THE ROLE OF THE MHV RECEPTOR AND RELATED GLYCOPROTEINS IN MOUSE HEPATITIS VIRUS INFECTION OF MURINE CELL LINES

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Abstract:

Title of Dissertation: The role of the MHV receptor and related glycoproteins in mouse hepatitis virus infection of murine cell lines.

Jinhua Lu, Doctor of Philosophy, 1995

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Mouse hepatitis virus strain A59 (MHV-A59), a murine coronavirus, infects different murine cell lines causing different levels of virus growth and virus-induced cell fusion. The role of the MHV receptor (MHVR) glycoprotein and related glycoproteins in determining the outcome of MHV infection in vitro was examined. A previously unknown murine CEA-related glycoprotein (now named Cea10) was discovered and found to be co-expressed with MHVR in the Cl 1 D and F40 lines of mouse fibroblasts. A monoclonal anti-MHVR antibody, MAb-CC1, protected the Cl 1 D and F40 cells from MHV infection. A chimeric molecule in which the N-terminal domain of MHVR was replaced with that of Cea10 did not bind to MAb-CC1 or MHV-A59 virions in a virus overlay protein blot assay. Neither the expression of this chimeric protein in MHV-resistant BHK cells nor the native Cea10

conferred MHV susceptibility. The Cea10 protein was shown to be an approximately 35-37 kDa secreted glycoprotein. These results showed for the first time that two murine CEA-related genes can be co-expressed in some cell lines from inbred mice, while MHVR was the only MHV receptor in these cell lines.

A sensitive FACS analysis was developed to compare the level of expression of MHVR in different cell lines. It was found that the cellular susceptibility to MHV-A59 infection is correlated with the level of expression of MHVR on the surface of cells. Transfection of an MHVR expression plasmid into Cl 1 D cells which have low level of MHVR expression increased the expression of MHVR in these cells and enhanced their susceptibility to MHV infection. This suggested that the only factor in these cells that affects susceptibility to MHV infection is the low level of MHVR on their cell surface. Five'-azacytidine, a demethylating reagent of DNA, enhanced MHV-A59 infection in Cl 1 D cells through increasing the level of expression of MHVR. This suggested that the control of MHVR expression in these cells may involve DNA methylation. Expression of high levels of recombinant MHVR on Cl 1 D cells also enhanced virus-induced cell fusion and increased the virus yield. MHV-A59 infection of L2 cells resulted in a cell population with a very low level of MHVR expression and a low susceptibility to MHV-A59 infection at a time before any virus mutants had

been selected. A cell line, #97, selected from persistently infected 17 Cl 1 cells had a majority of cells with no detectable MHVR while a small minority of the cells expressed MHVR at much lower levels than 17 Cl 1 cells. These studies showed that the level of expression of MHVR in cells may play a significant role in the establishment and maintenance of persistent MHV infection in vitro.

Other cellular factors, in addition to the MHV receptor may also affect virus-induced cell-cell fusion and virus yield. Cl 1 D and F40 cells expressed about the same levels of MHVR, yet the level of cell fusion and the virus yield following MHV infection was higher in F40 cells than in Cl 1 D cells. Saturated or unsaturated fatty acids regulated MHV-induced cell fusion, but did not affect cellular susceptibility to MHV-A59. The fatty acids had opposite effects on the production of virus antigen in Cl 1 D and F40 cells. FACS analysis indicated that at early times after MHV infection, F40 cells produced more virus antigen than did Cl 1 D cells. Electron microscopy showed that at 24 hours post infection F40 cells contained more virions than Cl 1 D cells. This suggested that the higher yield of virus from F40 cells probably was not just due to their relatively high level of virus-induced cell fusion, but was the result of higher virus productivity in F40 cells than in Cl 1 D cells.

The role of the MHV receptor and related glycoproteins in mouse hepatitis virus infection of murine cell lines

by

Jinhua Lu

Dissertation submitted to the Faculty of the Department of Microbiology and Immunology Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1995.

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I. INTRODUCTION

This PhD dissertation project focused on cellular factors that affect the infection process of mouse hepatitis virus (MHV) *in vitro*, with emphasis on the role of MHV receptor glycoproteins in this process. In this introduction, I will first discuss the pathogenesis of MHV infection and then describe the status of our understanding of MHV infection at the cellular and molecular levels at the time I began my research. Finally, topics related to virus receptors in general and to the MHV receptor in particular will be discussed.

Coronaviruses and MHV

Coronaviruses were originally recognized by their unique morphology in negative staining which included the large, well separated, petal-shaped glycoprotein spikes (Tyrrell, *et al.*, 1968). They are enveloped, single-strand, positive-sense RNA viruses (Malluci, 1965; Watkins, *et al.*, 1975; Sturman & Holmes, 1983; Spaan, *et al.*, 1988). Many coronaviruses have been isolated from different species (Wege, *et al.*, 1982). Based on serological studies, they have been divided into 3 antigenic groups (Table 1). Most coronaviruses naturally infect one species or a few closely related species and cause diseases of respiratory and/or enteric tracts.

Table 1. ***Coronaviruses: Names, natural hosts, and diseases***

Antigenic group	Virus[a]	Host	Respiratory Infection	Enteric Infection	Hepatitis	Neurologic Infection	Other[b]
I	HCV-229E	Human	X				
	TGEV	Pig	X	X			X
	CCV	Dog		X			
	FECV	Cat		X			
	FIPV	Cat	X	X	X	X	X
	RbCV	Rabbit					X
II	HCV-OC43	Human	X	?			
	MHV	Mouse	X	X	X	X	
	SDAV	Rat					X
	HEV	Pig	X	X		X	
	BCV	Cow		X			
	RbEVC	Rabbit		X			
	TCV	Turkey	X	X			
III	IBV	Chicken	X		X		X

[a] Abbreviations: HCV-229E, human respiratory coronavirus; TGEV, porcine transmissible gastroenteritis virus; CCV, canine coronavirus; FECV, feline enteric coronavirus; FIPV, feline infectious peritonitis virus; TCV, turkey coronavirus; HCV-OC43, human respiratory coronavirus; MHV, mouse hepatitis virus; SDAV, sialodacryoadenitis virus; HEV, porcine hemagglutinating encephalomyelitis virus; BCV, bovine coronavirus; RbCV, rabbit coronavirus; IBV, avian infectious bronchitis virus.

[b] Other diseases caused by coronaviruses include infectious peritonitis, immunological disorders, runting, nephritis, pancreatitis, parotitis, and adenitis.

Adapted from Holmes, 1989. Used with permission.

The first murine coronavirus described, mouse hepatitis virus (MHV), MHV-JHM, was isolated from a mouse during the study of disseminated encephalomyelitis in an infected mouse (Cheever, et al., 1949). Many additional murine coronavirus strains have been isolated under different conditions (Nelson, 1952; Dick, et al., 1956; Wege, et al., 1982). In general, MHV can only infect mice, although some strains like MHV-JHM can infect young rats after intracerebral inoculation, causing chronic demyelinating encephalomyelitis (Wege, et al., 1983). MHV infection in mice causes a variety of diseases. The primary target tissues of infection include the respiratory system, gastrointestinal tract, liver, nervous system, spleen and thymus. Besides acute infection, MHV can easily establish persistent infection in vivo, leading to subacute or chronic disease (LePrevost, et al., 1975; Sorenson, et al., 1984). Persistent MHV infection has also been demonstrated in vitro (Lucas, et al., 1977, 1978; Stohlman, et al., 1979).

The pathogenesis of MHV is influenced by both viral and host factors. Strains of MHV show different degrees of virulence and different patterns of diseases. MHV-S infection in weanling or adult Swiss mice is largely restricted to the mucosa of the upper respiratory tract. Secondary involvement of other organs can occur in neonates or immunocompromised hosts such as athymic nude mice (Barthold and Smith, 1983; Barthold, 1986). MHV-2 and MHV-3

are more virulent than MHV-S and cause fatal hepatitis in adult mice (Taguchi, et al., 1980; Wege, et al., 1982). MHV-JHM is considered to be neurotropic. In susceptible mice, infection with MHV-JHM causes acute and chronic demyelinating diseases. Oligodendrocytes are the main target cells for JHM virus, but especially in young mice virus can also infect neurons and endothelial cells (Fleury, et al., 1980). MHV-A59 is hepatotropic, and the infection of susceptible mice leads to the destruction of liver parenchymal and Kupffer cells. However, there is considerable overlap of target tissues between these strains of virus (Barthold and Smith, 1984). For example, intracerebral inoculation of MHV-A59 in weanling mice also causes demyelinating disease, although the target cells in this case are non-neuronal cells (Lavi, et al., 1984). On the other hand, MHV-JHM in infected mice is also found in tissues where MHV-A59 is easily detected. The outcome of MHV infection is also influenced by the route of inoculation and the dose of virus (Barthold, 1986). Among the host factors affecting MHV infection are the mouse strain, the age of the mice, and their immune status. Adult mice are more resistant to MHV infection than suckling mice (Taguchi, et al., 1979). Interferon and macrophages may be important determinants of resistance to MHV infection (Taguchi, et al., 1979). MHV infection in mice can be enhanced by the administration of cortisone, or by infection of the host

with other viruses (Lavelle and Starr, 1969; Gledhill, 1961)

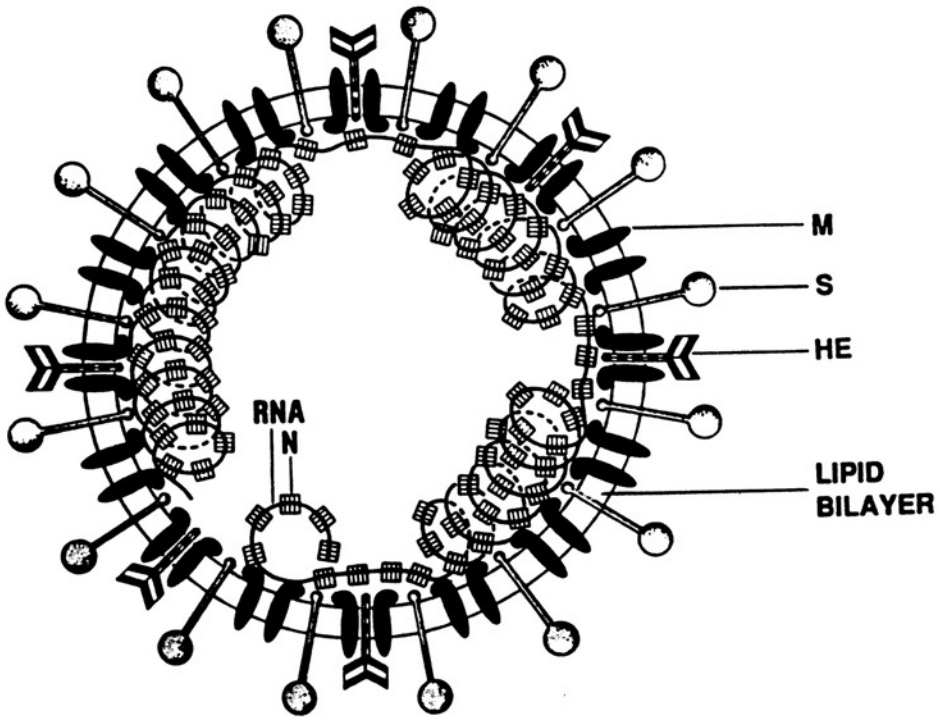
Strains of inbred mice show different degrees of susceptibility to infection by different MHV strains. In a classic experiment, Bang and Warwick (1960) demonstrated that MHV-2 infection in the PRI (Princeton) strain of mice caused fatal hepatitis, but the same virus did not kill C3H mice. MHV resistance was inherited as a single autosomal recessive gene. The susceptibility or resistance of mice to MHV-2 infection was reflected by the peritoneal macrophages isolated from each strain in culture. Other investigators extended these studies on host resistance to MHV by using different strains of MHV and different mouse strains. Three patterns of susceptibility to MHV-3 were found when 4-10 week old inbred mice were intraperitoneally inoculated, i.e., full susceptibility, semi-susceptibility, and resistance (LePrevost, et al., 1975). Inbred strain A/J mice survived high doses of MHV-3 infection. In contrast, the BALB/c and C57BL/6 mice were highly susceptible. C3H/He mice were of intermediate susceptibility to MHV-3, since more than 50% of mice survived the virus infection and chronic disease developed in some of the surviving animals (LePrevost, et al., 1975). The strain-dependent resistance to MHV-3 infection was expressed not only in macrophage cultures, but also in hepatocytes in primary monolayer cultures (Arnheiter, et al., 1982). Both H-2 or non-H-2 linked genes have been implicated in controlling the

development of disease in different inbred mice (Levy-Leblond, et al., 1979; Dindzans, et al., 1986). MHV-induced monocyte procoagulant activity (PCA) is believed to play an important role in MHV-3 pathogenesis, since it is co-expressed with the susceptibility to MHV-3 infection in different recombinant inbred mice (Dindzans, et al., 1986). All strains of mice were susceptible to MHV-A59 and MHV-JHM infection except for SJL/J mice (Stohlman and Frelinger, 1978; Smith, et al., 1984; Barthold and Smith, 1987). The MHV resistance of SJL/J mice has been mapped to a recessive gene in chromosome 7 (Smith, et al., 1984; Knobler, et al., 1985). Thus, various MHV strains exhibit different patterns of pathogenesis in strains of inbred mice and these difference are determined by both viral and host factors.

MHV Structure and Replication

Studies of the molecular biology of MHV have greatly enhanced our understanding of MHV pathogenesis. Several reviews have covered this subject (Sturman and Holmes, 1983; Spaan, et al., 1988; Lai, 1990). The general structure of MHV is shown in Fig. 1. The virus contains an approximately 27-30 kilobase (Kb) long infectious RNA genome. In virions, the RNA genome is complexed with the nucleocapsid protein (N). The envelope of the virus contains two glycoproteins in most MHV strains, i.e., the spike protein (S) and the membrane protein (M). Several MHV strains also express

Fig. 1. The structure of mouse hepatitis virus. MHV has a single strand, positive-sense RNA genome (27-30Kb) complexed with nucleocapsid protein (N). The virion is enveloped. The spike protein (S) projects from the envelop forming the spike. The M protein is an integral membrane protein and interacts with the nucleocapsid. In some MHV strains, another glycoprotein, the hemagglutinin esterase, is present on the membrane. Adapted from Holmes,1989. Used with permission.


M
S
HE
RNA
N
LIPID
BILAYER

another glycoprotein, called hemagglutinin-esterase, HE (Siddell, et al., 1983). In addition to the structural proteins, the virus genome also encodes several non-structural proteins. In this introduction, I will summarize what was known about MHV replication at the time my work began, focusing on the interaction of viral glycoproteins with cellular machinery for viral entry, release, and cytopathic effect.

MHV Proteins

M glycoprotein. This small membrane glycoprotein (20-30 kDa) is O-glycosylated in MHV (Holmes, et al., 1981; Niemann, et al., 1984; Locker, et al., 1992). The N-terminal domain of the M protein is exposed on the exterior of the virus envelope and the carboxyl-terminal domain is inside the virion membrane (Rottier, 1986). The accumulation site of M protein (Holmes, et al., 1981; Tooze, et al., 1988) and its ability to associate with isolated nucleocapsid in vitro suggested that the M protein may participate the virus assembly and budding process (Sturman, et al., 1980).

S glycoprotein. The spike protein is a large (180-200 kDa) N-glycosylated protein. It is synthesized as a large precursor protein, and during the virus maturation process in some MHV strains, it is cleaved by a cellular protease to generate two approximately 90 kDa proteins, the N-terminal

protein, S1, and the C-terminal protein, S2. The S protein projects outward from the virus envelope, forming the large spikes typical of coronaviruses. On the virus envelope, the S glycoprotein probably exists as a trimer (Delmas, et al., 1990).

Several functions have been attributed to the S protein. Virus infectivity can be neutralized with some monoclonal antibodies against S protein. The S protein is the only viral protein required to cause fusion of MHVR-expressing mouse cells. In MHV-infected cells, S protein, unlike M protein, is transported to the plasma membrane where it participates in cell fusion which is important for the cell-to-cell spread of infection (Sturman and Holmes, 1983). Recently, the receptor binding site has been mapped to the N-terminal 300 aa of the S1 protein (Kubo, et al., 1994). The spike protein of some MHV strains also has IgG Fc binding activity (Oleszak and Leibowitz 1990; Oleszak, et al., 1992). There is great diversity in the amino acid sequence and the length of S proteins among different MHV strains. Virus mutants which showed mutations in S protein appeared during infection in vitro and in vivo, suggesting that the S protein may play an important role in virus virulence (Parker, et al., 1989; Gallagher, et al., 1990; Hingley, et al., 1994).

N protein. Nucleocapsid protein is approximately 50-60 kDa. It is phosphorylated at serine residues and associates

with genomic RNA molecules (Siddell, et al., 1982). The role of the phosphorylation of N in virus replication is unknown. In vitro assay showed that the N protein is an RNA-binding protein and that it may associate with membranes (Robbins, et al., 1986; Baric, 1988; Anderson, et al., 1993). The N protein may regulate transcription, since an antibody against this protein inhibited the in vitro synthesis of genomic sized MHV RNA (Compton, et al., 1987).

HE protein. Some MHV strains, such as MHV-DVIM, or MHV-JHM express a third glycoprotein, called hemagglutinin-esterase (HE) (Luytjes, et al., 1988; Yokomori, et al., 1991). This 65-70 kDa protein forms a disulfide-linked dimer in the viral envelope (King, et al., 1985). The HE gene of MHV is closely related to the HE glycoprotein of influenza C virus (Luytjes, et al., 1988). The HE protein of MHV bind to 9-O-acetylated neuraminic acid residues on glycoproteins or glycolipids, as does that of bovine coronavirus (Vlasak, et al., 1988). The interaction of HE protein with its cellular ligand is not sufficient to mediate virus entry for MHV (Gagneten, et al., 1995). Some virus strains did not express the HE protein, but were infectious (Yokomori, et al., 1991).

Nonstructural proteins. The MHV genome includes 5 or more open reading frames (ORF), depending on the virus strain, that encode non-structural proteins. A virus-encoded p28 protein has been found in virus-infected cells

and by in vitro translation of the open reading frame 1a of the genomic RNA (Denison and Perlman, 1987). A 9.6 kDa product, now called sM, of the second ORF of mRNA 5 (Leibowitz, et al., 1988), originally identified as a nonstructural protein, has recently been shown to be in the virus membrane (Yu, et al., 1994). The functions of the non-structural proteins are not known.

Virus Genome

The infectious 27-30 kb, single-stranded RNA genome of coronaviruses, is the largest of the RNA viruses. The genome is capped and polyadenylated (Lai and Stohlman, 1981; Lai, et al., 1981). The gene order for the virus polymerase and three major structure proteins is Pol-S-M-N. Besides these genes, there are several ORFs encoding nonstructural proteins or HE. The order and number of these additional ORFs varies among coronaviruses (Spann, et al., 1988).

MHV Replication Cycle

MHV infection of a susceptible cell goes through the following processes: virus binding; fusion of virus envelope with cellular membrane; uncoating; translation, transcription and replication of RNA; virus assembly and budding into pre-Golgi vesicles; virus release and spread to uninfected cells. Host restriction and regulation of MHV replication could occur at any of the steps mentioned above.

Each of these events and the factors affecting them will be discussed.

Virus binding. Two of the virion glycoproteins, S and HE can bind to cell surface molecules. Spike protein bind specifically to cellular receptor glycoproteins. This property of S can be demonstrated in a virus overlay protein blot assay (VOPBA) in which the receptor molecule has been denatured by SDS, separated by electrophoresis, transferred to the membrane and blotted with MHV virions (Boyle, et al., 1987). Virus attachment to the MHV receptor can be blocked with antibodies against either S protein or the virus receptor (Compton, et al., 1992). Furthermore, the S protein interaction with the receptor can mediate virus infection, since transfection of the receptor cDNA clone into MHV-resistant hamster or human cell lines confers MHV susceptibility on these cells (Dveksler, et al., 1991; Yokomori and Lai, 1992a). In contrast, the binding of HE to its ligand is not able to mediate virus entry, although it is possible that this binding may enhance virus infection. The binding of the Fc portion of immunoglobulin to the spike glycoprotein of some MHV strains raises the question of whether this binding capability has any biological significance. For example, in mice with antibodies against host cell antigens, can MHV use the Fc binding activity to get into the cells independent of the MHV receptor? Or, alternatively, could the Fc binding activity of MHV spike

proteins increase virus infection by pre-concentration of virions on the cell surface?

