; Chen *et al.*, 1998; Hensley and Baric, 1998). This underscores the fact, first deduced from studies of MHV-resistant mouse cell lines, that more than one tissue-specific cellular factor is known to be required for successful MHV entry and/or uncoating (Asanaka and Lai, 1993; Yokomori *et al.*, 1993; Yokomori and Lai, 1993). Here, we report the infection of a human hepatocellular carcinoma cell line, HuH-7, with wild-type MHV strains JHM-DL and JHM-DS. We characterize the mechanism of viral infection. This finding raises the possibility that, under certain conditions, MHV may have the potential to infect humans.

## RESULTS

### Infection of HuH-7 cells by MHV strains

The human hepatocellular carcinoma cell line HuH-7 is generally thought to be slightly more differentiated than HepG2 cells, despite a similar origin (Yamamoto *et al.*, 1990). To examine the possibility that the differentiated hepatic factors may promote cellular susceptibility to MHV infection, we infected HuH-7 cells with various MHV strains. MHV was adsorbed to nearly confluent HuH-7 and mouse DBT cell monolayers for 1 h at 37°C. The cells were then washed and incubated in growth medium for 24 h at 37°C. Of all the MHV strains tested, the neurotropic strains JHM-DL and JHM-DS caused significant cytopathic effects in HuH-7 cells with the formation of syncytial plaques, indicating that these two strains were able to infect HuH-7 cells (data not shown). All other strains showed no evident cytopathic effect on HuH-7 cells. To detect possible virus replication in the absence of apparent cytopathic effects, infected cells were fixed and stained for the expression of the viral nucleocapsid (N) protein (Fig. 1). All virus strains replicated in DBT cells, as shown by the presence of N

protein staining. In contrast, N protein staining was detected only in HuH-7 cells infected with strains JHM-DL and JHM-DS. N protein was predominantly localized in the cytoplasm of fused cells and was most highly concentrated in the perinuclear region in both cell types. These results indicated that JHM-DL and JHM-DS can infect HuH-7 cells.

To rule out the possibility that HuH-7 cells were contaminated with mouse cells, monolayers were infected with JHM-DL at a low multiplicity of infection and stained with a rabbit polyclonal antibody against MHV structural proteins and a monoclonal antibody specific for human Ku antigen. The latter antibody can detect the Ku antigen in primate cells but not in mouse cells (Wang *et al.*, 1993). Cells were fixed at 8 h postinfection, before syncytia appeared in HuH-7 cells, ensuring that cells were infected individually rather than by cell fusion. As shown in Fig. 2, infected mouse DBT cells were not stained by the anti-Ku monoclonal antibody. In contrast, all the HuH-7 cells were positive for Ku antigen staining in the nucleus. The individually infected HuH-7 cell was positive for Ku staining in the nucleus and MHV structural protein staining in the cytoplasm. These results indicate that the infected HuH-7 cells were indeed of primate origin (Fig. 2).

To quantitate the levels of virus replication in HuH-7 cells, culture medium was harvested at 24 h postinfection and subjected to plaque assay on DBT cells. In order to negate variations among the titers of the original inocula, the number of plaque-forming units produced from HuH-7 cells is normalized to that produced from DBT cells infected with the same virus stock (Fig. 3). Cos cells infected with JHM-DL are included as a negative control. Surprisingly, strain JHM-DS produced nearly a 10-fold higher titer of virus from HuH-7 cells than from mouse DBT cells. JHM-DL also produced nearly the same virus titer from HuH-7 cells as from DBT cells. In contrast, strains JHM-X, A59, MHV-S, and At11f replicated only weakly in HuH-7 cells and produced virus titers that were between 10 and 100 times less than that from DBT cells, while strains MHV-1 and MHV-3 did not yield significant titers of virus from HuH-7 cells.

We next studied the kinetics of virus replication for strains JHM-DS, JHM-DL, and A59 on HuH-7 and DBT cells in parallel (Fig. 4). Consistent with the previous experiments, JHM-DS produced virus titers of almost  $10^5$  PFU/ml from the human HuH-7 cells, more than one  $\log_{10}$  higher than those from the mouse DBT cells at 12 h postinfection. Later in the infection, the virus titers from HuH-7 and DBT cells became almost the same. JHM-DL replicated at the same pace as JHM-DS in HuH-7 cells, but yielded a slightly lower virus titer from HuH-7 cells than from DBT cells. The growth curves for both JHM strains on HuH-7 cells mirrored their growth curves on DBT cells. In contrast, A59 yielded a significantly lower virus titer from HuH-7