Entry of MHV virus and fusion of virion envelope with cellular membranes. MHV may use either of two different ways to get into cells, i.e., fusion of the viral envelope with the plasma membrane or with endosomal membranes after endocytosis. Both modes of entry have some experimental support. For example, electron microscopy demonstrated the presence of MHV-3 virus particles within plasma membrane-proximal vesicles in hepatocytes after 1 hr incubation at 0°C followed by 10 min incubation at 37°C, suggesting entry via the endocytic pathway (Arnheiter, et al., 1982). The disruption of microtubules by colchicine or vinblastine had no detectable effect on the MHV-3 infection process, but the impairment of microfilament structure by cytochalasin B decreased the virus-induced cell fusion and converted the acute MHV-3 infection in peritoneal macrophages to a persistent infection (Mallucci and Edward, 1982). This suggested involvement of the cytoskeleton in MHV infection. MHV-3 or MHV-A59 infection was inhibited by lysosomotropic agents such as ammonium chloride (Krzystyniak and Dupuy, 1984; Mizzen, et al., 1985), which elevates the pH in endosomal/lysosomal vesicles and prevents low pH-dependent membrane fusion in other virus systems such as Semliki Forest virus and vesicular stomatitis virus (Matlin, et al., 1982; Helenius, et al., 1985). However, other studies

showed that low pH may actually inhibit cell fusion induced by MHV spike protein (Sawicki and Sawicki, 1986). The lysosomotropic agent apparently only delayed virus replication rather than inhibiting fusion (Mizzen, et al., 1985). These results suggest that MHV virions generally fuse with cells at the plasma membrane. The finding that the cytoskeleton-disrupting drugs colchicine and cytochalasin B have no effect on MHV-A59 uptake in L2 cells (Kooi, et al., 1988) also supports this idea. The spike protein of MHV may be an important factor in determining the entry pathway. In virions with mutant spike protein, the optimum pH required for membrane fusion was changed, and the entry of virions probably changed also (Gallagher, et al., 1991). Whether the MHV receptor plays any role in determining the pathway of virus entry is not known. In a recent study, MHV receptor-negative cells such as monkey kidney cells (Vero) or rat astrocytoma cells (C-6), were reported to internalize virions (probably through endocytosis), but did not undergo productive infection (Kooi, et al., 1991). Similar results have been reported in Chinese hamster ovary (CHO) cells (Asanaka and Lai, 1993).

The fusion event can be controlled at multiple levels. As mentioned above, some receptor-negative cells can internalize MHV virions, although they can not support productive infection. Resistant cells incubated with MHV become infected after membrane fusion induced by PEG,

suggesting that the limiting factor in these cells was the function before or during the fusion of the virus envelope with the cell membrane (Asanaka and Lai, 1993). Many cells are apparently able to bind virus but block infection at a post-adsorption stage (Shif and Bang, 1970; Knobler, et al., 1981; Arnheiter, et al., 1982; Beushausen, et al., 1987; Van Dinter and Flintoff, 1987). Based on these results, it was suggested that the cell tropism of and/or permissiveness to MHV are often determined at the virus penetration level. However, in all of these studies, the role of the MHV receptor in cellular susceptibility to MHV infection was not examined.

MHV receptor molecules may affect the membrane fusion process in virus infection either directly or indirectly. In the direct way, the MHV receptor molecule itself would participate in the formation of a fusion pore, as suggested in the universal model for membrane fusion events (White, 1992). In the indirect way, the MHV receptor might induce or regulate the formation of a fusion pore by other molecules. For example, the interaction of MHV spike protein with MHV receptor molecules may change the conformation of the spike protein and make the spike protein capable of mediating membrane fusion. So far, no experiment has directly discriminated between these two possibilities in the MHV system. The answer to this question is very important for understanding some aspects of the MHV

infection process. For example, if it is confirmed that the interaction of MHV spike protein with MHV receptor is necessary for mediating membrane fusion, it would explain why MHV receptor-negative cells can internalize MHV virions, but can undergo productive infection. It will be unnecessary to postulate factors other than MHV receptor involved in this process.

Besides the pH and the interaction of spike protein with MHV receptor, the fusion process of the MHV envelope with the cell membrane may also be regulated by other factors. Composition and distribution of membrane lipids have been suggested to affect the degree of cell fusion in several other viruses such as Sendai virus and vesicular stomatitis virus (MacDonald, et al., 1984; Herrmann, et al., 1990). Similarly, increasing the level of cholesterol in the cell membrane enhanced MHV-A59 mediated cell fusion in L2 cells and LM-K cells, but modification of fatty acids in these cell membranes had no obvious effect (Daya, et al., 1988). Other labs reported that the fatty acid composition of the cell membranes can alter the cell fusion induced by PEG or by viruses, including MHV (Roos, et al., 1990). The mechanism of this regulation is not clear. Since all the effects of lipid composition in these experiments were observed at a late stage of virus infection, i.e., after virions had already been produced in infected cells, it is not clear whether the change of lipid composition on cell

membrane also altered the fusion process at the virus entry stage. The protease activity of host cells can also affect the virus-induced fusion by controlling the cleavage of spike proteins in MHV virions and on the plasma membrane (Storz, et al., 1981; Frana, et al., 1985; Sturman, et al., 1985; Mizzen, et al., 1987)

Uncoating. As mentioned before, the MHV genome is associated with nucleocapsid protein, so an uncoating process may be necessary for the cellular machinery to translate the positive-strand genomic RNA. The mechanism of this uncoating step is not clear, but a specific phosphatase in the endosome has been suggested to dephosphorylate the viral N protein (Mohandas, et al., 1991). DbcAMP (N^6,2'-O-dibutyryladenosine-3',5'-cyclic monophosphate), an analog of 3',5'-cyclic AMP (cAMP) can induce cell differentiation and inhibit MHV-JHM replication in rat oligodendrocytes. Virus adsorption and internalization did not change after dbcAMP treatment, but the expression of viral RNA and protein were inhibited, implying that cAMP acted at the steps between penetration and initiation of translation, perhaps at the stage of uncoating (Beushausen, et al., 1987).

Translation, transcription and replication of the MHV RNA genome. The translation of the viral genomic RNA produces the RNA-dependent RNA polymerase which is required for subsequent virus transcription and replication. Treatment of virus-infected cells with cycloheximide, an

inhibitor of protein synthesis, immediately after virus adsorption inhibits virus RNA synthesis, probably by inhibiting the production of RNA polymerase (Sawicki and Sawicki, 1986). The positive-strand virus genomic RNA is transcribed into minus-strand RNA, which then serves as the template for synthesis of virus mRNA and genomic RNA. Two major alternative models suggested for coronavirus transcription and replication will be discussed here (Fig. 2).

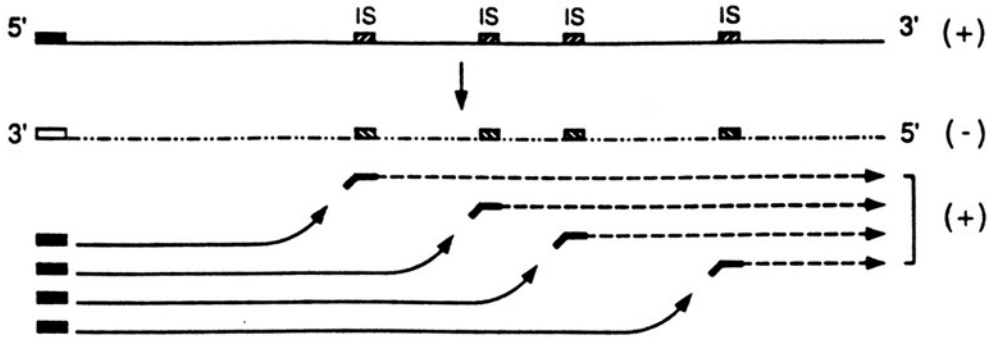
In the leader-primed transcription model (Lai, 1990), the transcription of the leader sequence begins at the 3'-end of the full-length, minus-strand RNA and terminates with the dissociation of the leader from the template. The transcribed leader sequence would then bind to intergenic sequences (IS) downstream on the minus-strand template which have homology to the 3' end of the leader. Thus, the leader-polymerase complex may serve as the primer for transcription of subgenomic mRNAs (Fig. 2). In this way, a set of nested mRNAs is generated with a common 3'-sequence, and polyadenylation and a common 5'-leader sequence. Each mRNA contains two or more ORFs except the smallest one. In most cases, only the ORF in the 5'-end is translated.

In the second model for MHV transcription and replication, the synthesis of minus-strand RNA is postulated to be discontinuous (Sawicki and Sawicki, 1990). The subgenomic minus-strand RNAs would be generated when the

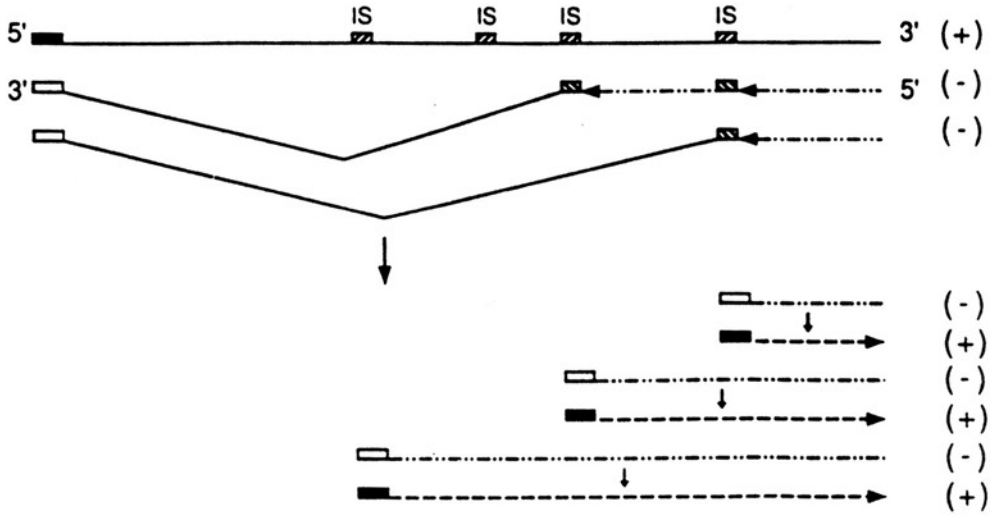
Fig. 2. Two alternative models for transcription of the plus strand RNA genome of MHV. a. Leader-primed transcription during plus-strand RNA synthesis: The plus-strand genomic RNA is transcribed into full length minus-strand RNA. The leader RNA (filled box) is transcribed from the 3′-end of minus strand RNA and somehow dissociates from the template. The leader RNA then binds to a down stream intergenic sequence (IS) and transcription is resumed and continue to the 5′-end of the template. b. Discontinuous transcription during minus-strand synthesis: Transcription of the minus strand RNA from the genomic RNA template may either continue to the 5′ end of the template or stop at intergenic sequence (IS). The subgenomic minus strand RNA would then dissociate from the template, be translocated to the leader sequence of the plus-strand RNA template and its transcription would be continued to generate a subgenomic minus strand RNA with an anti-leader sequence at the 3′-end. Each subgenomic minus strand RNA then serve as template for the transcription of plus strand RNA. Courtesy of K. V. Holmes and M. M. C. Lai.

Models of Discontinuous Transcription for Subgenomic mRNAs

a) Leader-primed transcription during positive-strand synthesis



b) Discontinuous transcription during negative-strand synthesis



polymerase halts at one of the IS sequences and is subsequently translocated to the leader sequence present at the 5'-end of the positive-strand genomic RNA template. The resulting subgenomic or genomic-size minus-strand RNAs would then serve as templates for the transcription of mRNAs or genomic RNA.

Both models have some experimental support (Baker and Lai, 1990; Sawicki and Sawicki, 1990). At this stage, it is not possible to rule out either model. The control of virus transcription and replication is poorly understood. The virus N protein has been shown to play a role in this process (Compton, et al., 1987), but possible cellular factors that could affect this process have not been identified.

Assembly and release of virions. The glycoproteins of MHV are synthesized in the rough endoplasmic reticulum. The S glycoprotein is co-translationally glycosylated with N-linked glycans, and oligomerized into non-covalently linked homo-trimers (Delmas and Laude, 1990). The proteins are transported to the Golgi apparatus for further processing including trimming of high mannose oligosaccharides, addition of terminal sugars, and fatty acylation of the S2 domain (Schmidt, 1982; Sturman, et al., 1985). When expressed from recombinant vaccinia, most S proteins accumulate in the Golgi, while some of the S proteins are transported to the plasma membrane where they play a role in

cell-cell fusion (Vennema, et al., 1990). The M protein of MHV is post-translationally O-glycosylated in the Golgi apparatus. Mature M proteins accumulate in the Golgi and are not transported to the plasma membrane. The N protein is translated on free polyribosomes and phosphorylated after translation.

The association of N proteins with the viral RNA genome is the beginning of the virus assembly process. N proteins may first bind to specific sequences in the genomic RNA and then additional N proteins bind to the remainder of the viral genome forming a helical nucleocapsid (Stohlman, et al., 1988). The nucleocapsid interacts with membrane containing the M proteins. The virus budding takes place on smooth membrane pre-Golgi elements referred to as the intermediate compartment or cis-Golgi network (CGN)(Tooze, et al., 1984; 1988). The M protein may dictate the site of virus budding (Holmes, et al., 1981; Tooze, et al., 1988). The S protein is not required for virion formation since tunicamycin-treated cells release virion containing normal amounts of M protein and nucleocapsid, but lacking S proteins (Holmes, et al., 1981; Rottier, et al., 1981). These virions are non-infectious. Depending on the time of infection, virions may also bud into the rough ER or nuclear envelope, but MHV does not bud at the plasma membrane. It is postulated that vesicles containing virions and vesicles containing secreted proteins may share the same exocytic

pathway until they exit from the trans-Golgi network (Tooze, et al., 1987). Since normal fibroblasts, which lack the regulated pathway, can be infected and release MHV virions, MHV virions may use the constitutive exocytic pathway to release virions by fusion of the vesicle membrane with the plasma membrane, in contrast with the secretion of some proteins such as hormones in hormone-secreting cells. However, the so-called constitutive exocytic pathway is also regulated by many factors such as small nucleotide-binding proteins and fatty acyl-coenzyme A (Rothman and Orci, 1992). So far, no host factors have been identified which can specifically affect the assembly/release of MHV.

MHV Receptor and Mouse Carcinoembryonic Antigen Family

In this section, I will first discuss some general topics related to the study of virus receptors and then results of studies related to the cellular receptors for MHV that led up to my work. I will also summarize what was known about the genes and proteins of the murine CEA family, because the MHV receptor(s) are members of this group.

Virus Receptors

A virus receptor is a component on the cell surface to which a viral attachment protein (VAP) binds. The following criteria have been used to test whether a molecule function as a virus receptor (Lentz, 1990).

a. The virion binds specifically to the receptor molecule.

b. At least some antibodies against the potential receptor molecule protect susceptible cells from virus infection.

c. Quantitatively, the surface density of the receptor should correlate with the susceptibility of cells to the virus.

d. The transfection of cloned receptor cDNA confer susceptibility to virus infection upon cells that are normally resistant to the virus.

Many approaches have been exploited for the identification of virus receptors. Competitive binding assays utilizing the labelled virions or virus attachment protein (VAP) with unlabelled virion, VAP or protective monoclonal antibody against cellular antigen can distinguish specific interaction between virion proteins and cell surface molecules from non-specific interactions. Screening of monoclonal antibodies (MAbs) against cell surface antigens to identify MAbs that protect susceptible cells from virus infection has been used to identify many virus receptors (Greve, et al., 1989; Yeager, et al., 1992; Wang, et al., 1992). Since infection with many viruses is very species-specific, a molecular cloning approach based on the functional transfer of viral susceptibility has also been used to identify some virus receptors (Mendelsohn, et al.,

1989). Some of the virus receptors are listed in Table II (Lentz, 1990; White, 1993). Virus receptors can be proteins, carbohydrates, lipids or complex macromolecules. Interestingly, many virus receptor glycoproteins belong to the immunoglobulin superfamily (White and Littman, 1989). Among these virus receptors, only a few meet all of the criteria listed above.

Some molecules can affect the virus entry process, but have not been recognized as virus receptors. For example, foot-and-mouth disease virus (FMDV) complexed with anti-viral antibodies can infect resistant cells via the Fc receptor on the host cells (Mason, et al., 1993). However, poliovirus and antibody complexes can not cause infection via the Fc receptor. It was reported that HSV-1 can bind the basic fibroblast growth factor (bFGF) and enter into the cells via bFGF receptor (Baird, et al., 1990). A recent report showed that expression of the epidermal growth factor receptor (EGFR) enhances the yield of reovirus and the expression of viral protein. A signal transduction pathway rather than virus binding or internalization is changed in host cells upon expression of the recombinant EGFR (Strong, et al., 1993).

A virus may bind to more than one type of receptor molecule. Human immunodeficiency virus (HIV) type 1 recognizes the CD4 glycoprotein which serves as its receptor (Sattentau and Weiss, 1988), but HIV can enter some CD4

Table 2: Examples of Virus Receptors

Cell surface molecule	Natural ligand	Virus	Family	Reference
Ig G superfamily members				
CD4	MHC Class II molecules	HIV	Lentivirinae	Maddon, et al., 1986
ICAM-1	LFA-1	Rhinovirus	Piconaviridae	Greve, et al., 1989
Carcinoembryonic antigen		MHV	Coronaviridae	Williams, et al., 1991
Class I HLA molecule	IgM k-chain	Human adenovirus	Adenoviridae	Chatterjee &Maizel,1984
Other proteins				
Aminopeptidase N		Human coronavirus 229E	Coronaviridae	Yeager, et al., 1992
Integrins	RGD-containing peptide	Foot-and-mouse disease virus	Piconaviridae	Fox, et al., 1989
(basic amino acid)transporter	Basic amino acids	Murine leukemia virus(ecotropic)	Retroviridae	Cunningham, 1992
Epidermal growth factor receptor	Epidermal growth factor	Vaccinia virus	Poxviridae	Eppstein, et al., 1985
Acetylcholine receptor	Neurotransmitter	Rabies virus	Rhabdoviridae	Lentz, et al., 1982
Carbohydrates and lipids				
Sialic acid-containing glycoprotins and glycolipids		Influenza virus	Orthomyxoviridae	Paulson, 1979
Heparin sulfate		HSV	Herpesviridae	Spear, et al., 1992
Galactosylceramide		HIV	Lentiviridae	Harouse, et al., 1991

Based on Lentz, 1990 and White, 1993.

negative cells using galactosyl ceramide which may serve as an alternative receptor (Bhat, et al., 1991). Herpes simplex virus glycoprotein C binds to heparan sulfate (Spear, 1993; WuDunn and Spear, 1989). However, this interaction is not sufficient to lead to infection. Interaction of glycoprotein D with a different molecule on the cell surface (Johnson, et al., 1990) and probably a cascade of interactions between glycoproteins and cell membrane receptors is needed to infect a cell. Recently, an integrin has been shown to serve as a receptor for the penton base that mediates adenovirus internalization (Wickham, et al., 1993). The binding of adenovirus to cells is not affected by monoclonal antibodies against integrins. The primary receptor that binds to the fiber proteins of adenovirus is unidentified. Integrins may serve as a secondary receptor for adenovirus in this case (White, 1993).

A receptor molecule may require association with other cell membrane molecules to form a functional receptor complex, to mediate virus infection. The HIV receptor, CD4, is an example. While the expression of recombinant human CD4 glycoprotein in CD4 negative human cells leads to productive infection of HIV, the expression of recombinant CD4 in murine cells and certain CD4-negative human cell lines did not confer HIV susceptibility (Maddon, et al., 1986; Levy, 1988; Harrington, 1993). Apparently the

fusion/penetration step is blocked in these non-permissive cells, since virus binds to the CD4 expressing cells, and the HIV is infectious for hybrid human-murine cells (Dragic, et al., 1992).

The binding of a viral attachment protein to a cellular receptor provides the physical linkage between a virion and the cell surface. Whether the virus receptor has any further role in virus entry depends upon the virus system studied. The receptor for poliovirus induces a conformational change of the virus capsid which may be necessary for virus entry (Fricks and Hogle, 1990). For enveloped viruses, membrane fusion is a necessary step for virus entry. Studies show that the continued presence of a specific cellular receptor is not needed after virus binding for the membrane fusion for influenza virus and alphavirus (Marsh and Helenius, 1989). It is not clear if this is true for other enveloped viruses also.

Virus receptors can play several important roles in virus pathogenesis. For instance, the cellular receptor for the viral hemagglutinin-neuraminidase glycoprotein of human parainfluenza virus type 3 is required for virus spread and virus-mediated membrane fusion (Moscona, et al., 1992). The relative affinity for sialic acid of the hemagglutinin-neuraminidase glycoproteins of different parainfluenza virus type 3 variants correlates with their virus-induced cell fusion activity (Moscona, et al., 1993). The species-

specificity and tissue tropism of infection of some viruses may be controlled at the level of expression of virus receptor (Crowell, et al., 1981; Compton, et al., 1992; Nomoto, et al., 1994). Few reports have addressed the relationship of virus receptors with other processes such as viral persistent infection.

Molecular mimicry has been suggested as the mechanism for the origin of molecules mediating cell interaction (Williams and Barclay, 1988). Since virus attachment to a cell surface is in many ways very similar to a typical cellular ligand-receptor interaction, the mimicry mechanism may also play a role in the selection of the cellular receptor for some viruses. The virus mimicry of cellular molecules has been discovered in various systems (Ahuja, et al., 1994). Molecular mimicry can cause autoimmune disease as a result of cross-reaction of antibodies against the virus with similar epitopes on host cell proteins and therefore alter the pathogenesis of virus infection (Oldstone, 1987; Lentz, 1990).

Mouse Hepatitis Virus Receptor

The identification and characterization of receptor glycoproteins for MHV was achieved using a combination of several strategies. Adult BALB/c mice are highly susceptible to MHV-A59 and MHV-JHM infection, while adult SJL/J mice are resistant to infection with these viruses.

This difference in susceptibility was also reflected *in vitro* in peritoneal macrophages from each strain of mice (Stohlman, *et al.*, 1980; Knobler, *et al.*, 1984). The genetic trait for this resistance has been mapped to a single gene, *hv2*, located 41.5 centimorgans from the albino locus on the end of mouse chromosome 7 (Smith, *et al.*, 1984; Knobler, *et al.*, 1985).

A solid phase virus-binding assay for MHV-A59 was developed to determine whether receptors played a role in determining the different MHV susceptibilities of different mouse strains. Brush border membranes (BBM) purified from the small intestine of adult mice were bound to nitrocellulose membrane; virus was allowed to interact with receptors on the membranes; and the bound virus was detected with anti-viral antibody (Boyle, *et al.*, 1987). MHV-A59 binds to BBM from adult BALB/c mice, but not to BBM from SJL/J mice. The potential receptor molecule from BALB/c mice was further characterized as a 110 to 120 kDa glycoprotein by virus overlay protein blot assay (VOPBA), in which the BBM proteins were separated by SDS-PAGE gel, and blotted with MHV-A59 transferred to a nitrocellulose membrane. Using this assay, the MHV-binding epitope was shown to be present only in mouse BBM and not those of other species (Compton, *et al.*, 1992).

A monoclonal anti-receptor antibody (MAb-CC1) was generated by immunizing SJL/J mice with BALB/c BBM.

Pretreatment with MAb-CC1 followed by virus challenge protected several cell lines from MHV-A59 infection. In immunoblots, MAb-CC1 detected a 110-120 kDa protein and an additional 58 kDa protein in liver from BALB/c mice, but not proteins from SJL/J liver (Williams, et al., 1990). The binding of MAb-CC1 to mouse tissues roughly correlated with the susceptibility of the tissues to MHV-A59 infection (Williams, et al., 1991). The administration of MAb-CC1 to susceptible BALB/c mice inhibited the yield of MHV-A59 virus from the target tissue (Smith, et al., 1990).

An affinity purification scheme for MHV receptor was developed based on MAb-CC1. The purified receptor from Swiss-Webster mouse liver with MAb-CC1 is a mixture of 110-120 kDa and 58 kDa protein (William, et al., 1990). The N-terminal sequences of the larger protein revealed that the receptor is a member of the murine carcinoembryonic antigen family within the immunoglobulin superfamily (Williams, et al., 1991). A full length cDNA clone encoding the MHV receptor (MHVR) was obtained from a library of cDNAs from BALB/c mouse liver. The cDNA encodes a glycoprotein with 4 immunoglobulin-like domains, a transmembrane domain, and a short cytoplasmic tail (Dveksler, et al., 1991). When this MHVR cDNA was expressed in MHV-resistant cells such as baby hamster kidney cells (BHK-21) or human RD cell lines, the cells became susceptible to infection by MHV-A59 as well as by other MHV strains (Dveksler, et al., 1991). The

infection was blocked by MAb-CC1. These data conclusively showed that the MHVR is a receptor for MHV.

In SJL/J mice, as in BALB/c mice, a polyclonal antibody against the synthesized peptide of the first 15 amino acids of MHVR detected a 110-120 kDa and a 55-58 kDa protein from mouse liver and BBM membranes, suggesting that non-functional homologs of MHVR glycoprotein are present in SJL/J mice (Williams, et al., 1990). A cDNA clone from SJL/J mice encoding a homologous protein of MHVR was isolated (Yokomori and Lai, 1992b; Dveksler, et al., 1993a). Surprisingly, when this cDNA was transfected into BHK cells, they became susceptible to MHV-A59 infection. This infection can not be blocked by MAb-CC1 (Yokomori and Lai, 1992b; Dveksler,et al., 1993a). Furthermore, a cell line from SJL/J mice that was resistant to MHV-A59 became susceptible after transfection with either the MHVR cDNA clone or cDNA clones encoding the SJL/J homolog (Dveksler, et al., 1993a).

Several isoforms of MHVR generated through alternative splicing of transcribed mRNA can also serve as MHV receptors. These isoforms contain either 4 or 2 immunoglobulin-like domains, a transmembrane domain, and different cytoplasmic tails. Different isoforms can be expressed together, and the pattern of expression of different isoforms may vary in different tissues (Huang, et al., 1990; Williams, et al.; 1991, Yokomori and Lai, 1992a).

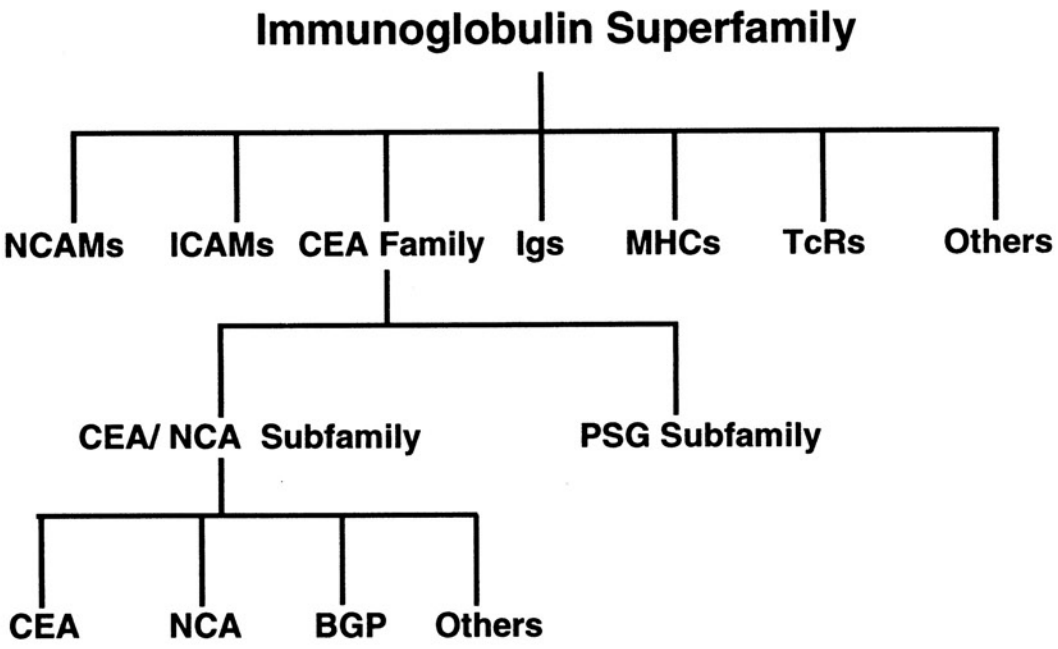
The possible effects of these differences in receptor expression on MHV pathogenesis are not yet clear.

Carcinoembryonic Antigen Family (CEA): Structure, Expression, Regulation and Function

The first member of the family of carcinoembryonic antigen (CEA) was originally identified as a marker for human tumors (Gold and Freedman, 1965). This family includes approximately 20 genes in humans. Based on sequence comparison, the CEA family can be divided into two main subgroups, i.e., CEA and its antigenitically related proteins such as non-specific crossreacting antigen (NCA), and the pregnancy-specific glycoproteins (PSG)(Thompson, et al., 1991; Williams and Barclay, 1988)(Fig. 3). PSGs are secreted, while CEA/NCA glycoproteins are mainly membrane-bound. The mRNAs encoded by each CEA-related gene undergo extensive splicing, which generates numerous protein isoforms from each gene. The CEA-related proteins are usually highly glycosylated.

The CEA/NCA subgroup can be further subdivided into the biliary glycoprotein (BGP), CEA, NCA, and other groups. The different CEA/NCA groups differ in their patterns of expression. Immunological methods show that NCA is present not only in tumors, but also in a variety of normal fetal and adult tissues including colonic mucosa, lung, liver et al. (Huitric, et al., 1976). The BGP proteins are found in

Fig. 3. The relationship of the CEA family and immunoglobulin superfamily. The immunoglobulin superfamily has been divided into several families based upon sequence and structure analysis. NCAM: neural cell adhesion molecules. ICAM: intercellular cell adhesion molecules. CEA: carcinoembryonic antigen. Ig: immunoglobulin. MHC: major histocompatibility complex. TcR: T cell receptor. PSG: pregnancy specific glycoproteins. BGP: biliary glycoprotein. NCA: non-cross reacting antigen. Adapted from Thompson, et al., 1990 and Williams and Barclay, 1988.


Immunoglobulin Superfamily
NCAMs
ICAMs
CEA Family
Igs
MHCs
TcRs
Others
CEA/ NCA Subfamily
PSG Subfamily
CEA
NCA
BGP
Others

epithelium of bile canaliculi (Svenberg, 1976). PSGs are expressed in increasing amounts in the placenta during pregnancy. Although all the CEA and CEA-related glycoproteins exhibit structural features of Ig variable-like domains and internal Ig constant-like domains of the immunoglobulin superfamily, the proteins in different groups have distinct features. BGP proteins usually have either long or short cytoplasmic tails and are integral membrane proteins (Hinoda, et al., 1990; Barnett, et al., 1993). In humans, the CEA and NCA glycoproteins are linked to the cell membrane by glycosyl phosphatidylinositol (GPI) anchors as a result of a post-translational modification (Hefta, et al., 1988; Takami, et al. 1988).

At the time when the receptor for MHV was cloned (Dveksler, et al., 1991), the rodent counterparts of the human CEA genes were just beginning to be identified. Using the human CEA gene as a probe and screening the cDNA library in low stringency conditions, several CEA-related genes were isolated from both mouse and rat (Beauchemin, et al., 1989a; Kodelga, et al., 1989; Rebstock, et al., 1990). Dr. Beauchemin first reported the isolation of 4 cDNA clones related to human CEA from a CD-1 mouse colon cDNA library. Since CD-1 are outbred mice, it was not clear whether the 4 clones represented 4 individual gene products, or were generated by alternative splicing of transcripts from a single gene (Beauchemin, et al., 1989). Therefore it was

unclear how many genes were expressed in inbred mice. The identification of rodent CEA-related genes greatly facilitated the study of the expression and function of CEA-related genes. In situ hybridization showed that murine BGP genes are expressed in cartilage, bone, muscle layers of stomach and intestine, and blood vessel walls at different stages of mouse embryonic development, suggesting a role for BGP in morphogenesis (Huang, et al., 1990). A closely related protein from rat has been identified as rat ecto-ATPase (Lin and Guidotti, 1989). This protein was later found to be the same as a bile acid transporter (Sippel, et al., 1993) and a previously identified cell adhesion molecule (C-CAM105) (Culic, et al., 1992). Other reports have shown that C-CAM105 binds calmodulin and is a homophilic cell adhesion molecule (Tingstrom, et al., 1990; Blikstad, et al., 1992). Tyrosine phosphorylation of biliary glycoprotein has been reported (Afar, et al., 1992). The full spectrum of the natural function(s) of murine BGPs is not yet known.

The regulation of expression of the CEA-related glycoproteins has been studied. Human tumor cells express high levels of CEA and show hypomethylation of CEA-related genes, while these genes are hypermethylated in normal fibroblast cell lines (Tran, et al., 1988). The analysis of the promoter regions of the human CEA, NCA and PSG genes showed that all of these genes lacked the typical TATA and

CAAT-box which are present in many other genes (Cordon, et al., 1980; Schrewe, et al., 1990). Cellular trans-acting factors that interact with the promoter region of CEA-related genes have not yet been identified.

The regulation of expression of CEA glycoproteins may also occur at the post-transcriptional level. The alternative splicing of gene transcripts is one of the major mechanisms for generating different proteins from the same BGP gene (Barnett, et al., 1989). Although the level of CEA proteins in some colonic tumor cells is higher than that in normal colonic mucosa, the steady state levels of CEA transcripts are comparable, indicating regulation at the translational or post-translational level (Kuroki, et al., 1988; Cournoyer, et al., 1988). Many substances have been reported which can regulate the expression of CEA-related molecules in vitro. For example, cAMP can stimulate the synthesis and release of CEA in human colon cancer cells (Hwang, et al., 1986). In other studies, interferon-γ was found to stimulate the synthesis of CEA to different degrees in various human colorectal tumor cell lines, depending on the degree of differentiation of cells (Guadagni, et al., 1990). Other factors such as transforming growth factor-β (TGF-β), or retinoic acid can also affect the expression of CEA in cells (Chakrabarty, et al., 1988; Niles, et al., 1988). However, compared to human CEA and its related genes, little is known about the regulation of expression of

rodent CEA-related genes.

As discussed above, the replication cycle of MHV is a complex process. Several host factors have been postulated to play a role in controlling cellular susceptibility. Since our laboratory identified and characterized the receptor molecules for MHV, it is feasible now to address several questions related to the role of MHV receptor in MHV infection. How are MHV receptor molecules involved in the membrane fusion process during MHV entry? What is the mechanism for the regulation of membrane fusion by lipid composition? Do fatty acids function through the regulation of expression or function of MHV receptor? What is the role of the MHV receptor in MHV-induced cell-cell fusion? What is the effect of regulation of expression of MHV receptor upon MHV infection?

To answer these questions, we need to know the nature of the receptor(s), the amounts of the receptors and the locations of the receptors in the cells. In this project, a systematic approach was used to address some of these questions. First I characterized the MHV receptor in the cell lines I used. Then using the expression of MHV antigen in the cells as a criterion for cellular susceptibility to MHV, the effect of level of expression of MHV receptor on the cell surface on MHV susceptibility was studied. The regulation of expression of MHV receptor and its effect on

viral persistent infection were also examined. Based on these studies, I postulate that the major determinant for cellular susceptibility is the level of expression of MHV receptor on the cells, but that the levels of virus yield and virus-induced cell fusion are also affected by MHV receptor as well as other cellular factors.

II. MATERIALS AND METHODS

Cells

Mouse Cl 1 D and F40 cells were obtained from Dr. Roos, University of Pennsylvania. Cl 1 D cells were sub-cloned from mouse fibroblast LM-TK$^-$ cells. F40 cells were derived from Cl 1 D cells by treating the cells with polyethylene glycol for 40 cycles (Roos, 1984). The L2 and 17 Cl 1 lines of murine fibroblasts, N-BALB-3T3 cells, a nontransformed mouse cell line and SV-BALB-3T3 cells, an SV40 virus-transformed cell line were obtained from Dr. Sturman (Sturman and Takemoto, 1972), New York State Department of Health, Albany, NY. These cell lines were all propagated in Dulbecco's-modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2% penicillin-streptomycin-fungizone mixture (PSF)(Gibco BRL, Gaithersburg, MD). The cells were passaged twice each week at a 1:5 dilution for Cl 1 D cells and 1:10 for F40, L2 and 17 Cl 1 cells. Since Cl 1 D cells give higher yields of virus at high passages, only cells passaged less than 15 times after thawing from liquid nitrogen were used for these studies. One lot of FBS which yielded the lowest plaque number when compared to other lots of FBS was used for Cl 1 D cells. Baby hamster kidney cells (BHK-21), MHVR-transfected BHK cell (Pan-2) generated in this lab which were susceptible to MHV infection, and Vero cells (from Dr.

Jenkins, University of Pittsburgh) were grown in Eagle's modified essential medium (EMEM) supplemented with 10% FBS and 2% PSF. #97 cells were obtained from Dr. S. Sawicki, Medical College of Ohio. This cell line was derived by single cloning from the 97th passage of a carrier culture of 17 Cl 1 cells persistently infected with MHV-A59. No viral antigen or infectious MHV was detected in these cells. The #97 cells were grown in DMEM medium with 6% FBS, 2% PSF and 5% tryptose phosphate broth (Gibco, BRL). The cells were split at a 1:5 dilution, twice each week. When 17 Cl 1 cells were used for comparison with #97 cells, they were also grown in the same medium as used for #97 cells.

Virus

The MHV-A59 strain of MHV was obtained from Dr. L. Sturman, New York State Department of Health, and propagated in 17 Cl 1 cells. The supernatant from infected cells, which contained released MHV-A59 virions was harvested at 20-24 hours post inoculation (p.i.). The supernatant was cleared from cell debris by centrifugation at 1000 rpm for 5 min (500x g) and then frozen at -70°C.

The virus titer was determined by plaque assay on L2 cells (Sturman and Takemoto, 1972). Briefly, ten-fold dilutions of virus stock were made and 0.5 ml of diluted virus was inoculated onto L2 cells in 6 well plates. After 1 hour adsorption at 37°C, the cells were washed twice and

overlaid with 3 ml a final concentration of 0.95% Noble agar in minimum essential medium (MEM) supplemented with 4% FBS and 2% PSF. The plaques were allowed to develop for 36 to 48 hours. Then 1.5 ml of 0.95% Noble agar in MEM containing 0.02% neutral red was added onto each well. The cells were incubated at 37°C for another 2 hours to stain the plaques and the plaques were counted.

To study virus growth in L2, Cl 1 D, F40 and MHVR cDNA transfected Cl 1 D or F40 cells, all of these cells were infected with MHV-A59. The virus in the supernatant medium was harvested at different times and quantitated by plaque assay on L2 cells. In some experiments, the monoclonal anti-MHVR antibody MAb—CC1 or a control MAb of the same isotype directed against an irrelevant antigen (at 1:5 dilution of hybridoma cell supernatant) was added into cells immediately after virus inoculation to prevent-MHV induced cell fusion.

Antibodies

Anti-MHVR monoclonal antibody MAb—CC1, an IgG_1 of mouse origin, was generated in this laboratory (Williams, et al., 1990). The antibody recognizes an epitope in the N-terminal domain of MHVR glycoproteins (Dveksler, et al., 1993) and can protect 17 Cl 1 and L2 cells from MHV-A59 infection. Monoclonal antibody JL anti-N obtained from Dr. Leibowitz, J., University of Texas, Health Sciences Center, recognizes

the nucleocapsid protein (N) of MHV-A59. Monoclonal antibody against cholera toxin, an IgG_1 MAb from Dr. R. Holmes, Uniformed Services University of Health Sciences, was used as a control monoclonal antibody. In this study, MAbs were used as hybridoma supernatants unless otherwise stated. For fluorescences activated cell sorting (FACS) analysis of MHVR expression on the cells, the antibody was concentrated as described below.