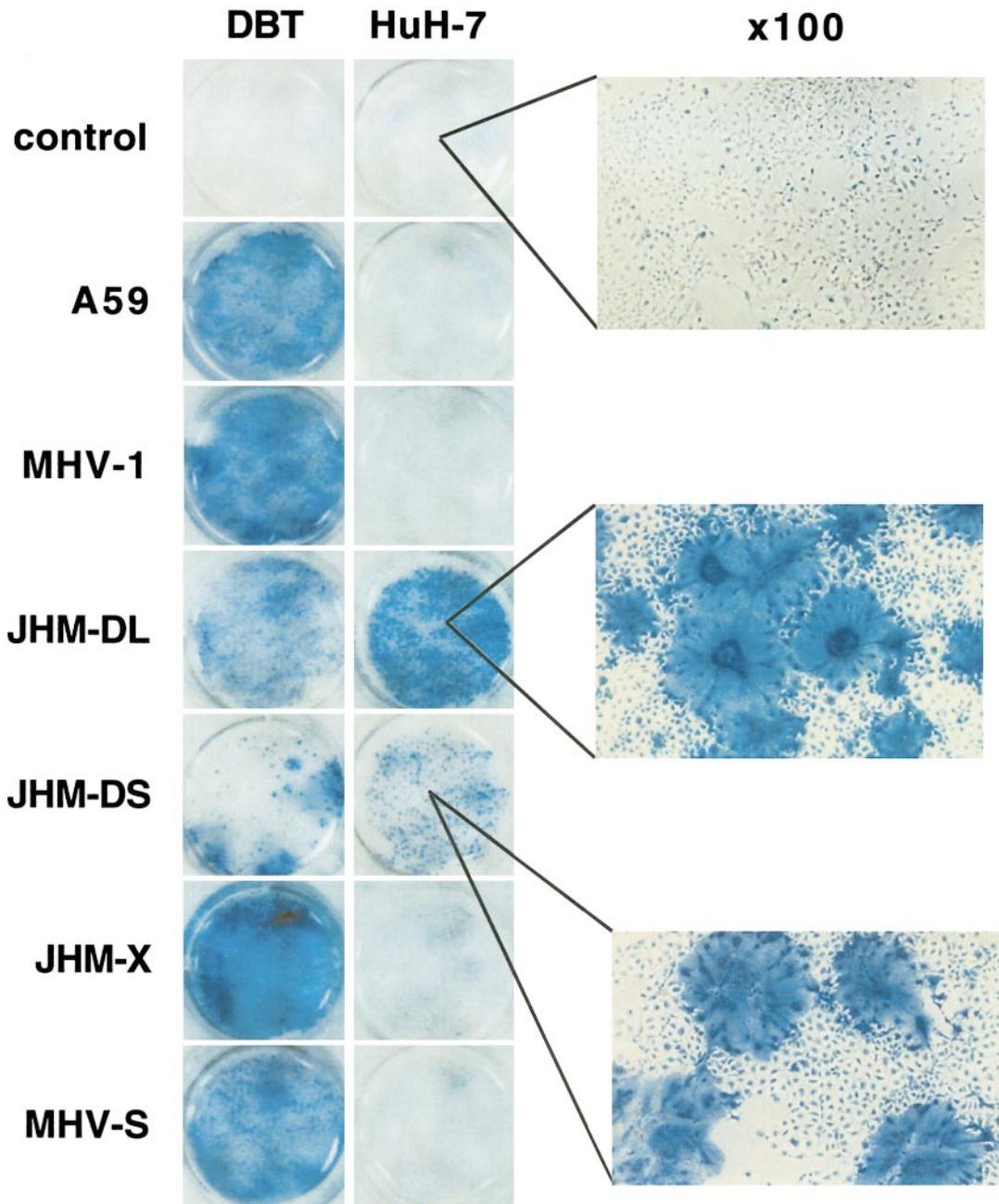


FIG. 1. Infection of HuH-7 and DBT cells by MHV strains. Nearly confluent monolayer cells on 6-well plates were exposed to MHV strains for 1 h at 37°C. Twenty-four hours after infection, cells were stained for N protein expression.

cells than from DBT cells (Figs. 3 and 4), and the viral growth kinetics demonstrated that the virus titer for A59 peaked at a later time in HuH-7 cells than in DBT cells and never reached the peak titers of strains JHM-DS and JHM-DL. Thus, A59 can also infect HuH-7 cells, but with low efficiency. Unlike its effect on DBT cells, and unlike the effect of JHM strains on HuH-7 cells, A59 did not cause any detectable cytopathic effect in the infected HuH-7 cells (Fig. 1 and data not shown).

#### The role of the S protein in MHV infection of HuH-7 cells

To determine whether the JHM infection of HuH-7 cells is mediated by the S protein, we incubated JHM-DL with the neutralizing monoclonal antibody J.7.2, which is specific for the JHM S protein (Fleming *et al.*, 1983), before infection of HuH-7 or DBT cells. This monoclonal antibody has been previously shown to block JHM infection and syncytium formation (i.e., cell fusion) in mouse cells

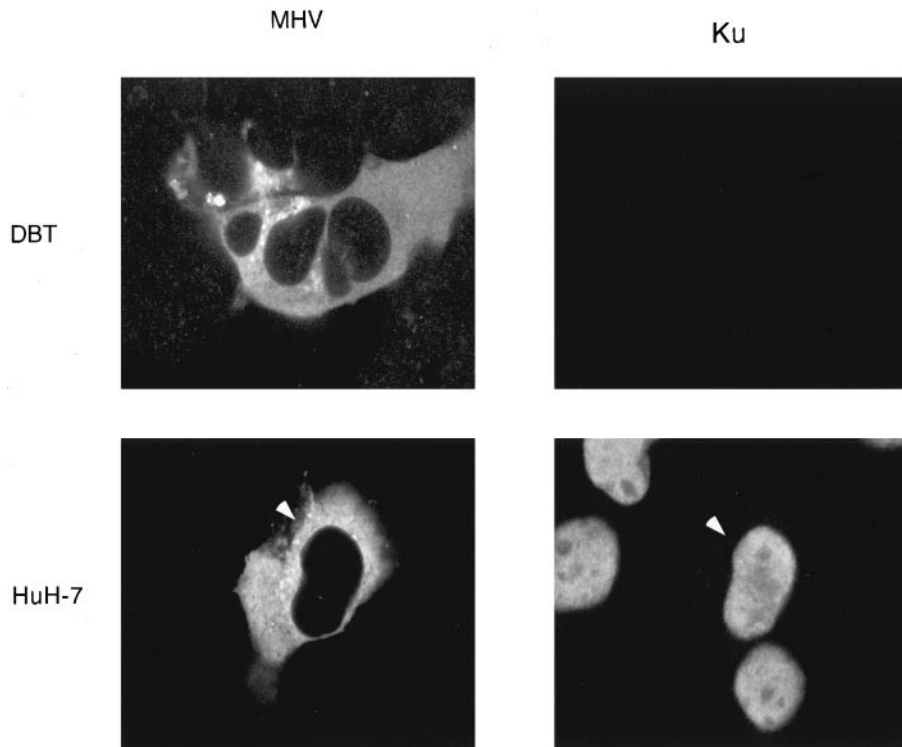


FIG. 2. Double staining of Ku antigen and MHV by confocal fluorescent microscopy. Cells were infected with MHV as in Fig. 1. After 8 h, cells were double stained using a primate-specific monoclonal antibody against the Ku antigen and a rabbit polyclonal antibody against the MHV structural proteins. Arrowhead, MHV-infected cell.

and can protect mice from lethal challenge with JHM by passive immunization (Fleming *et al.*, 1983, 1987). J.7.2 completely blocked infection of DBT cells at 24 h post-inoculation when assayed by immunostaining of viral N protein (Table 1). Infection of HuH-7 cells was also significantly inhibited by J.7.2 antibody; the number of

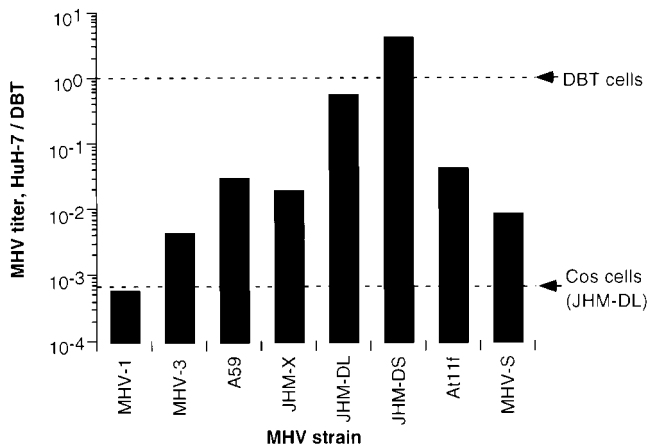


FIG. 3. Replication of MHV strains on HuH-7 cells. HuH-7 and DBT cells were infected with various MHV strains as in Fig. 1. Culture media were harvested 24 h after infection and used for plaque assay on DBT cells to determine the titer of virus produced. The data are shown as the ratio of PFU/ml produced from HuH-7 cells to PFU/ml produced from DBT cells. Background titer of JHM-DL on MHV-resistant Cos-7 cells is shown for comparison (lower dotted line).

plaques was significantly reduced, but the blockade of infection was not complete, possibly suggesting slightly different mechanisms of infection for these two cell types (Table 1). Similarly, the virus titers produced from DBT and HuH-7 cells were also reduced to approximately the same extent. These results suggest that the same functional moiety of the viral S protein is involved in the infection of DBT cells and HuH-7 cells.

HuH-7 cells were observed to form syncytia at a rate slower than that of DBT cells after infection with JHM. DBT cells began to fuse around 6 h after infection, whereas HuH-7 cells began to fuse only after about 9 h with slow expansion of the plaque over a 24-h period (data not shown). When J.7.2 was added to virus-infected DBT cells at 1 h postinfection and maintained throughout the experiment, the number of plaques was not affected, but the size of each plaque was reduced to less than half the diameter of the control after 24 h (Table 1). However, in HuH-7 cells, J.7.2 had no significant effect on plaque size. These results suggest that the MHV-induced fusion of HuH-7 cells may not be mediated by the same mechanism as that for DBT cells.

By contrast, an antibody against human CEA which has been shown to inhibit the infection of hCEA-expressing BHK cells by an MHV variant (Baric *et al.*, 1999) did not have effects on either virus infection or cell fusion. This point will be discussed below.



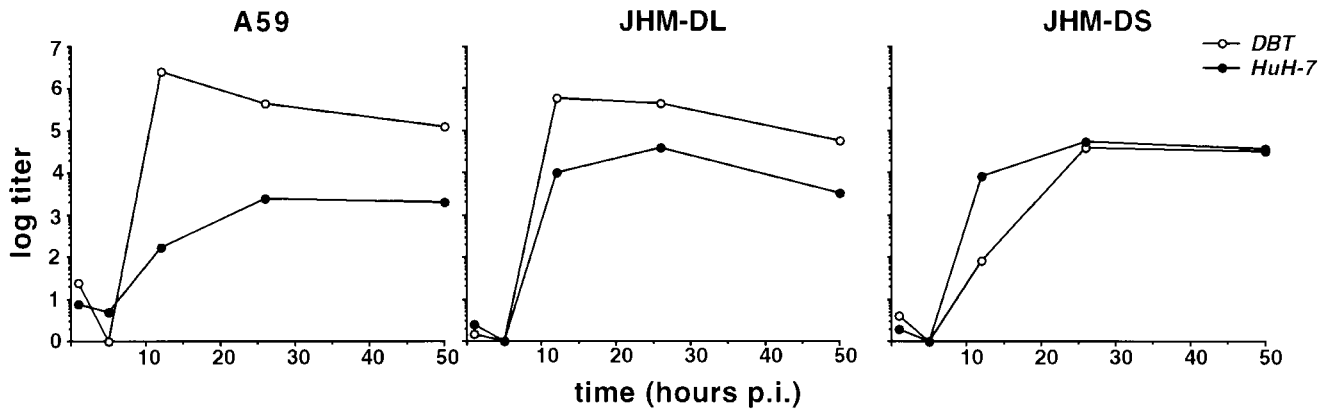


FIG. 4. Time course of replication of selected MHV strains in HuH-7 and DBT cells. Cells were infected as in Fig. 1. At various time points after infection, samples of cell culture media were collected and used for plaque assay on DBT cells. Results are shown as  $\log_{10}$  (PFU/ml).

### Growth of JHM/A59 recombinants in HuH-7 cells

The difference in the ability of strains JHM and A59 to replicate in HuH-7 cells led us to examine the growth of recombinant viruses between JHM and A59 strains in these cells (Fig. 5). The genomic structures of these recombinant strains have previously been characterized (Lai, 1992). At 24 h postinfection, all recombinants tested produced virus titers between  $1 \times 10^4$  and  $2 \times 10^5$  PFU/ml from DBT cells (data not shown). Replication in HuH-7 cells was highly variable, however, among recombinant viruses. Each of the recombinants B1, IL27, EL3, IL6, and EL7, which were derived from JHM-DL and A59,

grew very poorly, if at all, in HuH-7 cells. The virus titers produced from HuH-7 cells were lower than those of either parental strain. In contrast, the recombinants CA13 and CA43, which were derived from JHM-X and A59, grew at least as well as their parental strains. Recombinant CA13 especially rivaled the growth of JHM-DL and JHM-DS, consistently achieving virus titers more than 10-fold greater than those of its parental strains JHM-X and A59. These patterns of infectivity displayed by different MHV recombinants are not consistent with the idea that any single viral gene is the sole determinant of infectivity on HuH-7 cells. These results thus suggest that several viral genetic loci may contribute to the replication ability of MHV on HuH-7 cells and that interaction between these loci or their encoded proteins may affect this biological property.