Hybridoma cells secreting MAb-CC1 and a control antibody with same IgG1 isotype were grown in HT medium (RPMT 1640, 10% fetal bovine serum, 10% NCTC-109 medium, 2 mM L-glutamine, 1 mM oxalacetic acid, 100 U of insulin, 0.1 mM hypoxanthine, 0.016 mM thymidine, 50,000 U of penicillin, 50,000 U of streptomycin, 125 ug of fungizone, 50 ng of gentamicin). The culture supernatant was collected and saturated ammonium sulfate solution was added to a final volume of 0.38 (V:V). The solution was adjusted to pH 7.0 using 10 N NaOH and put at 4°C overnight. The protein precipitate was pelleted by centrifugation at 6000 rpm for 25 minutes in a Sorvall GSA rotor. The pellet was suspended in phosphate balanced saline (PBS) (Ca^{2+} and Mg^{2+} free), and reprecipitated with 50% ammonium sulfate. After centrifugation at 15,000 rpm for 20 minutes, the pellet was subjected to the same precipitation again. The final protein pellet was suspended in 5 ml PBS (Ca^{2+} and Mg^{2+} free) and the salt was separated from protein using a PD10 column

(Pharmacia) according to the manufacturer's instructions. The concentration of protein was determined by the Bradford assay (Bio-Rad).

Rabbit polyclonal anti-MHVR antiserum 655 was generated in this laboratory by immunization of rabbits with affinity purified MHVR glycoproteins with MAb-CC1 from Swiss–Webster mouse liver cells. Rabbit polyclonal anti–serum 669 against rat ectoATPase was a gift from Dr. Sue-Hwa Lin, University of Texas, M. D. Anderson Cancer Center, Houston, TX. Rabbit anti-Cea10 anti–serum, anti-CEA10 K, and preimmune serum were provided by Dr. W. Zimmermann, University of Freiburg. The anti–serum was obtained by immunization of rabbits with a 14 amino acid synthesized peptide at the C-terminus of the deduced Cea10 protein sequences coupled to keyhole limpet hemocyanin. This anti–serum detected the synthesized peptide coupled with ovalbumin in a ELISA assay and also had high reactivity with ovalbumin. In some experiments, anti–serum 655, 669 and control normal rabbit anti–serum were preadsorbed 3 times with paraformaldehyde-fixed BHK cells or vaccinia-infected BHK cells to remove antibodies directed against epitopes shared by hamster and mouse cells. Goat anti–serum AO4 against purified MHV-A59 spike protein was generated in this lab (Sturman, et al., 1980). Mouse anti-mouse A59 anti–serum is from convalescent BALB/c mice infected with MHV-A59. The affinity-purified rabbit anti-mouse IgG antibody and rhodamine-conjugated rabbit anti-

mouse IgG antibody, goat-anti rabbit IgG antibody and peroxidase-conjugated goat anti-mouse IgG $F(ab')_2$ were purchased from Cappel (Cappel, Chester, PA). The phycoerythrin-conjugated goat anti-mouse IgG_1 antibody was obtained from Boehringer Mannheim.

RNA Extraction

Total RNA from cells or tissues was extracted using RNAzol B solution (Tel-Test"B" Inc. Friendswood, TX.) according to the manufacturer's instructions (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was prepared by passage through oligo(dT) cellulose columns (Invitrogen, San Diego, CA), precipitation with 70% ethanol (final concentration) and 0.3 M sodium acetate and resuspension in DEPC (diethyl pyrocarbonate) treated water. The RNA was stored at -70°C.

RT-PCR Amplification of MHVR and Cea10

cDNAs were synthesized from either 1 ug of poly(A)$^+$ RNA or 4 ug of total RNA and reactions were primed with either random primers or oligo(dT). The cDNA synthesis reaction was carried out using Moloney murine leukemia virus RNAse H$^-$ reverse transcriptase according to manufacturer's instructions (Gibco BRL, Gaithersburg, MD). The total 25 ul reaction mixture contained 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM of each deoxynucleotide triphosphates, and 300 units of M-MLV reverse transcriptase.

For PCR amplification, a 50 ul total volume containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.3 mM each of deoxynucleotide triphosphates, 0.5 ug each of sense and anti-sense primers (see primer list below), 1 unit of Taq DNA polymerase and was supplemented with 2 ul of cDNA reaction mixture (Boeringer Mannheim). The PCR reactions were carried out at 94°C for 1 min, 45°C for 1 min, and 72°C for 2 min for 30 cycles.

DNA Sequencing

Sequencing of double stranded DNA was performed on DNA obtained either from PCR reactions or from subcloned PCR products using the dideoxy method (Sanger, et al., 1977) with Taq DNA polymerase (Applied Biosystems, Columbia, MD). The sequencing reactions were run at 96°C for 30 sec, 50°C for 15 sec, and 60°C for 4 minutes for 25 cycles. DNA and unincorporated nucleotides were separated by Sephedex G-50 column (5 prime→3 prime, West Chester, PA). After vacuum-drying, the DNA was separated on polyacrylamide sequencing gel and sequences were determined using the model 373A DNA sequencing system (Applied Biosystems).

To purify the DNA for sequencing from PCR reaction mixtures, the DNA was first separated by electrophoresis on 2% agarose gels. The band containing the required DNA was cut out and the DNA was extracted from the gel using silica matrix (Bio 101 Inc. La Jolla, CA). Plasmid DNA was

purified in small scale as described (Sambrook, et al., 1989), except that after lysis of bacteria and precipitation of DNA with isopropanol, the plasmid DNA was suspended in water and re-precipitated with an equal volume of PEG-8000. Large scale plasmid DNA preparations were purified by double centrifugation in cesium chloride-ethidium bromide gradients (Clewell and Helinski, 1972). Briefly, the bacterial cells were collected and suspended in 9 ml SET solution (25% sucrose, 50 mM Tris pH 8.0, 40 mM EDTA), treated with 1 mg/ml lysozyme for 5 min at 4°C, and lysed in Triton solution (0.40% triton X-100, 62.5 mM EDTA, pH 8.0, 50 mM Tris). After centrifugation at 25,000 rpm using a Beckman SW 28 rotor for 2 hrs to remove cell debris, the DNA in solution was mixed with CsCl (d=1.47 g/ml) and spun at 47,000 rpm for 18 hrs in a Sorvall Ti50 rotor at 20°C. The DNA was purified a second time under the same conditions. Finally, the ethidium bromide in the DNA was extracted with butanol and the DNA was precipitated with 70% ethanol. The concentration of DNA was determined using optical absorbance at 260 nm.

Oligonucleotide Primers Used in This Study

1. 685: CGGCAGAGAGATAATATACAG (MHVR 357-377)
2. 253: ACTGGTACAAGGGAAACAGTGG (Cea10 253-274)
3. 1671: CCTCCTGGAGCCCTG (MHVR 146-160)
4. 865: GTTCTTCTACTTGTTCACAATCT (MHVR 220-242)

5. Cea10: AGTGGGGCTGGACACAAT (Cea10 270-287)
6. GCS2: TGAATTTCACGGGGCAAG (MHVR 332-349)
7. GCS1: ACGCTACGGCTATAGACAAAG (MHVR 281-301)
8. 1295: AGACTACAACAGGCCTG (mmCGM2 283-300)
9. 980: TGGAAGGAGGTGAAGCAAGG (Cea10 980-999)
10. DELB: CGGATGTACATGAAATCG (MHVR 478-495)

Southern Hybridization

After separation on 1 to 2% agarose gels depending on the experiment, DNA in the gel was denatured in 1.5 M NaCl, 0.5 M NaOH for 15 minutes and neutralized with 1 M Tris-HCl pH 7.4, 1.5 M NaCl for 15 minutes. The DNA was then transferred to nylon membranes (Schleicher and Schuell Co., Keene, NH) and UV-cross linked before baking at 80°C for 1 hour. The membrane was prehybridized with 5 X SSPE (1 X SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA pH 7.4), 5 X Denhart's solution (1 X Denhart's solution is 0.1 g Ficoll[type 400], 0.1 g polyvinylpyrolidone, 0.1 g bovine serum albumin in 10 ml water), 0.2% SDS (sodium dodecyl sulfate) at 42°C for at least 3 hours. Synthetic oligonucleotides were end-labelled with ^{32}P using T4 DNA kinase (Sambrook, et al., 1989). Probes were allowed to hybridize with DNA overnight at 42°C. The membranes were washed with 2X SSPE, 0.1% SDS at room temperature and 5X SSPE, 0.1% SDS at 50°C. The membranes were air dried and autoradiographed. In some experiments, DNA was digested

with appropriate restriction enzymes according to the manufacturer's recommendation before electrophoresis.

Subcloning of PCR Products and Construction of Plasmids for Expression of Cea10 and Cea10-MHVR Chimeric Glycoproteins

The PCR product made from a Cl 1 D cell RNA template using primer pair (1671-delB) was purified from an agarose gel as described above. The DNA was treated with T4 DNA polymerase to remove any overhanging nucleotides added by Taq, then ligated with the Sma I-digested Bluescript SK(+) plasmid (Stratagene, La Jolla,CA) to yield plasmid SK4N3. The Sac I and Ban II segment of a partial cDNA clone which contains the 5' untranslated region and signal peptide of Cea10 (Dr. N. Beauchemin, personal communication) were ligated to Sac I and Ban II double-digested SK4N3 to obtain plasmid SKLN3 which contains the entire 5'- untranslated sequence, leader sequence, and the whole N terminal domain of Cea10. Plasmid SKLN3D234 was derived by recombination of SKLN3 with plasmid Bam-Nsi in which the sequences between the BamH1 site and the end of N-terminal domain (AA H106) of MHVR was replaced by that of the Cea10 sequence so that plasmid SKLN3D234 has the signal peptide and N-terminal domain of Cea10 with Ig domains 2,3,4, the transmembrane domain and cytoplasmic tail derived from MHVR. Plasmid pUC-Cea10 containing the full length cDNA of Cea10 was obtained from Dr. Zimmermann's laboratory. The cDNA insert was

excised using Kpn I and EcoR I enzymes. This insert was ligated with Kpn I and EcoR I-digested pSP72 plasmid (Promega, Madison, WI) to yield the plasmid pSP72-Cea10 which can express Cea10 under T7 promoter.

Growth and Titration of Vaccinia VTF7-3 Virus in Cells

A recombinant vaccinia virus, VTF7-3 which can express a T7 RNA polymerase (Fuerst, et al., 1987) was a gift from Dr. B. Moss, National Institute of Health. BHK cells grown in T150 flasks were inoculated with 5 ml of VTF7-3 at multiplicity of infection (MOI) of approximately 1 PFU/cell. After virus adsorption at 37°C for 1 hour with occasional shaking, 25 ml of fresh medium was added to the cells. At 3 days post-infection, infected cells were harvested and centrifuged at 1500 rpm for 5 min. Cell pellets were resuspended in TE buffer (10mM Tris-HCl, pH 9, 1 mM EDTA), and frozen and thawed 3 times before storing at -70°C (approximately 1ml/flask).

For titration, vaccinia virus stocks were thawed at 37°C, mixed with 0.1 volume of 2.5 mg/ml trypsin in Hank's balanced salt solution (HBSS) (Gibco) (10 X Buffer contains NaCl 80 g/L, KCl 4 g/L, Na_2HPO_4 4.75 g/L, KH_2PO_4 0.6 g/L, Glucose 10 g/L) and incubated at 37°C for 30 min with vortexing every 10 minutes. Serial 10-fold dilutions of virus in EMEM medium without fetal bovine serum were prepared and inoculated onto Vero cells in 6 well plates for

1 hour at 37°C. The virus inocula were removed and the cells were washed twice with medium. Cells were refed with 2.5 ml fresh medium. After incubation for 2 days at 37°C, the medium were removed and the cell monolayers were washed twice with PBS. Cells were stained with 1 ml of 0.2% crystal violet in 20% ethanol at room temperature for 5 min. The crystal violet was removed, cells were air dried and the plaques were counted (Mackett,1991).

Expression of Recombinant Proteins Using VTF7-3 Virus

BHK-21 cells were inoculated with VTF7-3 virus at an MOI of 10. Three hours later, the cells were washed twice with medium. DNA transfection was performed on the cells using either lipofectin or lipofectAMINE as suggested by the manufacturer (Gibco). Transfected cells were incubated at 37°C for 12-14 hours. The medium was removed and the cells were washed once with PBS and lysed in 200-300 ul of lysis buffer (0.1 M NaCl, 10 mM EDTA, 10 mM Tris-HCl pH7.4, 1.0% NP40, 0.5% sodium deoxycholate). To detect Cea-related glycoproteins in the supernatant medium, the medium was collected and cells were removed by centrifugation. Proteins in the supernatant were concentrated 20 fold using a centricon 10 (Amicon, Beverly, MA) before loading onto acrylamide gels for electrophoresis.

Immunoblot and Virus Overlay Protein Blot Analysis

Protein samples were mixed with sample treatment buffer (STB)(4 X STM is 0.25 M Tris-HCl pH6.8, 8% sodium dodecyl sulfate (SDS), 20% β-mercaptoethanol, 40% glycerol and 0.005% bromophenol blue) and heated at 95°C for 3 minutes before being applied to SDS–polyacrylamide gel (8% or 10% polyacrylamide) separation. After electrophoresis, the gels were soaked for 15 minute in transfer buffer (25 mM Tris, 192 mM glycine [pH 8.3], 20% methanol). Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Immobilon P. Millipore Corp. Bedford, MA.) at 100 V for 2 hours as described by Towbin (1979), using a mini-trans-blot electrophoretic transfer cell (Bio-Rad, Richmond, CA). The membrane was treated overnight in 1 X B3 buffer (0.15 M NaCl, 0.05 M Tris hydrochloride, 1 mM EDTA, 0.05% Tween-20, 0.1% bovine serum albumin) with an additional 2% bovine serum albumin (BSA) to saturate all nonspecific protein binding sites. The membranes were then incubated for one hour at room temperature with 15 ml of either MAb–CC1 (diluted 1:80), polyclonal rabbit anti–serum 655 (diluted 1:500) preabsorbed with vaccinia virus-infected BHK cells, polyclonal rabbit anti-rat ectoATPase 669 (diluted 1:500) or rabbit anti–serum anti-Cea10 K (diluted 1:1000). After washing 5 times with 1 X B3 buffer, the membranes were either incubated with a 1:500 dilution of rabbit anti-mouse IgG antibody (for blots with MAb-CC1) and then with ^{125}I-

labeled staphylococcal protein A, 1 X 10^5 cpm/ml (Du Pont, NEN Research Products, Boston, MA) for one hour, or directly with ^{125}I-labeled staphylococcal protein A (for blots using rabbit anti—serum). The membranes were washed, dried and autoradiographed on XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) at -70°C.

For the virus overlay protein blot assay (VOPBA), membranes were incubated with 15 ml of supernatant medium from MHV-A59 infected 17 Cl 1 cells supplemented with 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.4) for one hour at room temperature. After washing with 1 X B3 buffer, blots were incubated with 15 ml of 1:200 diluted goat anti—serum directed against the spike glycoprotein of MHV-A59 (Boyle, et al., 1987), followed by ^{125}I-labeled staphylococcal protein A, and autoradiographed.

MAb-CC1 Blockade of MHV Receptor

Two types of MAb-CC1 blockade assay were used. In the ELISA assay format, Cl 1 D and F40 cells grown in 96 well plates were treated with supernatant medium from a hybridoma producing MAb-CC1, or with an isotype-matched control MAb to an irrelevant protein at different dilutions for one hour. MHV-A59 was adsorbed onto the cells at 37°C for one hour in the presence of the anti-receptor or control antibodies. The virus inocula were removed and the cells were washed twice with medium. At 13 hours post-inoculation (p.i.), the

cells were fixed with -20°C methanol for 10 minutes and air dried. To detect viral antigens in infected cells, fixed cells were soaked with 1 X B3 buffer containing 2% BSA for one hour at room temperature. Monoclonal antibody, JL anti-N, or an isotype-matched control MAb were added to each well at 1:2 dilution. After one hour of incubation at room temperature, antibodies were removed and the cells were washed 5 times with blocking buffer. Peroxidase-conjugated goat anti-mouse IgG $F(ab)_2$ (1:1000 dilution.) was added to each well. The relative levels of N protein in the wells were measured by adding 100 ul peroxidase substrate (KPI, Gaithersburg, MD) to each well. The reaction was stopped at the approximately 5 minutes by adding 50 ul of 1 M phosphoric acid. The optical density (OD) was read at 450 nm using Dynatech MR700 ELISA reader (Dynatech Lab., Chantilly, VA).

For immunofluorescence assays, the cells were pre-treated with MAb-CC1 hybridoma supernatant at a 1:2 dilution for 1 hour then challenged with MHV-A59 in the presence of MAb-CC1. After one hour adsorption, the virus inocula were removed. The cells were washed and refed with medium. Viral antigen in the cells was detected by immunofluorescence using polyclonal mouse anti—serum against MHV-A59.

MHV Superinfection of Vaccinia VTF7-3 Infected Cells

BHK-21 cells on coverslips were transfected with plasmid DNA by lipofectin or lipofectAMINE according to manufacturer's recommended protocols (Gibco). After 12-14 hours, cells were inoculated with vaccinia VTF7-3 at an MOI of 10 PFU/cell for one hour at 37°C. Virus was removed and cell culture medium supplemented with cytosine-1-b-D-arabinofuranoside (ara-C), final concentration of 40ug/ml, was put to cells for 3 hours to inhibit vaccinia DNA production. Cells were then challenged with MHV-A59 at MOI 5 PFU/cell. At 12 hours post-infection, the cells were washed with PBS and fixed with acetone at -20°C for 20 minutes. The samples were stored at -20°C until immunofluorescent labeling was performed.

Immunofluorescent Labeling of Viral Antigens or Membrane Protein

To detect antigens on the cell surface, the cells on coverslips were fixed with 2% paraformaldehyde. To detect intracellular virus antigen using anti—serum against MHV-A59, the cells were fixed with cold acetone. For labeling, cells were soaked in PBS with 2% normal goat serum (NGS). Mouse anti-MHV-A59 anti—serum (diluted 1:50) or rabbit anti-MHVR 655 anti—serum absorbed 3 times with vaccinia-infected BHK cells (diluted 1:50) was added to cells. After incubation at 4°C for 1 hour, the cells were washed 3 times

with PBS. The samples were then subjected to labelling with affinity-purified rhodamine-conjugated goat anti-rabbit antibody or goat anti-mouse antibody. After washing 3 times with PBS (containing 2% goat serum), the coverslips were mounted onto slides by Aqua-mount (Lerner Lab., Pittsburgh, PA) and examined using a Zeiss photomicroscope.

Concentration of Proteins from Conditioned Medium and Detection of Cea10 Protein by Rabbit Anti-Cea10 Peptide Anti—serum

Cl 1 D cells were grown in Opti-MEM serum-free medium (Gibco BRL, Gaithersburg, MD) for two days in 60 mm dishes. The supernatant medium was collected and cell debris was removed by centrifugation at 1000 rpm for 5 min at 4°C. The 4 ml supernatant was diluted with 6 ml water and concentrated with Centriprep-30 (Amicon, Beverly, MA) by centrifugation at 1500 X g for 15 min. The resulting approximately 2.5 ml of concentrate was diluted with an equal volume of water and spun again under the same conditions. The 1 ml of concentrate was further concentrated with a Centricon-30 by centrifugation at 3000 X g for 90 min. The final sample was concentrated for approximately 100 fold. As control, the same volume of Opti-MEM medium was treated as described above, and concentrated approximately 80 fold. To detect the protein, 5 ul (25 ug) concentrated cell conditioned supernatant or

7.5 ul concentrated Opti-MEM medium was loaded onto SDS-polyacrylamide gel. In some experiments, the Opti-MEM medium was concentrated using Centricon-10 (Amicon, Beverly, MA) and the same amount of protein (25 ug) from concentrated Opti-MEM medium as from concentrated cell conditioned supernatant was loaded onto the protein gels.

Preparation of Membrane Vesicles from Cl 1 D and F40 Cells

The Cl 1 D and F40 cells were grown in 150 cm^2 flasks. Cells were washed with PBS (Ca^{2+} and Mg^{2+} free) twice after removing the culture medium. PBS solution with 0.5mM EDTA (PBS-EDTA) (5 ml) was added onto cells for 15 min and dissociated cells were transferred to 50 ml tubes. After spinning at 1000 rpm for 5 min, the cells were washed with PBS once more and resuspended in cell disruption buffer (10 mM KPO_4, pH7.4, phenylmethyl-sulfonyl fluoride (PMSF), 0.1 mg/ml). Cells were put on ice for at least 5 min and then homogenized with a 15 ml Dounce homogenizer ('A' pestle) with 30 strokes. The homogenate was diluted with cell disruption buffer and spun at 1,000 X g for 5 min. The supernatant was collected and spun at 25,000 rpm in an SW28 rotor (Beckman) for 1 hour. The membrane pellet was suspended in PBS with 1% aprotinin and stored at -70°C. Protein concentration was determined by the Bradford method using a BSA standard.