TABLE 1

Effects of Antibodies on JHM-DL Infection: HuH-7 vs DBT Cells

	Infection <sup>a</sup>		Cell fusion <sup>b</sup>		Titer <sup>c</sup>	
	DBT	HuH-7	DBT	HuH-7	DBT	HuH-7
Control	+++	+++	+++	++	$3.4 \times 10^5$	$1.7 \times 10^4$
J.7.2	—	+	+	++	$7.2 \times 10^2$	$3.1 \times 10^2$
$\alpha$ -hCEA	+++	+++	+++	++	$3.0 \times 10^5$	$6.5 \times 10^3$

Note. Cells were incubated with JHM-DL for 1 h at 37°C. Antibodies J.7.2 (anti-S) or anti-hCEA were added during the first hour only (to study infection) or after the first hour and maintained throughout the experiment (to study cell fusion). After three washes, cells were incubated for 24 h in MEM at 37°C and then stained for N protein expression to visualize plaques.

<sup>a</sup> Assayed by the number of plaques formed at 24 h postinfection. +++, confluent plaques; ++, subconfluent plaques; +, scattered plaques; —, no plaques. JHM inoculum was preincubated with antibody for 20 min at room temperature prior to infection. Antibody was present during the first hour of infection only.

<sup>b</sup> Assayed by plaque size at 24 h postinfection. +++, complete fusion; ++, medium-size plaques (> ~20 nuclei); +, small plaques (< ~20 nuclei); —, no fusion. Antibody was added at 1 h postinfection and was present throughout the remainder of the experiment.

<sup>c</sup> Media collected at 24 h postinfection under the same conditions as in (a) were used for plaque assay on DBT cells. Titers are given in PFU/ml.

### MHV receptor utilization in HuH-7 cells

MHV strain A59 has been shown to use a wider variety of CEA-related receptors than JHM (Chen *et al.*, 1995, 1997). However, HuH-7 allowed more efficient infection and replication of strains JHM-DL and JHM-DS than strain A59. Therefore, we sought to analyze the potential role of the known MHV receptors, all of which allow entry of A59, in the infection of HuH-7 cells. A purified soluble form of the virus-binding domain of the prototype MHV receptor mBGP (sMHVR) (Gallagher, 1997) was tested for its ability to block the infection of HuH-7 cells by JHM-DS. Infection of both HuH-7 and DBT cells was inhibited to approximately the same extent by the addition of sMHVR in the range of 0.5–2.0  $\mu$ g/ml when assayed for N protein production as a marker of MHV infection (Fig. 6). These data suggest that the same receptor-binding domain of the S protein is involved in the infection of HuH-7 and DBT cells by JHM.

To determine whether the resistance of HuH-7 cells to infection by MHV strains other than JHM is due to the lack of an appropriate receptor or to some other factor, we transiently transfected HuH-7 and Cos-7 cell mono-