Northern Hybridization

Northern hybridization was done according to the procedure described by Sambrook, et al., (1989). Briefly 25 ug of total RNA from BHK cells, Pan-2 cells, Cl 1 D and F40 cells were treated with 50% formamide before loading onto an 1.2% agarose gel. After separation, one lane was excised and stained with ethidium bromide to locate the 18S and 28S rRNA. The RNA in the other lanes was blotted onto a Nytran membrane and prehybridized overnight at 40°C using hybridization buffer (50% formamide, 5 X Denhart's solution, 6 X SSPE, 100 ug/ml tRNA). The MHVR cDNA probe was labeled with ^{32}P-dATP by nick-translation. The probe was separated from unincorporated nucleotide with a G-25 Sephadex column and allowed to hybridize with membrane at 40°C overnight. The membrane was washed with 2 X SSPE, 0.1% SDS at room temperature and then 0.1 X SSPE, 0.1% SDS at 55°C. The membrane was air dried and autoradiographed at -70°C on Kodak XAR-5 film.

Infectious Center Assay

The infectious center assay used to determine the number of cells infected with virus was conducted essentially as described by Lucas, (1977). Briefly, the L2, Cl 1 D and F40 cells were inoculated with MHV-A59 at an MOI of approximately 2 PFU/cell. Virus was allowed to adsorb for 1 hour at 37°C and then the cells were washed with PBS,

refed with fresh medium, and incubated at 37°C for 2 hours. The cells were then trypsinized and counted. Ten fold serial dilutions of cells were plated onto confluent L2 monolayers. Cells were incubated for 2 hours at 37°C and then overlaid with 0.95% Noble agar in MEM medium supplemented with 4% FBS, 2% PSF. The plaques were stained 2 days later and counted as described for plaque assay.

Detection of MHVR in Cells

The expression of MHVR in various cell lines was assessed by flow cytometry analysis. The cells were harvested by treating cells with PBS-EDTA for about 3 min followed by 30 sec of trypsinization. The cells were washed using the appropriate cell culture medium and counted. The cells were then transferred to labeling buffer (HBSS with 3% fetal bovine serum). MAb-CC1 or control MAb were added at a final antibody concentration of 40 ug/ml into 1 million cells in 100 ul of labeling buffer. After 30 minutes of incubation at 4°C, cells were washed twice with 2 ml of labeling buffer and phycoerythrin-coupled goat anti-mouse IgG_1 antibody (Boehringer Mannheim Biochemica) was added to the cells followed by incubation at 4°C for 30 minutes. The cells were washed twice and resuspended in 0.5 ml of labeling buffer. The fluorescence intensity on the cell surface was assessed by EPICS Elite cell sorter (Coulter Electronics, FL.).

FACS Analysis of Virus Nucleocapsid Antigen

A FACS analysis was developed to assess the percentage of cells infected at early time points of MHV infection. L2, Cl 1 D, and F40 cells were infected at an MOI of 2 PFU/cell. After removing the virus inoculum and washing the cells twice, the cells were refed with fresh medium. At 4 hours p.i., the cells were harvested, fixed with 2% paraformaldehyde and then permeabilized with 0.2% Triton X-100 in PBS (Ca^{2+} and Mg^{2+} free) at 4°C for 30 min. After extensive washing with HBSS with 3% FBS, the cells were subjected to FACS analysis as described for the detection of MHVR on the cells. MAb JL anti-N was used as the first antibody followed by phycoerythrin-coupled goat anti-mouse IgG_1 antibody (Boehringer Mannheim). The same sample was labelled with an irrelevant MAb as the control.

To detect the level of N protein in single infected Cl 1 D and F40 cells, cells were grown in flasks and infected with MHV-A59 at MOI of 10 PFU/cell. After virus inoculation, MAb-CC1 (1:5) was put into the cells to prevent cell fusion. The cells were harvested at 3, 5, or 8 hours p.i. and fixed with 2% paraformaldehyde at 4°C. The cells were permeabilized and the N protein in the infected cells was measured using FACS as described above. The level of N protein was expressed as the mean intensity of immunofluorescence of JL anti-N labelling.

Transfection of Cl 1 D and F40 Cells with MHVR Plasmid

The plasmid pCMVneoMHVR (gift from Dr. Dveksler, Uniformed Services University of Health Sciences) in which the MHVR cDNA was expressed under the control of CMV promoter and the neo gene by SV40 early promoter (Invitrogen Co.) was used to transfect Cl 1 D and F40 cells grown in 60 mm dishes. Cl 1 D cells were washed in Opti-MEM twice. One hundred ul Lipofectin (Gibco BRL) and 9 ug of plasmid pCMVneoMHVR were mixed in 600 ul Opti-MEM for 15 min and this mixture together with another 2.4 ml Opti-MEM were added onto the cells. Geneticin (G418 from Gibco) was added onto cells at a concentration of 800 ug/ml 2 days later. Single clones were picked and amplified. The cells were labelled with MAb-CC1 for detection of expression of MHVR as described before. Cells showing high levels of MHVR expression were sorted and one clone (called 9B1) was selected for further analysis. An independent cell clone (called 9A7) obtaining the G418 resistance, but not showing the increase of MHVR was selected as a control.

The transfection of F40 cells was done essentially the same as that of Cl 1 D cells, except that the concentration of G418 was increased to 1200 ug/ml. Five clones were picked and after FACS analysis to determine the level of MHVR expressed, two of them were used for virus infection.

Treatment of Cells with DbcAMP or 5-azacytidine

Cl 1 D cells were treated with 5'-azacytidine at a concentration of 5 uM, 10 uM, 20 uM for 60 hours and then challenged with MHV-A59 (MOI approximately 10 PFU/cell). Cells were fixed with acetone at 7 hours p.i. To see if MAb-CC1 protected the treated cells from MHV infection, MAb-CC1 (1:5 diluted) was added before and during MHV virus inoculation. To see the effect of dbcAMP on MHV infection of Cl 1 D cells, cells were treated with DbcAMP at 0.01 mM, 1 mM for 48 hours before virus infection. Infected Cl 1 D cells were fixed with acetone at 8 hours p.i. and stained with anti-MHV anti—serum followed by rhodamine conjugated goat anti-mouse antibody. The level of MHV infection was assessed by survey of randomly selected fields using immunofluorescence microscopy.

Determination of MHVR Expression in Cells Surviving the MHV-A59 Infection

L2 cells were inoculated with MHV-A59 at an MOI of approximately 5 PFU/cell. Twenty hours later, fused and dead cells were washed away with PBS. Half of the surviving cells (approximately 3% of the original cells) was harvested and fixed with paraformaldehyde and the level of MHVR expression was determined by FACS analysis using MAb-CC1. The other half of the surviving cells was incubated at 37°C for 3 days and then passaged once more. MHVR expression was

assessed 3 days later by FACS. At the same time, a small aliquot of the cells was put on coverslips for 10 hours and then fixed by acetone. The cells were labelled with anti-MHV anti-serum to detect of cells expressing viral antigen.

Electron Microscopy Study of MHV Infection of Cl 1 D and F40 Cells

Four cell lines were used in this study, L2, Cl 1 D, F40, MHVR-transfected Cl 1 D cell (9B1). The cells were either sham-inoculated or inoculated at an MOI of approximately 25 PFU/ml. The infected cells were harvested at 6 hours p.i. or 24 hours p.i., washed with PBS and fixed with 1% glutaraldehyde in a round-bottomed centrifuge tube for 15 min. After rinsing with PBS, the cells were treated with 1% OsO_4 for 30 min at room temperature. The sample were then dehydrated by passing through 70%, 80%, 95% ethanol, 15 min for each step. The samples were then dehydrated with 100% ethanol twice, 15 min each time, cut into pieces about 2 mm in size, and embedded in Epon at 60°C for 2 days. After thin sectioning, the cells were stained with lead and uranyl acetate (Hayat, 1989). The samples were observed using JEM-100CX electron microscope and photographed using Kodak electron microscopy film.

Preparation of Fatty Acids

Linolenic acid (18:3) and nonadecanoic acid (19:0) were

obtained from Nu-CheK (Elysian,MN). The 10 mM stock solutions were made by dissolving the fatty acids in 30 mM NaOH. The stock solutions were sealed tightly and stored in dark at -20°C.

Treatment of Cells with Fatty Acids

Cl 1 D or F40 cells were grown in 96 well plates in DMEM medium containing fatty acids at 100 uM in addition to 10% FBS and 2% PSF for 24 hours at 37°C. The cells were then inoculated with MHV-A59 10^6 PFU in 100 ul medium at 37°C for 1 hour. The virus inoculum was removed, and the cells were washed, and refed with fresh medium with or without fatty acids. The cells were fixed with methanol at 14 hours p.i. and the level of intracellular MHV N protein was detected using an ELISA assay as described above. To detect the effects of fatty acids on cellular susceptibility to MHV infection, F40 cells grown in 60 mm dishes were pre-treated with fatty acids for 24 hours then inoculated with MHV-A59 at 37°C for 1 hour. Virus inoculum was removed and cells were incubated at 37°C for 2 hours. The cells were harvested and diluted cells were seeded onto L2 monolayers. After 2 hours, the cells were then overlaid with 0.95% Noble agar in MEM medium and plaques were counted two days later after staining with 0.02% neutral red. In another assays for cellular susceptibility (plaque assay), F40 cells were treated with fatty acids or control medium for 24 hours and

then 10-fold serial dilutions of MHV-A59 virus were used to infect the treated F40 monolayers. Plaques were allowed to develop in F40 cells as described previously and counted two days later.

III. IDENTIFICATION OF CEA10 AND CHARACTERIZATION OF MHV RECEPTORS IN CELL LINES

Introduction

Most murine cell lines are susceptible to MHV-A59 infection. At the time I began my studies on the role of MHV receptor in MHV infection of cell lines, it was not known whether there was more than one murine CEA gene in the inbred mouse genome. If there were more than one gene, I wanted to know if MHV could use multiple gene products as its receptors, either isoforms encoded by spliced transcripts from the same gene, or related proteins encoded by different genes.

Our laboratory previously found that the virus yield and virus-induced cell fusion in MHV-A59 infected F40 cells were much higher than those in Cl 1 D cells (F40 and Cl 1 D cells differed in other aspects also, as will be discussed in the next chapter). It was not known which molecule(s) were used as an MHV receptor in these cells and whether different cells expressed different MHV receptors. As the first step toward understanding the mechanism underlying the differences of MHV infection between Cl 1 D and F40 cells, I characterized the MHV receptor used in the Cl 1 D and F40 cell lines. I found that the mRNA of MHVR was expressed in both cell lines. Besides MHVR, I discovered that these cells expressed an additional, so far not described cDNA

sequence (now named Cea10), which is highly homologous to the known MHV receptor (MHVR) both at the nucleotide sequence and at the predicted amino acid sequence level.

This section describes experiments to characterize the new CEA-related gene and to determine whether it could serve as an MHV receptor. A recombinant chimeric protein with the N-domain of Cea10 replacing that of MHVR did not bind either MHV-A59 virus in viral overlay protein blot assay or the anti-MHVR monoclonal antibody MAb-CC1, which protected the Cl 1 D and F40 cells from MHV infection. Transfection of the DNA encoding the chimeric protein into MHV-resistant hamster cells did not confer MHV-A59 susceptibility.

In collaboration with Dr. W. Zimmermann's laboratory, Germany, we characterized the Cea10 protein expressed in the vaccinia T7 system as a 37 kDa glycoprotein. A soluble protein with a molecular size of about 35 kDa in Cl 1 D cells was detected by anti-Cea10 peptide antibody. Thus, we showed for the first time that a soluble murine CEA-related glycoprotein was coexpressed with MHVR in Cl 1 D and F40 cells, but that only MHVR served as a functional receptor for MHV-A59 in these cells.

Results

Detection of MHVR by RT-PCR

To learn if Cl 1 D and F40 cells used MHVR-related glycoproteins as MHV-A59 receptors, polyclonal anti-MHVR

antibody 655 and monoclonal anti-MHVR MAb-CC1 were used to detect MHVR proteins in the cells. Immunoblots of proteins from membrane preparations of both Cl 1 D and F40 cell lines and immunofluorescent labelling of fixed cells failed to detect the expression of MHVR proteins in both cell lines. The Northern hybridization also failed to detect the mRNA of MHVR in both cells (Fig. 4), although the mRNA for MHVR was detectable in BHK cells transfected with MHVR cDNA (Fig. 4). These observations suggested that either the level of MHVR expression was very low in these cells or that these cell lines may use other molecules as MHV receptors. The more sensitive method, reverse transcription-polymerase chain reaction (RT-PCR), was therefore used to detect MHVR mRNA. The mRNAs from both Cl 1 D and F40 cells were reverse transcribed with random primers and the resulting cDNAs were amplified with primers 1671 and delB. Primer 1671 corresponds to nucleotides 146 to 160, and delB corresponds to nucleotides 478 to 445 in the MHVR cDNA clone. As shown in Fig. 5A, this set of primers yielded a product of approximately 350 bp, which was the size predicted from the sequence of the MHVR cDNA clone. The same product was obtained using RT-PCR of F40 mRNA (data not shown). The products were hybridized with the oligomer 865 or 685 located in the middle of the N-terminal domain of MHVR (Fig. 5B).

Concomitantly with these experiments, the MHVR homolog,

Fig. 4: Northern blot to detect MHVR mRNA in Cl 1 D and F40 cells. Twenty five ug of total RNA from each cell line was loaded on each lane (except the last lane which was 50 ug) of a formamide agarose gel (1%). The RNAs were separated by electrophoresis and then transferred to a nytran membrane. MHVR cDNA labelled with ^{32}P-dATP was used as the probe. A band of approximately 2.7Kb was detected in MHVR-transfected BHK cells (Pan-2 cells), but not in BHK-21, Cl 1 D or F40 cells. The positions of 18S and 28S RNA are indicated.

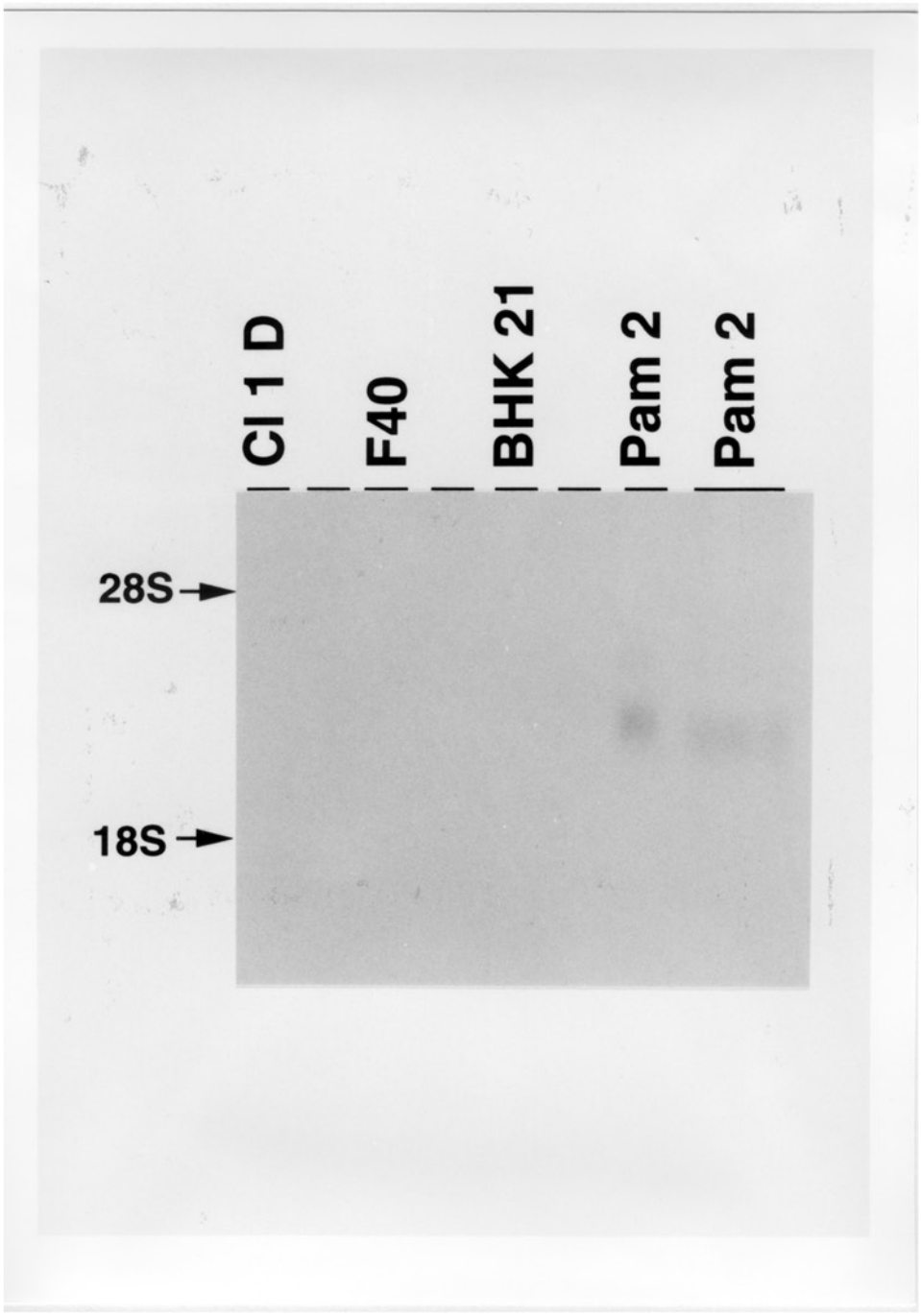
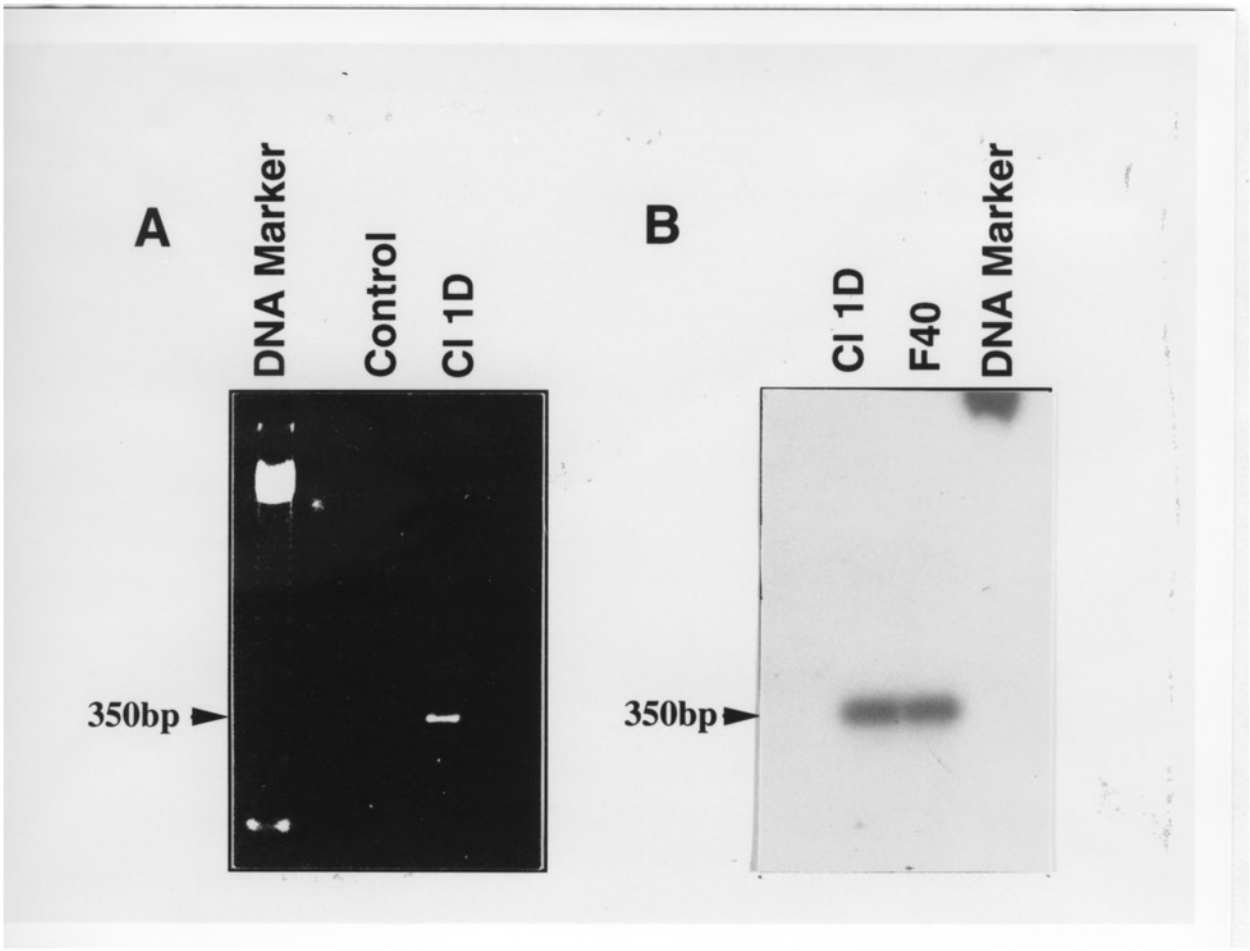

Cl 1 D
F40
BHK 21
Pam 2
Pam 2
28S
18S

Fig. 5. RT-PCR amplification and detection of MHVR mRNA in Cl 1 D and F40 cells. A. One ug of poly(A) RNA from Cl 1 D cells was reverse transcribed into cDNA. Two ul of each cDNA reaction mixture was used as the template for the PCR reaction using primers 1671 and delB. Ten ul of each PCR sample was electrophoresed on a 2% agarose gel. A band of about 350 bp was detected in Cl 1 D cells; and in F40 cells (data not shown). The control lane was the sample from a PCR reaction without a cDNA template. B. The 1671-delB RT-PCR products from Cl 1 D and F40 cells were separated on 2% agarose gels, transferred to nytran and membrane, hybridized with ^{32}P-labelled oligomer 865, which is located in the N-terminal domain of MHVR. The 350 bp product was detected in PCR products of Cl 1 D and F40 mRNAs.


A
DNA Marker
Control
Cl 1D
350bp
B
Cl 1D
F40
DNA Marker
350bp

mmCGM2 (now called Bgp1^{b}) from SJL/J mice was cloned (Yokomori and Lai, 1992b; Dveksler, et al., 1993). The mmCGM2 protein isoforms were found to be highly homologous to those of MHVR. Even within the N-terminal domain, where most of the differences between different members of the CEA family occur, mmCGM2 and MHVR share about 70% identity at the amino acid level (Dveksler, et al., 1993a). To determine whether the RT-PCR products from Cl 1 D and F40 cells were MHVR or mmCGM2, I hybridized the PCR products with probes designed to be specific for either MHVR or mmCGM2. Specific hybridization with the MHVR-specific probe (oligomer GCS1) was detected in the 350 bp PCR products from both Cl 1 D and F40 cells (Fig. 6A), indicating that MHVR was expressed in these cell lines. However, under the washing and hybridization conditions of this experiment, the oligomer 1295 probe was not specific for mmCGM2 (Fig. 6B), but recognized both MHVR and mmCGM2 standards and the 350 bp PCR products from Cl 1 D and F40 cells.

Discovery of A New CEA-related Glycoprotein, Cea10

The sequences of the primer 1671-delB PCR products from Cl 1 D and F40 cells were determined and found to be identical to each other. Surprisingly, this sequence was neither that of the N-terminal domain of MHVR nor of mmCGM2. The new sequence, however, was highly homologous to both MHVR and mmCGM2 (Fig. 7). This new sequence, now called

Fig. 6. Hybridization of the 1671-delB RT-PCR products from Cl 1 D and F40 cells with probes for MHVR or mmCGM2. Ten ul of each PCR sample and 0.75 ug of plasmid DNA containing MHVR or mmCGM2 cDNA were loaded on 2% agarose gels in duplicate. After transfer to a membrane, the DNA was hybridized with end-labelled oligomers specific for MHVR (primer GCS1, A) or mmCGM2 (primer 1295, B). After hybridization, the membranes were washed with 2 X SSC, 0.1% SDS at room temperature and then washed with tetramethylammonium chloride at the Tm (melting temperature) of each oligomer.

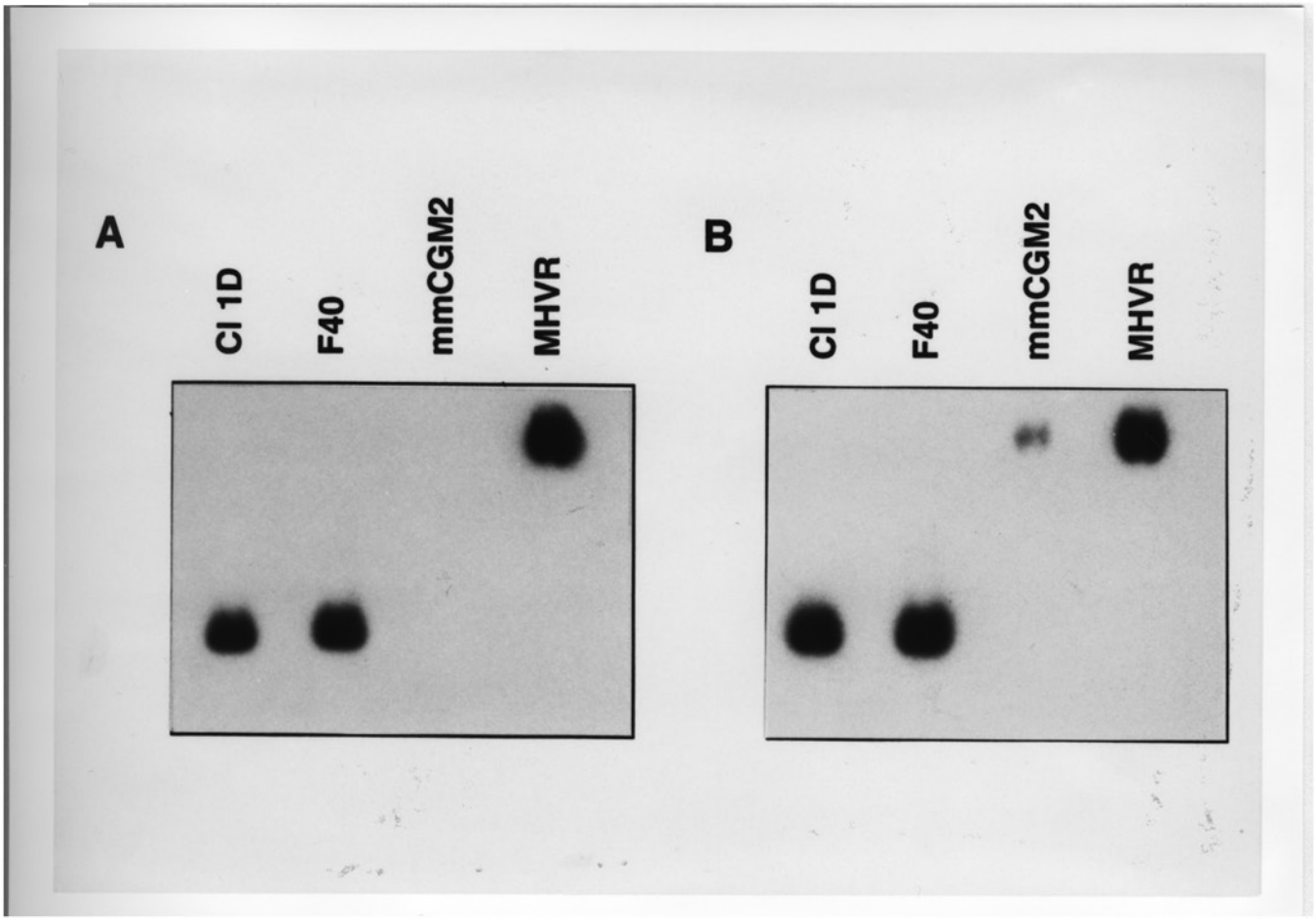
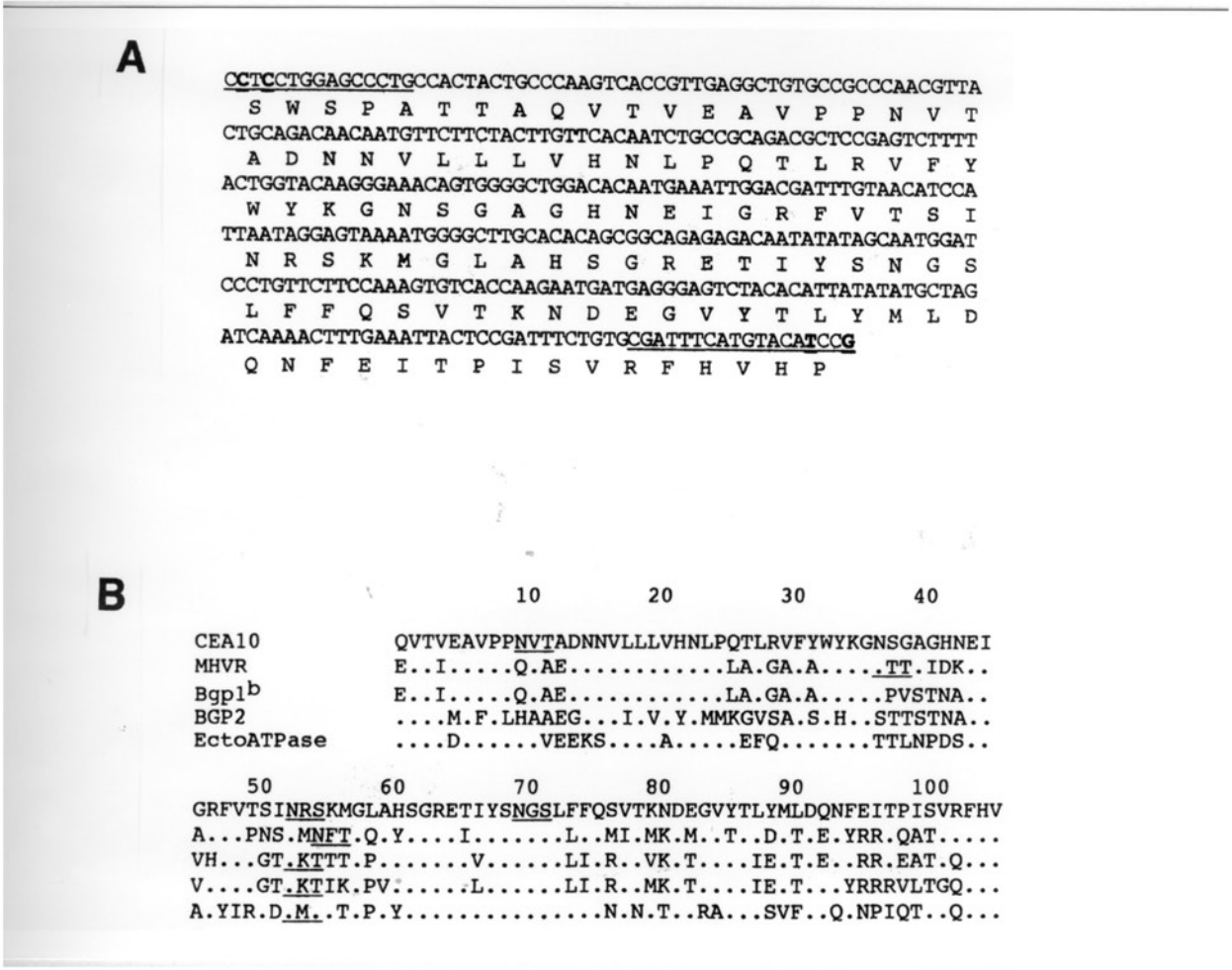

A
Cl 1D
F40
mmCGM2
MHVR
B
Cl 1D
F40
mmCGM2
MHVR

Fig. 7. The sequence of the RT-PCR product of primer 1671-delB from Cl 1 D cells aligned with the sequences of the N-terminal domain of other CEA-related proteins. A. The nucleotide sequence and predicted amino acid sequence of 1671-delB PCR product. The sense primer (1671) and antisense primer (delB) are underlined. The nucleotides in primers that differ from sequence of Cea10 are presented in bold. B. Amino acid sequence alignment of N domain of Cea10 with other CEA-related proteins. The potential glycosylation sites are underlined. The residues identical to those of Cea10 were represented as dots. The N domain of Cea10 showed 61% identity to that of MHVR, Bgp1^{b}(mmCGM2) or rat-ectoATPase, but only 48% identity to that of BGP2.

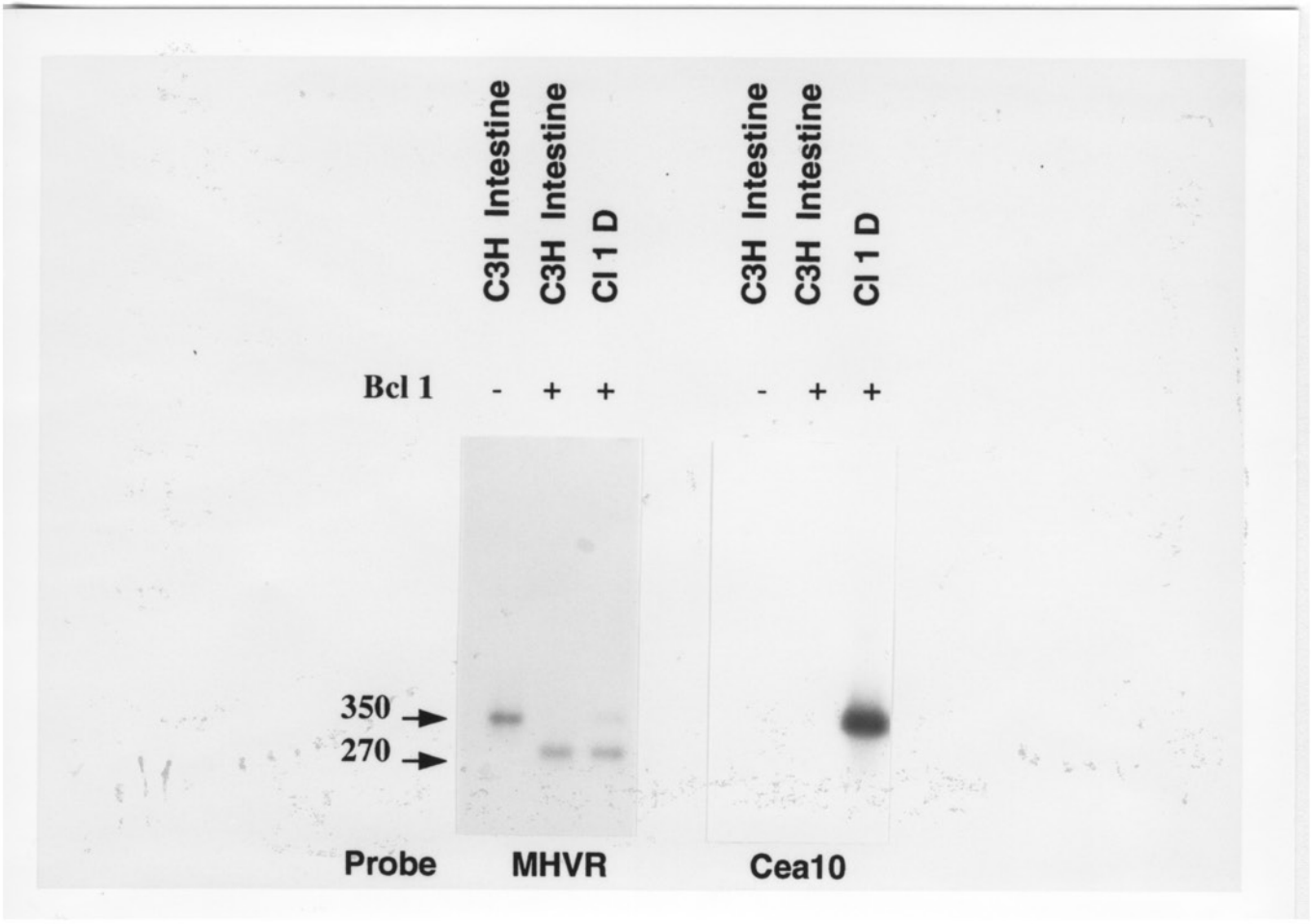

A
CCTCCTGGAGCCCTGCCACTACTGCCCAAGTCACCGTTGAGGCTGTGCCGCCCAACGTTA
S W S P A T T A Q V T V E A V P P N V T
CTGCAGACAACAATGTTCTTCTACTTGTTCACAATCTGCCGCAGACGCTCCGAGTCTTTT
A D N N V L L L V H N L P Q T L R V F Y
ACTGGTACAAGGGAAACAGTGGGGCTGGACACAATGAAATTGGACGATTTGTAACATCCA
W Y K G N S G A G H N E I G R F V T S I
TTAATAGGAGTAAAATGGGGCTTGCACACAGCGGCAGAGAGACAATATATAGCAATGGAT
N R S K M G L A H S G R E T I Y S N G S
CCCTGTTCTTCCAAAGTGTCACCAAGAATGATGAGGGAGTCTACACATTATATATGCTAG
L F F Q S V T K N D E G V Y T L Y M L D
ATCAAAACTTTGAAATTACTCCGATTTCTGTGCGATTTCATGTACATCCG
Q N F E I T P I S V R F H V H P
B
10 20 30 40
CEA10 QVTVEAVPPNVTADNNVLLLVHNLPQTLRVFYWYKGNSGAGHNEI
MHVR E..I.....Q.AE.....LA.GA.A....TT.IDK..
Bgp1b E..I.....Q.AE.....LA.GA.A.....PVSTNA..
BGP2 ....M.F.LHAAEG...I.V.Y.MMKGVSA.S.H..STTSTNA..
EctoATPase ....D.....VEEKS....A.....EFQ.....TTLNPDS..
50 60 70 80 90 100
GRFVTSINRSKMGLAHSGRETIYSNGSLFFQSVTKNDEGVYTLYMLDQNFEITPISVRFHV
A...PNS.MNFT.Q.Y....I.....L..MI.MK.M..T..D.T.E.YRR.QAT.....
VH...GT.KTTT.P.....V.....LI.R..VK.T....IE.T.E..RR.EAT.Q...
V....GT.KTIK.PV.....L.....LI.R..MK.T....IE.T...YRRRVLTGQ...
A.YIR.D.M..T.P.Y.....N.N.T..RA...SVF..Q.NPIQT..Q...

Cea10, predicted an ORF of 116 amino acids if the primer sequences were included. The predicted amino acid sequences are 60% identical to that of either MHVR or mmCGM2. Cea10 had strong homology (about 85%) to the N-terminal domain of a partial cDNA clone called mmCGM3, originally cloned from CD1 mouse colon (Beauchemin, et al., 1989). After our sequence was obtained, a sequencing error in the partial mmCGM3 clone was corrected and it was found to be identical to Cea10.

Coexpression of MHVR and Cea10

Since our hybridization experiment detected MHVR in the 1671-delB PCR products from both cell lines, I investigated whether the PCR products were a mixture of MHVR and Cea10. Comparison of sequences of MHVR and Cea10 indicated that there is a unique Bcl I site in the nucleotide sequence of the N-terminal domain of MHVR, but not in that of Cea10. Fig. 8 shows that after digestion of the PCR product from Cl 1 D cells with Bcl 1, a new band was generated and it hybridized with MHVR specific probe. The same material still shows the 350 bp band after hybridization with a Cea10 specific probe (Cea10). This probe did not hybridize with MHVR. Interestingly, PCR amplification of transcripts from adult C3H mouse liver or intestine with the same primers did not show the expression of Cea10, suggesting that in these tissues more MHVR than Cea10 mRNA is expressed. This result

Fig. 8. Co-expression of MHVR and Cea10 in Cl 1 D cells. The RT-PCR products of primer 1671-delB from Cl 1 D cells or C3H mouse small intestine were digested with the restriction enzyme Bcl I. The digested and undigested PCR products were analyzed on 2% agarose gels in duplicate. After transferring to a membrane, one panel was hybridized with an MHVR-specific probe, oligomer GCS2. The other was hybridized with a Cea10 specific probe, oligomer Cea10. The Bcl I site is present only in the MHVR sequence and the cleavage generates a 270bp band. The RT-PCR products from mouse intestine contained only MHVR, while PCR products from Cl 1 D cells contained both MHVR and Cea10. The same results were obtained using samples from F40 cells (data not shown).