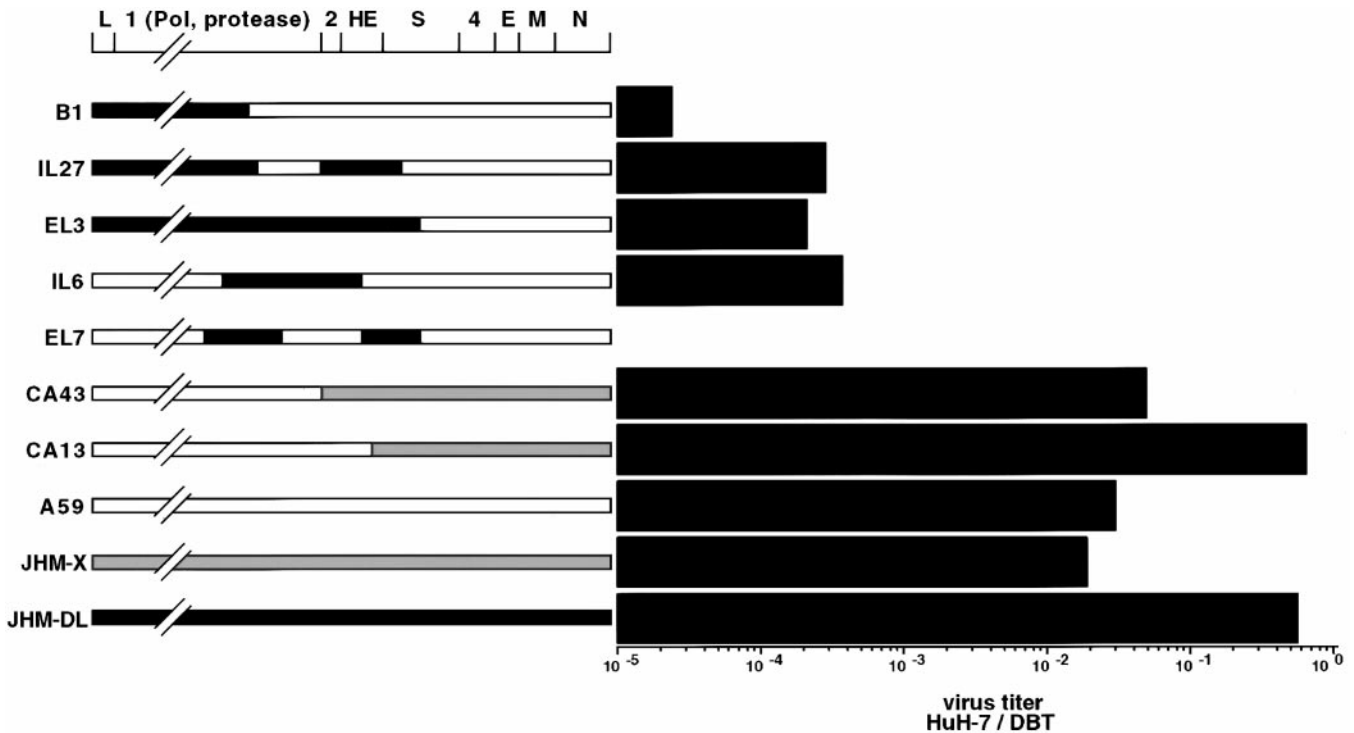


FIG. 5. Replication of recombinant MHV strains in HuH-7 cells. Various recombinant MHV strains between A59 and two JHM strains were used to infect DBT and HuH-7 cells as described in the legend to Fig. 1. After 24 h, supernatant was collected for plaque assay on DBT cells. Results are shown as the ratio of virus released from HuH-7 cells to virus released from DBT cells. The genomic structures of the recombinants are shown on the left (Lai, 1992): white areas denote genomic sequences derived from A59, gray areas those from JHM-X, and black areas those from JHM-DL. A simple map of the MHV genome is provided for reference. The length of gene 1 is not to scale.

layers with a mammalian expression plasmid encoding mBGP (MHVR1). This plasmid has previously been shown to render Cos-7 cells susceptible to infection by all MHV strains (Chen *et al.*, 1997; Yokomori and Lai, 1992). At 72 h posttransfection, cells were infected with strains A59, MHV-2, and JHM-DS and were assayed for virus production, plaque formation, and viral N protein production 24 h later. Similar to the effects on Cos-7 cells, the exogenous expression of mBGP in HuH-7 cells increased the numbers of infected cells as assayed by N protein immunostaining (Fig. 7) and increased the virus titers produced from the infected cells (Table 2). These results suggest that HuH-7 cells lack only sufficient amounts of the proper receptor to allow entry of A59 and MHV-2. It is notable that the infectivity of HuH-7 cells by JHM also increased following the transfection of mBGP (MHVR1), suggesting that the endogenous receptor responsible for JHM entry may be present only in a subset of the cultured HuH-7 cells at a level sufficient to allow infection. It is also worth noting that the mBGP-expressing HuH-7 cells infected with A59 or MHV-2 remained mostly as single cells, in contrast to the syncytia seen in JHM-DS-infected cells. This finding further confirms that A59 did not cause fusion in HuH-7 cells. Thus, the mechanism of JHM-induced cell fusion in HuH-7 cells is likely different from that in mouse DBT cells.

Finally, in order to verify that endogenous hCEA is not

acting as the receptor for JHM in HuH-7 cells, a polyclonal antibody directed against hCEA was tested for its ability to block infection (Table 1). This antibody has been demonstrated to block infection of hCEA-expressing BHK cells by MHV variants that utilize CEA as receptor (Baric *et al.*, 1999). The antibody caused only slight inhibition of virus production in HuH-7 cells and did not affect cell-cell fusion. Thus, hCEA may play only a minor role in the infection of HuH-7 cells by JHM.

#### MHV infection of other human cell lines

Besides HuH-7 cells, a number of other human cell lines derived from tissues relevant to MHV infection were tested for their ability to be infected by strains of MHV (Table 3). None of the cell lines tested allowed significant replication of any of the strains shown in Fig. 1. However, HepG2 cells, another human hepatocellular carcinoma cell line, did form a few rare plaques that stained positively for viral N protein when infected with JHM-DL and JHM-DS (Fig. 8).

#### DISCUSSION

The results of the experiments performed in this study demonstrate that a human hepatocellular carcinoma cell line, HuH-7, can be infected by some strains of mouse hepatitis virus, resulting in productive infection and the

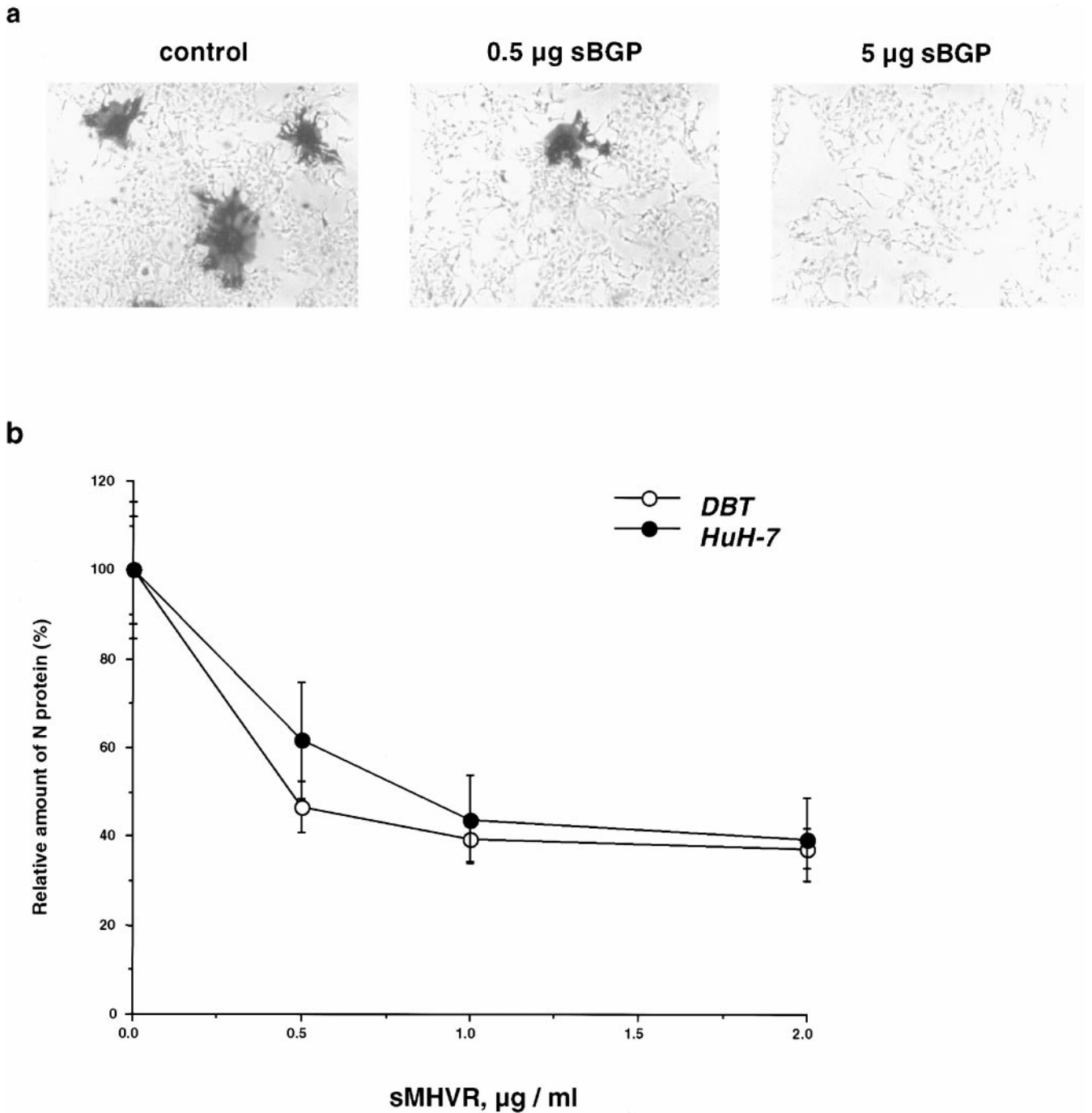


FIG. 6. Effects of a soluble form of the MHV receptor (sMHVR) on infection of HuH-7 and DBT cells. sMHVR was added to JHM-DL for 1 h at room temperature before infection. After removal of the inoculum, cells were further incubated for 24 h and assayed for virus. (a) Immunostaining for viral N protein in HuH-7 cells. (b) Quantitative enzyme-linked immunoassay for N protein.

formation of well-defined plaques. HuH-7 cells apparently possess the ability to be infected by MHV strain JHM whereas other human cell lines (with the possible exception of HepG2 cells) derived from tissues known to be infected by MHV in mice essentially lack this ability.

Due to effects of MHV on susceptible mice, there has been for decades a good deal of speculation about its potential, especially strain JHM, to cause human neuro-

logical disease (Lane and Buchmeier, 1997; Murray *et al.*, 1992a, 1993, 1990; Tanaka *et al.*, 1976). Despite experiments designed to test just such a hypothesis, coronaviruses have been neither established nor ruled out as a potential viral cause of multiple sclerosis in humans (Dalglish, 1997; Johnson, 1994; Kurtzke, 1993; Poser, 1994). Indeed, Murray *et al.* (1992a) found that 12 of 22 brains from multiple sclerosis patients and none of the



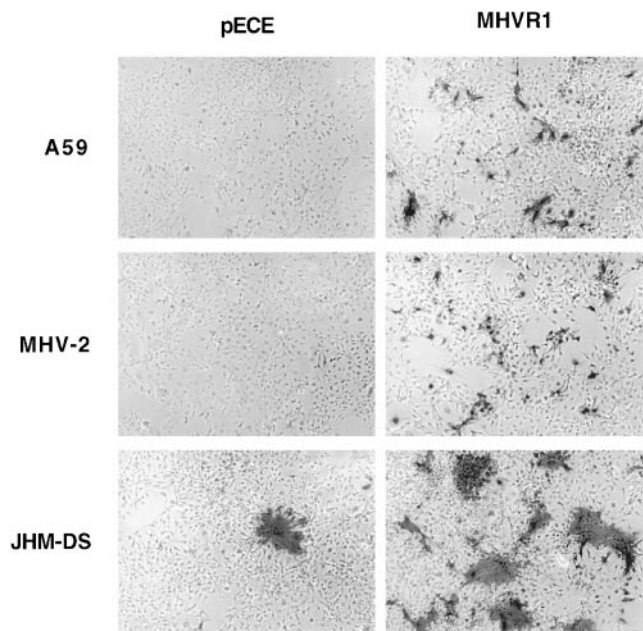


FIG. 7. MHV infection of HuH-7 cells transfected with mBGP (MHVR1). HuH-7 cells were transfected with 5  $\mu$ g mBGP (MHVR1) or control pECE vector (Yokomori and Lai, 1993) and infected with MHV strains 48 h after transfection. Cells were stained for viral N protein at 24 h postinfection as in Fig. 1.

controls showed hybridization to a murine-like coronavirus probe. The most likely role, if any, of coronaviruses in human multiple sclerosis would be through the activation of autoreactive T cells that could result in demyelination. This process has been described to occur in JHM-infected rodents, as evidenced by active demyelination by otherwise healthy rats following the adoptive transfer of T cells from JHM-infected animals (Watanabe *et al.*, 1983).

While the actual infection of a human by MHV has not been documented, a coronavirus (strain SD), which most closely resembles MHV (Gerdes *et al.*, 1981; Weiss, 1983) and can replicate in DBT cells, has been indirectly isolated from the brain of a human multiple sclerosis patient after passage in suckling mice (Burks *et al.*, 1980). Indeed, both this virus and the JHM strain were found to

TABLE 2  
Effects of Transfected mBGP on MHV Infection

Transfected plasmid	Virus titer	
	A59	JHM-DS
Vector control	$9.2 \times 10^1$	$1.2 \times 10^2$
mBGP	$2.0 \times 10^4$	$4.2 \times 10^3$

Note. Supernatant from virus-infected HuH-7 cells in Fig. 7 was harvested at 9 h postinfection and used for plaque assay on DBT cells. Values are given in PFU/ml.