C3H Intestine
C3H Intestine
Cl 1 D
C3H Intestine
C3H Intestine
Cl 1 D
Bcl 1
-
+
+
-
+
+
350
270
Probe
MHVR
Cea10

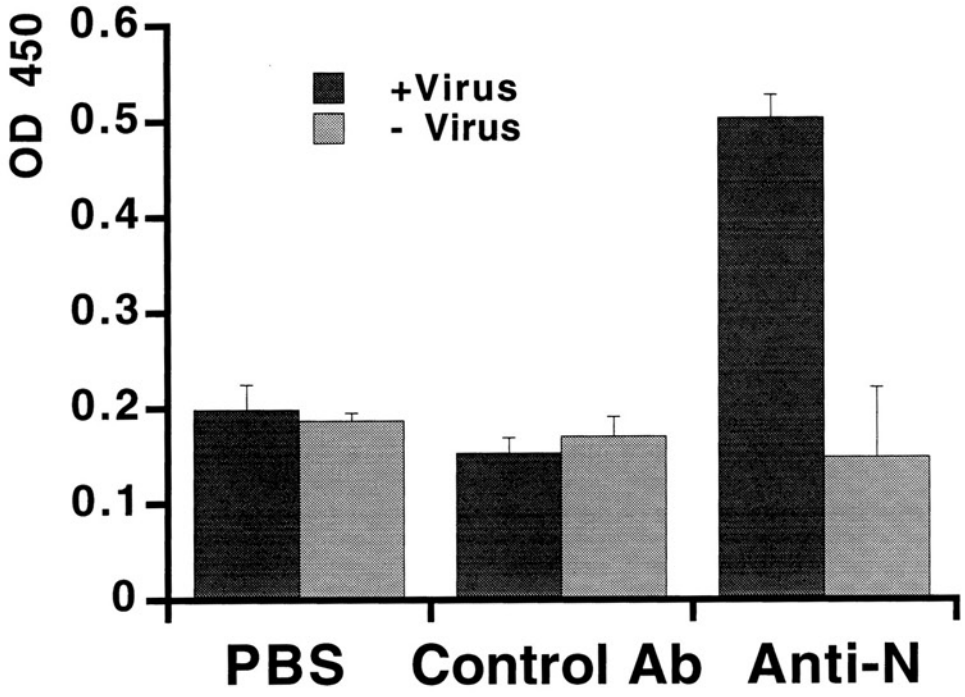
was later confirmed by Zimmermann's and Beauchemin's laboratories (Keck, et al., 1995).

MHV Receptor Blockade of Cl 1 D and F40 Cells with MAb-CC1

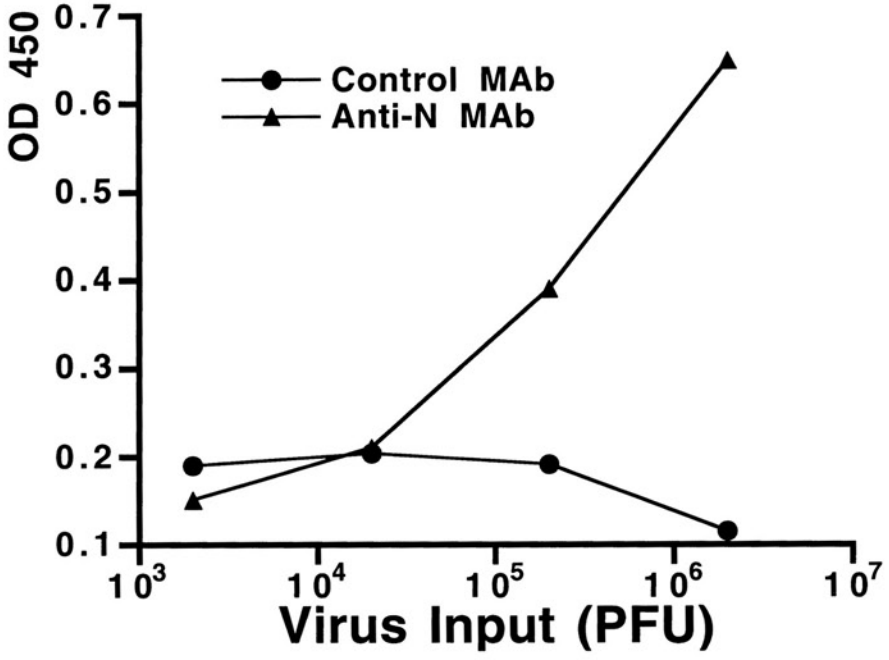
To learn which molecules were used as MHV receptor(s) in Cl 1 D and F40 cells, I performed a MAb-CC1 receptor blockade assay on both cell lines. Anti-MHVR MAb-CC1 binds to the N-terminal domain of MHVR and protects several murine cell lines from MHV-A59 infection (Dveksler, et al., 1993b). In contrast, MAb-CC1 does not bind to mmCGM2 glycoproteins. The cells were treated with MAb-CC1 at different dilutions before inoculation with MHV-A59. At 13 hours p.i., the cells were fixed and the viral nucleocapsid protein (N) in the cytoplasm of infected cells was detected using a monoclonal antibody against protein N by an ELISA assay. As shown in Fig. 9, anti-nucleocapsid antibody detected virus antigen in MHV-infected F40 cells, but only showed background signal in mock-infected cells. A control antibody showed only background signal, demonstrating the specificity of the JL anti-N antibody used in this assay. A similar result was obtained in Cl 1 D cells (data not shown). The signal is correlated with the level of MHV inoculation in F40 cells (Fig. 9). Therefore, this ELISA assay was used to compare the level of MHV infection in the Cl 1 D and F40 cells, and to see if MAb-CC1 protected Cl 1 D and F40 cells from MHV-A59 infection. The control antibody

Fig. 9. The specificity and dose-dependence of an ELISA assay to detect the nucleocapsid protein in MHV-A59 infected cells. To show the specificity of this assay, F40 cells grown in 96 well plates were inoculated with MHV-A59 at a MOI of 10 PFU/cell. After 1 hours adsorption at 37°C, the virus inoculum was washed away and the cells were refed with fresh medium. The cells were fixed with cold methanol at 13 hours p.i. and the nucleocapsid protein (N) in infected cells was detected using an ELISA with a MAb against N protein. An isotype matched MAb was used as the control. The data shown are the means of triplicate readings from three samples and standard deviation. To show the dose-dependence of the assay upon the virus infection level, the confluent F40 monolayers in 96 well plates were inoculated with different amounts of MHV-A59. The cells were then fixed and the level of N protein in the cells were determined as described.

Specificity of Anti-N Assay



Dose-dependence of Anti-N Assay



did not protect cells from MHV-A59 infection, but the MAb-CC1 pre-treatment completely protected cells from infection, and no N antigen above background levels could be detected (Fig. 10). The immunofluorescent labelling with JL anti-N antibody in MAb-CC1 pre-treated Cl 1 D and F40 cells failed to show MHV antigen positive cells (data not shown), confirming the results from the ELISA assay . Thus, the receptor molecules in both Cl 1 D and F40 cells can be blocked by MAb-CC1, which recognizes only MHVR. This observation suggested that MHVR is expressed in these cell lines and serves as the sole functional receptor in them. Interestingly, the level of MHV N protein expressed in F40 cells was much higher than that in Cl 1 D cells. I will discuss this observation in chapter V of this dissertation.

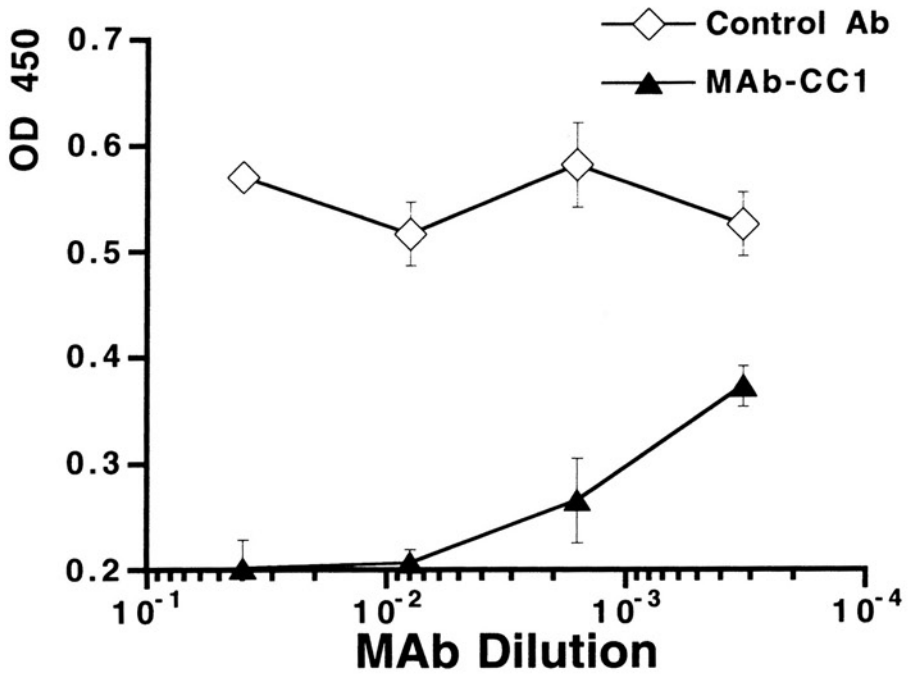
Reaction of Cea10-MHVR Chimeric Protein with Antibodies

To determine whether MAb-CC1 or virus could bind to the Cea10 glycoprotein as well as to MHVR, the N-terminal domain of Cea10 was cloned from PCR products from Cl 1 D cells and a chimeric construct was made in which the N-terminal domain of MHVR was replaced with that of Cea10 as detailed in Fig. 11.

The chimeric protein was expressed from pSKLN3D234 in BHK cells using the vaccinia VTF7-3 virus. BHK cells were first infected with VTF7-3 virus which expresses a T7 RNA polymerase. The infected cells were then transfected with a

Fig. 10. Anti-MHVR MAb-CC1 protected Cl 1D and F40 cells from MHV-A59 infection. Cells grown in 96 well plates were pre-treated with MAb-CC1 or a control MAb at different dilutions for 1 hour. The cells were then inoculated with MHV-A59 at an MOI of 10 in the presence of antibody. After 1 hour adsorption at 37°C, cells were washed and refed with fresh medium. At 13 hours p.i., the cells were fixed and the amounts of N protein in infected cells was determined using MAb against N protein. The data shown are the means of triplicate readings from three samples and standard deviation.

MAb-CC1 Protection of F40 Cells



MAb-CC1 Protection of Cl 1 D Cells

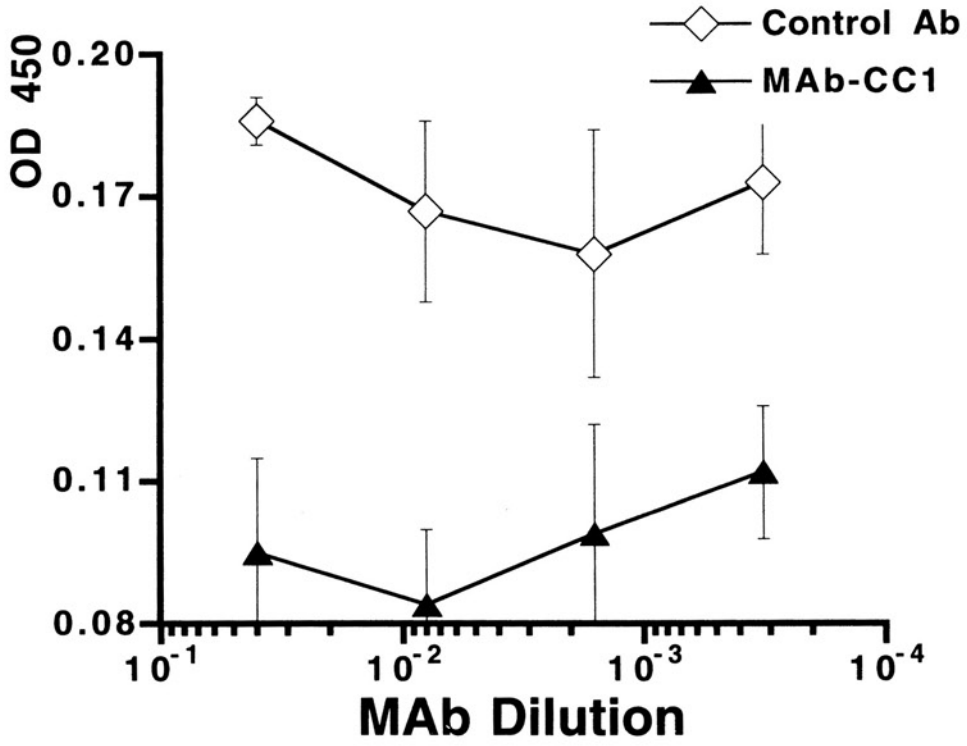
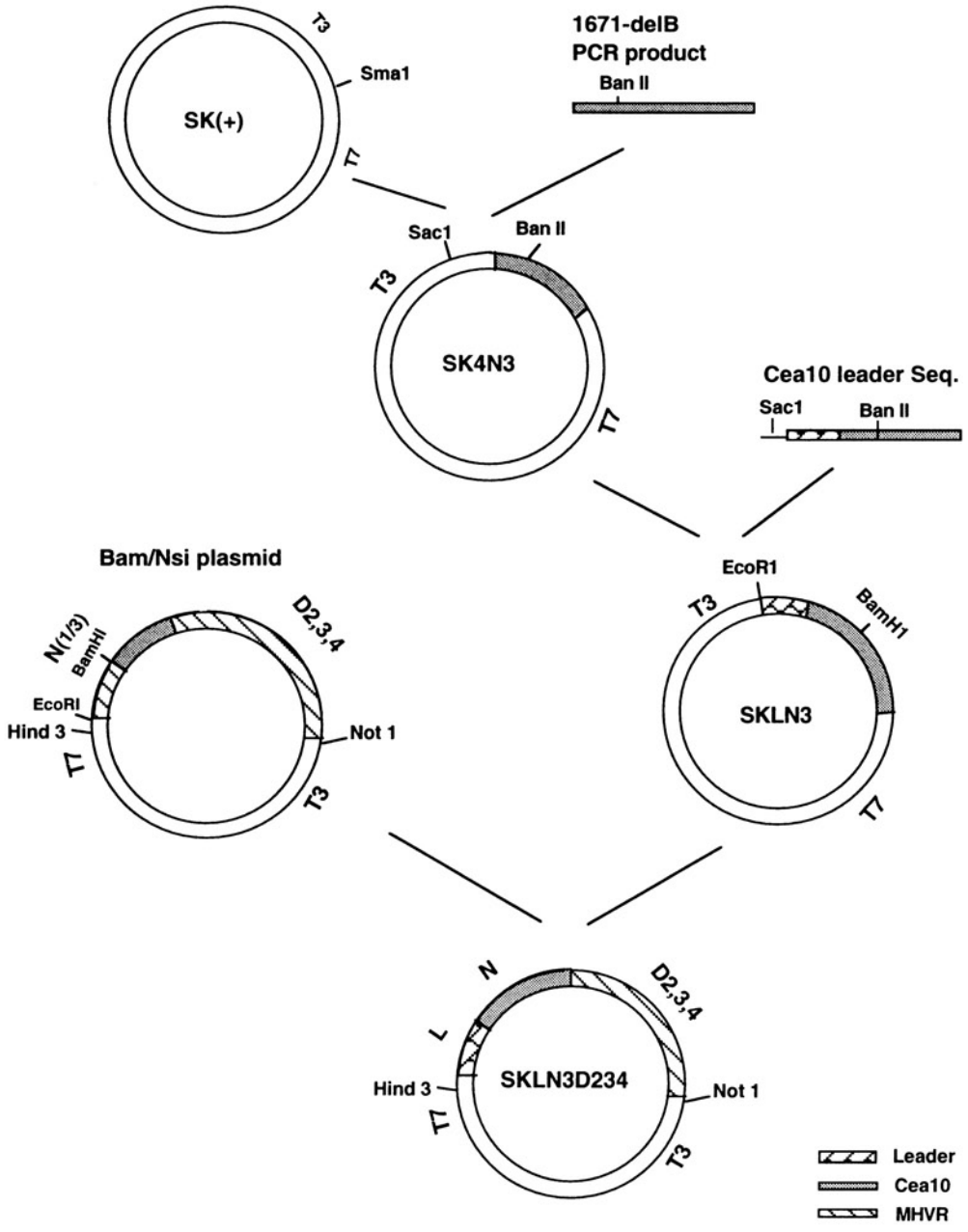


Fig. 11. Construction of the Cea10-MHVR chimera for analysis of virus binding, antibody binding, and MHV receptor activities of the N domain of Cea10. The 1671-delB PCR product from Cl 1 D cells was subcloned into bluescript plasmid to yield SK4N3. A partial cDNA clone of Cea10 from Dr. Beauchemin's lab was used to obtain Sac I and Ban II fragment which contains the 5'-untranslated sequence, leader sequence and part of the N domain sequence. This fragment was used to replace the Sac I-Ban II portion of plasmid SK4N3 to obtain the plasmid SKLN3. The plasmid Bam-Nsi made by Dr. Dveksler, has the portion of N domain of MHVR from BamH I site to the end of N domain replaced with that of Cea10. The EcoR I-BamH I fragment of the SKLN3 plasmid was used to replace the corresponding fragment in the Bam-Nsi plasmid to obtain plasmid pSKLN3D234. In this construct, the leader peptide and N domain of MHVR were replaced with that from Cea10.



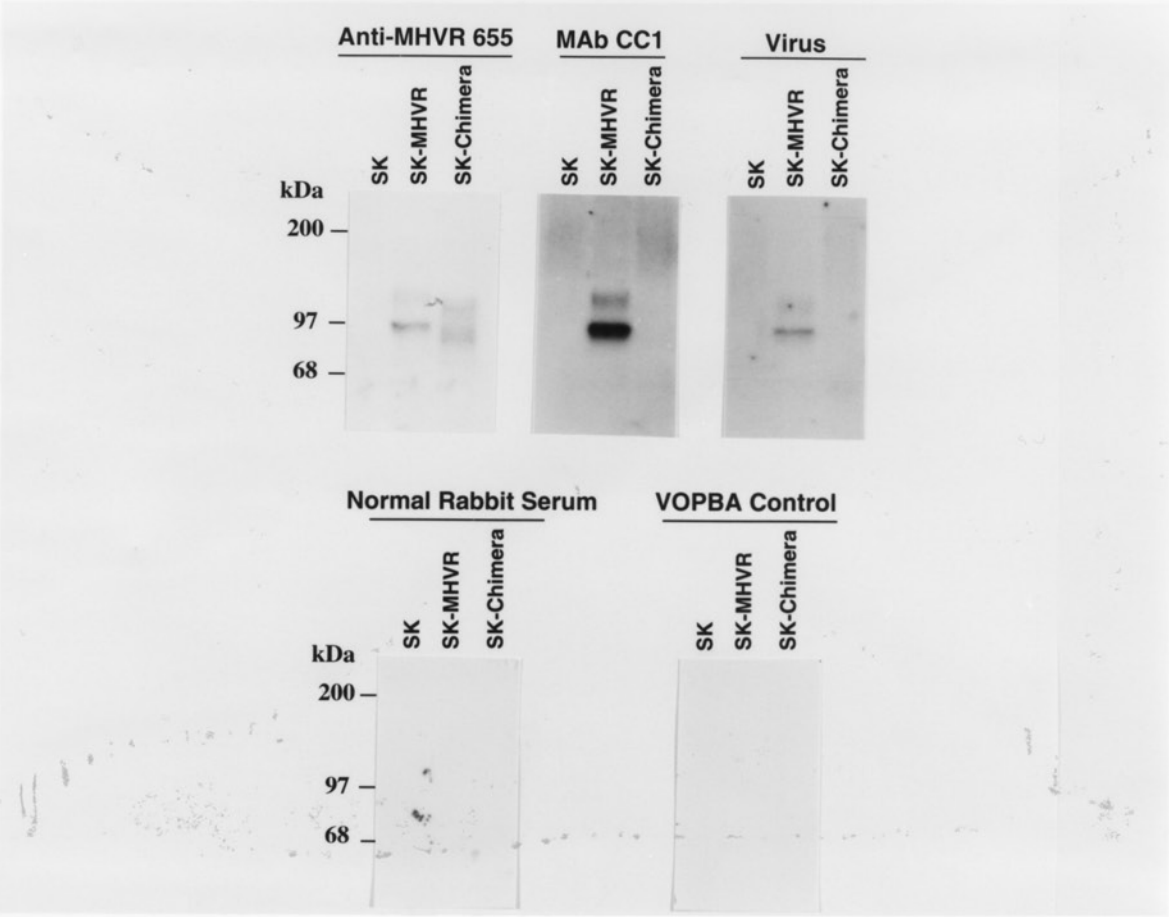
Cloning of Cea10 N-domain into MHVR

plasmid that had either MHVR cDNA or Cea10-MHVR chimera as the insert under the control of the T7 promoter. The plasmid with no insert served as the control. The proteins expressed from each plasmid insert were harvested 12 to 14 hours after DNA transfection. The expression of the chimeric protein as well as the MHVR protein was detected by immunoblots using polyclonal anti-MHVR antibody 655 (Fig. 12). The double band detected in MHVR and chimeric proteins expressed may result from heterogeneity of glycosylation of the recombinant proteins and is very common in protein samples using the vaccinia expression system (Bernhardt, et al., 1994). In contrast to anti-serum 655, MAb-CC1 did not recognize the chimeric protein (Fig. 12). Thus, MAb-CC1 is specific for an epitope present on MHVR, but not on Cea10. To determine if MHV-A59 virions could bind to Cea10 protein, a virus overlay protein blot assay (VOPBA) was used. This assay has been used to show the virus binding activity of MHVR (Fig. 12). Analysis of the Cea10-MHVR chimeric protein by this VOPBA assay also showed that it did not bind MHV-A59 virus (Fig. 12).