TABLE 3  
MHV Infection of Several Human Cell Lines

Cell line	Tissue of origin	Cell fusion	Replication <sup>a</sup>
HuH-7	Hepatocellular carcinoma	+++ <sup>b</sup>	+++ <sup>b</sup>
Hep-G2	Hepatocellular carcinoma	+ <sup>b</sup>	+ <sup>b</sup>
SW608	Astrocytoma	—	—
SW1088	Astrocytoma	—	—
HRT-18	Colon carcinoma	—	—
Intestine 407	Normal fetal small intestine	—	—

<sup>a</sup> Assayed by immunostaining for viral N protein.

<sup>b</sup> JHM-DS and JHM-DL only.

replicate in primate brain, causing demyelination and encephalitis (Murray *et al.*, 1992b). Unfortunately, since antibodies made against the nearly ubiquitous human coronaviruses OC43 and 229E will cross-react with MHV antigens, it has not been practically feasible to determine whether or not humans have been exposed to MHV based on antibody-based tests.

Recent data have shown that variants of MHV strain A59 can be artificially selected for the phenotype of an extended host range and that such a variant (MHV-H2) can infect human cells, including HepG2 cells (Hensley and Baric, 1998). This extended host range is believed to occur through altered interactions with CEA-related proteins that can serve as receptors (Hensley *et al.*, 1998; Schickli *et al.*, 1997). In contrast, we show in this study that MHV strains JHM-DS and JHM-DL, which have not undergone any intentional artificial selection, are able to infect HuH-7 cells without any additional manipulation. Notably, HepG2 cells remain essentially resistant to this virus, indicating that the infection of HuH-7 cells by the JHM strains used in this study is not due to the same relaxation of CEA-related receptor specificity seen with MHV-H2. The actual receptor for JHM on HuH-7 cells remains unknown. It has been previously demonstrated in Cos-7 cells that although A59 and MHV-2 can utilize human BGP and/or human CEA as a receptor, JHM cannot (Chen *et al.*, 1997). Consistent with this finding, a neutralizing polyclonal antibody against hCEA was unable to significantly inhibit the infection of HuH-7 cells by JHM-DL. Thus, hCEA and hBGP are probably not used as receptors by JHM for the infection of HuH-7 cells. Furthermore, transfection of HuH-7 cells with mBGP allows infection by A59 and MHV-2, as well as by JHM, still more evidence that the susceptibility of HuH-7 cells to JHM infection occurs at the level of a (yet unidentified) receptor. The identification of this putative receptor might shed light on the characteristic cellular tropism of JHM in mouse brain.

We demonstrate in this study that the infection of HuH-7 and DBT cells by JHM can be blocked almost to the same extent by a neutralizing antibody directed

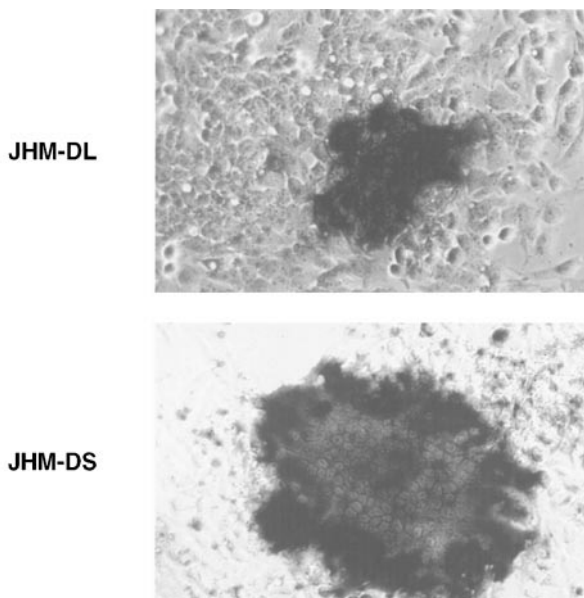


FIG. 8. Infection of HepG2 cells by JHM-DL and JHM-DS. HepG2 cells were infected and stained as in Fig. 1.

against the viral S protein or by a soluble form of the MHV receptor mBGP. Thus, the same epitope of the S protein involved in the infection of mouse cells appears also to be involved in the infection of HuH-7 cells. However, the formation of syncytia could be inhibited by an anti-S antibody only in DBT cells and not in HuH-7 cells (Table 1). Also, A59 did not cause syncytia to form in infected HuH-7 cells either after direct infection or following transfection of mBGP (MHVR1). This result suggests that, at least in this cell line, the processes of virus-cell fusion (leading to initial infection) and cell-cell fusion (leading to syncytia formation and cell-cell spread of virus) may not share the same mechanism. This conclusion is consistent with the finding that some monoclonal antibodies directed against the S protein will block infection but not fusion of mouse cells by MHV, while others will block fusion but not infection (Kubo *et al.*, 1993).

The infection of HuH-7 cells with MHV recombinants between A59 and JHM strains yielded little specific information about the sequence requirements for infection. However, it appears that most of the recombinants containing the leader sequence as well as genes 1 and 2 from A59 (roughly two-thirds of the genome) are capable of infecting HuH-7 cells, but those containing the remaining 3' end of the A59 genome appear to infect poorly, suggesting that some of these 3' end genes from A59 may suppress the infection or replication of MHV in HuH-7 cells. Conversely, the 5' two-thirds of the genome from strain JHM-X also appear to suppress efficient infection, since their replacement with A59 sequences (as demonstrated by recombinant CA13) results in a significant increase in titer. These results imply that several

loci within the viral genome may contribute to the efficiency of infection and/or replication of MHV in HuH-7 cells and that recombination between MHV strains can result in *cis*-complementation between these loci.

The observation that the antibody against the S protein inhibited the infection of HuH-7 cells by JHM, yet did not affect syncytia formation (Table 1), suggests that JHM may cause cell-cell fusion in HuH-7 cells by a mechanism not directly involving the S protein. This theory is consistent with the observation that HuH-7 cells infected with A59 produced detectable levels of virus particles (Fig. 4) but produced no plaques (Fig. 1). The hemagglutinin esterase (HE) protein will agglutinate red blood cells and is necessary for infection by some coronaviruses, e.g., bovine coronavirus (Deregt *et al.*, 1989). HE expression may also alter the tropism and virulence of MHV in mice (Yokomori *et al.*, 1995; Zhang *et al.*, 1998). Therefore, it is conceivable that the HE protein might contribute to the formation of syncytia and/or virus infection in HuH-7 cells.

It is known that JHM expresses HE, whereas A59 does not, in direct correlation with their ability or inability to cause fusion in HuH-7 cells. However, we have been unable to directly demonstrate fusion activity by the HE protein in HuH-7 cells (data not shown). Likewise, we could not demonstrate a significant difference in fusion activity or infectivity in HuH-7 cells between variants of JHM which express different amounts of HE (data not shown). Thus, the viral gene product responsible for plaque formation in HuH-7 cells remains unclear. The question of whether humans can be (or have been) infected by JHM may prove difficult to answer. The identification of a human liver cell line that is susceptible to JHM infection is an important step, however. The possibility arises that JHM might be capable of causing human hepatitis. Equally important is the prospect of identifying a JHM-specific receptor in HuH-7 cells that might help to explain the peculiar pathology of JHM in rodents.

## MATERIALS AND METHODS

### Viruses and cells

MHV strains A59, JHM-DL, JHM-DS, JHM-X, MHV-1, MHV-2, MHV-3, MHV-S, and At11f have been previously described (Hierholzer *et al.*, 1979; Lai *et al.*, 1981; Lai and Stohlman, 1981; Makino *et al.*, 1984; Morris *et al.*, 1989; Stohlman *et al.*, 1982). Recombinant strains B1, IL27, EL3, IL6, EL7, CA43, and CA13 have been described elsewhere (Lai, 1992).

DBT cells, a mouse astrocytoma cell line (Hirano *et al.*, 1974), was cultured in Eagle's minimal essential medium (MEM) supplemented with 5% newborn calf serum and 10% tryptone phosphate broth. DBT cells were used to propagate all virus strains and for all plaque assays, as previously described (Yokomori and Lai, 1992). HuH-7 cells (Nakabayashi *et al.*, 1982) were grown in Dul-

becco's minimal essential medium supplemented with 10% fetal calf serum (FCS) and nonessential amino acids. The human astrocytoma cell line SW608 (kindly provided by Dr. Alan Epstein, University of Southern California School of Medicine) was cultured in RPMI 1640 supplemented with 10% FCS. HeLa cells stably transfected with a plasmid expressing mBGP were maintained as previously described (Gallagher, 1996). Cell lines HepG2, SW1088, HRT-18, and Intestine 407 were purchased from the American Type Culture Collection (Manassas, VA) and were cultured in Dulbecco's minimal essential medium plus 10% FCS.

### Infection and immunostaining

Cells were infected with MHV at an m.o.i. of less than 1 as previously described (Yokomori *et al.*, 1993). After 1 h, the inoculum was removed and the cells were washed three times with serum-free MEM before being incubated for various lengths of time (typically 18–24 h) in MEM supplemented with 1% newborn calf serum and 10% tryptone phosphate broth (virus growth medium). Staining for the viral N protein was conducted using the anti-nucleocapsid protein monoclonal antibody J.3.3 (Fleming *et al.*, 1983) as previously described (Yokomori and Lai, 1992) except that the cells were seeded on 12-well plates and the secondary antibody was a goat anti-mouse IgG covalently linked to the enzyme  $\beta$ -galactosidase (American Qualex, San Clemente, CA). Cells were then stained with 2 mg/ml X-gal as substrate in PBS with 2 mM MgCl<sub>2</sub>, 3 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, and 3 mM K<sub>3</sub>Fe(CN)<sub>6</sub> overnight at 37°C. For quantitative analysis of relative N protein levels, the soluble substrate ONPG was used instead at 1.33 mg/ml in PBS with 2 mM MgCl<sub>2</sub> and 100 mM  $\beta$ -mercaptoethanol. Cells were incubated in 0.3 ml substrate solution at 37°C until color development occurred, whereupon 0.5 ml 1M sodium carbonate was added to stop the reaction and the OD<sub>420</sub> determined by spectroscopy.

For double staining of Ku antigen and MHV, cells were seeded on multichamber culture slides, infected as described above, and incubated for 8 h at 37°C. The cells were probed with polyclonal rabbit serum against MHV strain A59 (1:500 dilution) and a monoclonal antibody that specifically detects primate Ku antigen (1:100 dilution). TRITC-linked anti-mouse IgG and FITC-linked anti-rabbit IgG were used for detection by confocal fluorescence microscopy.

### Transfection–infection analysis

The transfection of cultured cells with mBGP (i.e., MHVR1, mmCGM1) cloned into the mammalian expression vector pECE has been previously described (Yokomori *et al.*, 1993).

### Antibodies and purified proteins

The neutralizing anti-S monoclonal antibody J.7.2 has been previously described (Fleming *et al.*, 1987; Wang *et al.*, 1992) and was used as hybridoma supernatant at a dilution of 1:30 in MEM for neutralization of infection (when added during initial virus infection) or syncytium formation (when added after initial virus infection). Anti-hCEA monoclonal antibody was purchased from Dako (Carpenteria, CA) and was used at a dilution of 1:10. The monoclonal antibody against human Ku antigen (Ku Ab-2 (111)) was purchased from NeoMarkers (Fremont, CA). sMHVR, a soluble form of the MHV receptor mBGP composed of the virus-binding domain fused to the Fc domain of human immunoglobulin G1, has been described elsewhere (Gallagher, 1997). To assess the effects of sMHVR, the recombinant protein was added to the virus inoculum in MEM for 1 h at room temperature prior to the infection of cells.

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