Analysis of Virus Receptor Activity of Cea10 Protein

In the process of analyzing the receptor activity of the N-terminal domain of Cea10 protein, work by Dr. Dveksler showed that mmCGM2 cannot be detected by MAb-CC1 in immunoblots and that it did not show virus binding activity

Fig. 12. Analysis of Cea10-MHVR chimeric protein using antibodies: BHK-21 cells grown in 60 mm dishes were infected with VTF7-3 at a MOI of 10. At 3 hours p.i., the cells were transfected with 10 ug of MHVR-expressing SK-MHVR, chimeric protein-expressing SK-Chimera, or control SK plasmid DNA. The cells were lysed in 300 ul buffer at 14 hours after transfection. Twenty five ul sample (approximately $1X10^5$ cells) was loaded to each lane on 8% SDS acrylamide gel. The blots were detected by antibodies as indicated or MHV virus followed with antibody against MHV spike protein (VOPBA). For control, the blot was stripped with buffers containing 2% SDS, 100 mM mercaptoethanol, 62.5 mM Tris-HCl, pH 6.7 at 50°C for 30 min. The membrane was then reblotted with normal rabbit serum or antibody against viral S protein. The molecular size markers are indicated at left side.

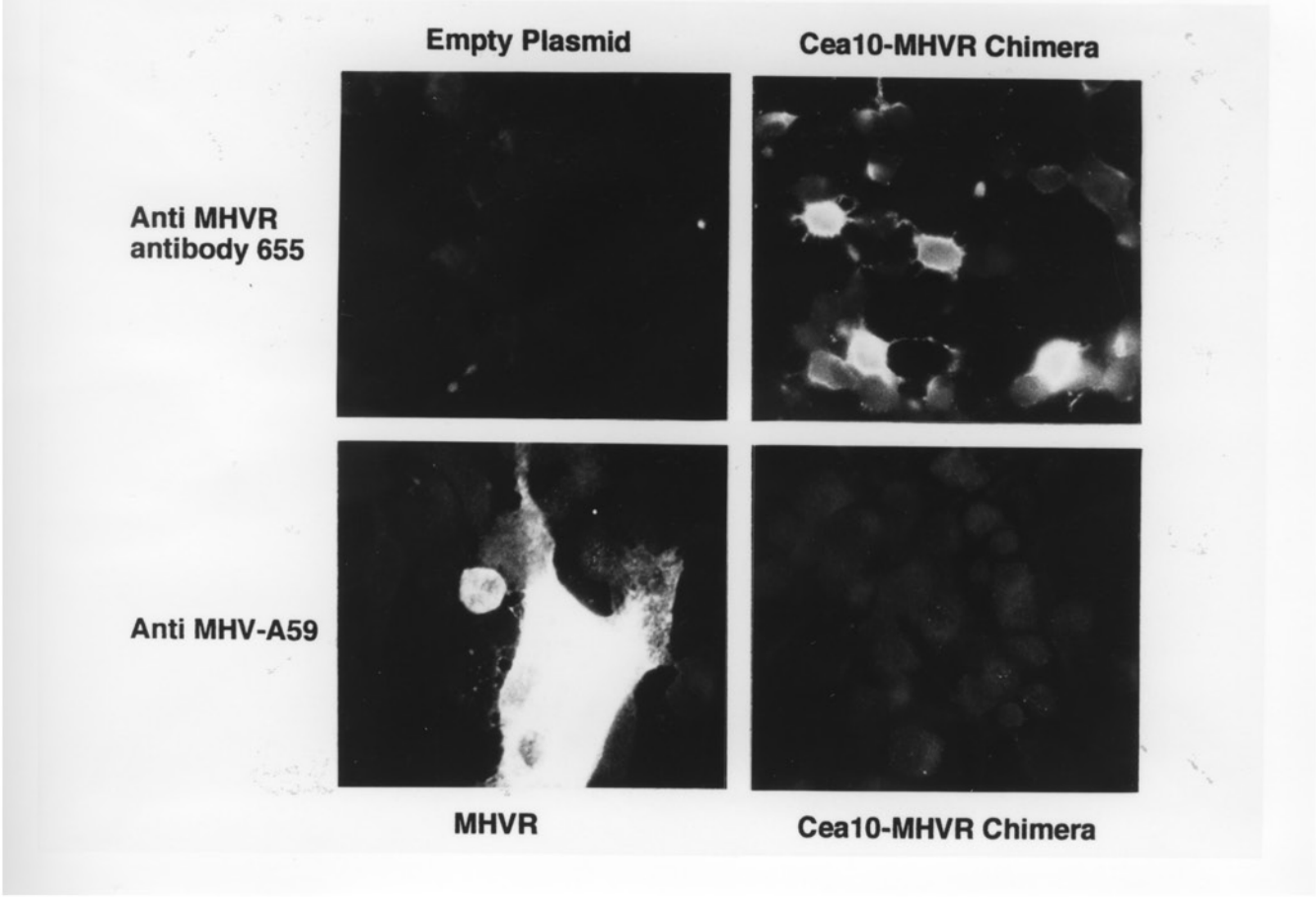

Anti-MHVR 655
MAb CC1
Virus
SK
SK-MHVR
SK-Chimera
kDa
200
97
68
Normal Rabbit Serum
VOPBA Control

in VOPBA assay, yet mmCGM2 protein can confer MHV susceptibility when expressed in virus-resistant BHK cells (Williams, et al., 1990; Dveksler, et al., 1993b). To determine if the Cea10-MHVR chimeric glycoprotein can also serve as an MHV receptor, the chimeric protein was expressed in BHK cells using VTF7-3 virus. The cells were then challenged with MHV-A59. Expression of the chimeric protein on the surface of transfected BHK cells was detected by immunofluorescence with rabbit polyclonal anti-MHVR anti-serum 655 (Fig 13). Although the expression of MHVR protein on BHK cells made the cells susceptible to MHV infection, BHK cells expressing the Cea10-MHVR chimeric protein remained resistant to virus infection (Fig. 13). This result was consistent with Dr. Dveksler's result indicating that the N domain of MHVR is the virus binding site (Dveksler, et al., 1993). The data strongly suggested that MHVR is the only functional receptor in the Cl 1 D and F40 cells, even though they express both MHVR and Cea10.

Western Blot Analysis of Cea10 Protein

A full length cDNA clone of Cea10 was obtained from a cDNA library of CD-1 mouse colon (Keck, et al., 1995). The sequence of this 1.0 Kb cDNA clone predicted a protein of 265 amino acids with 4 potential N-linked glycosylation sites and no apparent transmembrane sequences (Fig. 14). This clone had one nucleotide difference in the N-terminal

Fig. 13. Expression of Cea10-MHVR chimeric protein on the BHK cell surface and analysis of its MHV receptor activity. Recombinant MHVR and Cea10-chimeric proteins were expressed using the VTF7-3 vaccinia virus in BHK cells. To detect the surface expression of proteins, the cells were fixed with paraformaldehyde and stained with anti-MHVR antibody 655 followed with rhodamine-coupled goat anti-rabbit antibody. To detect the virus antigens in infected cells, BHK cells transfected with either the pSK-MHVR plasmid or the pSK-chimera (pSKLN3D234) plasmid were challenged with MHV-A59. At 13 hours p.i., the cells were fixed with acetone and virus antigens in cells was detected using anti-MHV-A59 anti—serum followed by rhodamine-coupled goat anti-mouse antibody. Controls for immunolabelling were negative (data not shown).


Empty Plasmid
Cea10-MHVR Chimera
Anti MHVR antibody 655
Anti MHV-A59
MHVR
Cea10-MHVR Chimera

domain of Cea10 that I sequenced from Cl 1 D cells. This difference resulted in a change of one amino acid from leucine in the colon to methionine at position 91 in Cl 1 D cells).

I subcloned the full length cDNA clone of Cea10 into the pSP72 plasmid under the control of the T7 promoter, and the Cea10 protein was expressed in BHK cells using vaccinia VTF7-3 virus. The expressed protein was analyzed in immunoblots using MAb-CC1 and two other antibodies, a polyclonal rabbit anti-MHVR antibody 655 and a polyclonal rabbit anti-rat-ectoATPase antibody 669. Anti-serum 669 detected several bands in both empty or Cea10 plasmid-transfected BHK cells. Anti-serum 669 detected a protein of approximately 37 kDa in Cea10-transfected cells, which was not present in cells transfected with empty plasmid (Fig. 15B). This protein was not detected by MAb-CC1 (Fig. 15A), confirming the results obtained with the Cea10-MHVR chimeric protein. After longer exposure, antibody 655 also detected this protein (Fig. 15C), as well as several BHK cell proteins (data not shown).

To detect the Cea10 protein more specifically, immunoblots were also performed with a rabbit anti-serum (Anti-Cea10 K) directed against the 14 amino acids at the C-terminal of the deduced Cea10 amino acid sequence. This sequence differed from that of MHVR in the same region. As shown in Fig. 16, this anti-serum detected an approximately

Fig. 14. The nucleotide sequence of the Cea10 cDNA and analysis of the hydrophobicity of Cea10 protein. A. The sequence of Cea10 cDNA from CD-1 mouse colon and its predicted amino acid sequence (Keck, et al., 1995). The potential glycosylation sites in the protein are underlined. The stop codon is designated as *. The polyadenylation signal is highlighted and underlined. The sequences for primer 253 and primer 980 are italicized and underlined. One amino acid that differed between this sequence and the sequence that I cloned from Cl 1D cells is highlighted. This difference resulted from one nucleotide difference between the two sequences. However, the sequence at this position in Dr. Beauchemin's cDNA clone was identical to that in Cl 1 D and F40 cells. B. Hydrophobicity analysis of Cea10 protein. Kyte-Doolittle plot was done using GCG program with a window of 9 amino acids. For comparison, the MHVR protein was included (upper panel). Values above the x-axis are hydrophobic and those below are hydrophilic.

A

```
CATCAGGGCAGCCTGGCTTAGCAGTAGTGTTGGAGAAGCTAGCAGGCAGCAGAGACATGG
                                                          M  E
AGCTGGCCTCAGCACATCTCCACAAAGGGCAGGTTCCCTGGGTTGGACTACTGCTCACAG
  L  A  S  A  H  L  H  K  G  Q  V  P  W  V  G  L  L  L  T  A
CCTCACTTTTAACTTACTGGAGCCCTGCCACTACTGCCCAAGTCACCGTTGAGGCTGTGC
  S  L  L  T  Y  W  S  P  A  T  T  A  Q  V  T  V  E  A  V  P
CGCCCAACGTTACTGCAGACAACAATGTTCTTCTACTTGTTCACAATCTGCCGCAGACGC
  P  N  V  T  A  D  N  N  V  L  L  L  V  H  N  L  P  Q  T  L
TCCGAGTCTTTTACTGGTACAAGGGAAACAGTGGGGCTGGACACAATGAAATTGGACGAT
  R  V  F  Y  W  Y  K  G  N  S  G  A  G  H  N  E  I  G  R  F
TTGTAACATCCATTAATAGGAGTAAATTGGGGCTTGCACACAGCGGCAGAGAGACAATAT
  V  T  S  I  N  R  S  K  L  G  L  A  H  S  G  R  E  T  I  Y
ATAGCAATGGATCCCTGTTCTTCCAAAGTGTCACCAAGAATGATGAGGGAGTCTACACAT
  S  N  G  S  L  F  F  Q  S  V  T  K  N  D  E  G  V  Y  T  L
TATATATGCTAGATCAAAACTTTGAAATTACTCCGATTTCTGTGCGATTTCATGTACACC
  Y  M  L  D  Q  N  F  E  I  T  P  I  S  V  R  F  H  V  H  P
CCTCACTTTTACCCTCCTTGAGCCCTCCCACCACTGGCCAAGTCACCGTTGAGGCTGTGC
  S  L  L  P  S  L  S  P  P  T  T  G  Q  V  T  V  E  A  V  P
CGCCCAACGTTGCCGAAGGGGAAAACGTTCTTCTACTTGTTCACAATCTGCCACGGACGC
  P  N  V  A  E  G  E  N  V  L  L  L  V  H  N  L  P  R  T  L
TCCGAGCAATTTACTGGTACAGGGGAACCACTGCGGGTGAAAGAAATGAAATTGCACGAT
  R  A  I  Y  W  Y  R  G  T  T  A  G  E  R  N  E  I  A  R  F
TTATAACAGCCTCCAATAAGATTATATTGGGGCCTGCACACAGCGACAGAGAGATCATAT
  I  T  A  S  N  K  I  I  L  G  P  A  H  S  D  R  E  I  I  Y
ACAACAATGGATCCCTGTTCTTCCAAGGTGTCACCAAGAATGATGAGGGAGCCTACGCAC
  N  N  G  S  L  F  F  Q  G  V  T  K  N  D  E  G  A  Y  A  L
TAGATATGCTATTTCAAAACTTCGATCATACCCTAATGCCTGTGCAATTTAATGTTCACG
  D  M  L  F  Q  N  F  D  H  T  L  M  P  V  Q  F  N  V  H  A
CTAAAAAGCAGTAGGAATTTTTTTTCCAGCCCCCTTGCATTTAATTCTTCTTTCATTGCT
  K  K  Q  *
CTTTCTAACTGGAACCGAACTCTGGGAAAGAGGGCTGATGCTCACTAGTGAACCCCAGGG
 ATTTCATCAGCTGACGGTGCCTTGCTTCACCTCCTTCCACCAGCAATAAAGTCCGCATAA
CTTCTGCA
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B

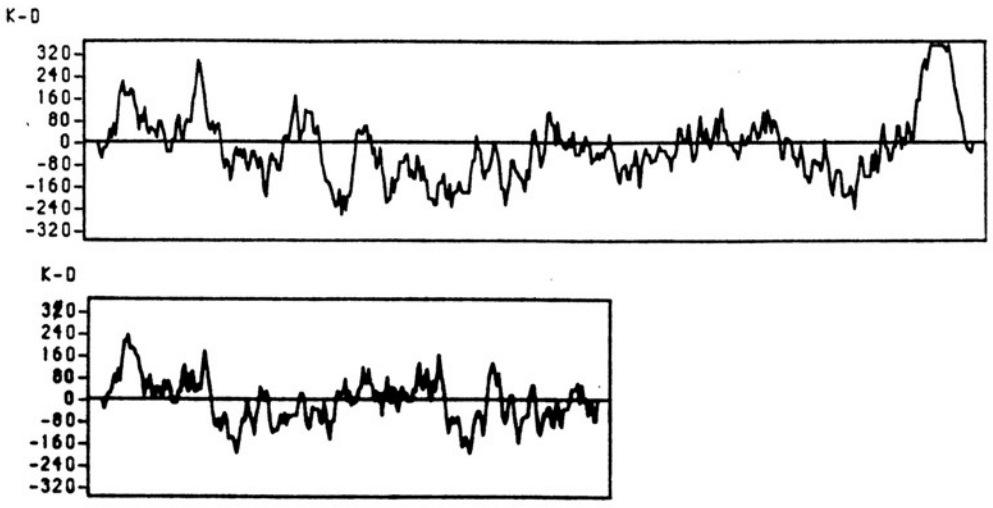
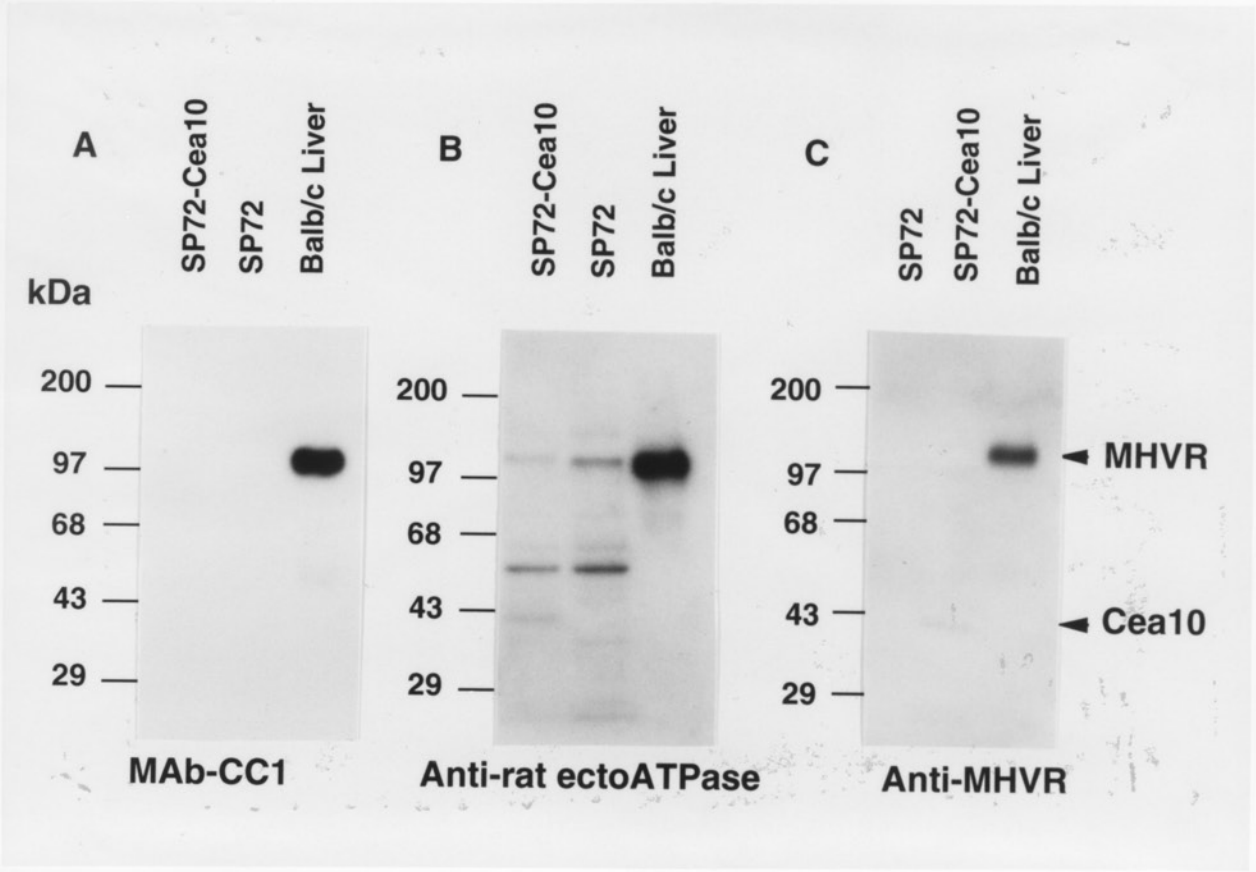


Fig. 15. Detection of Cea10 protein using different antibodies: BHK-21 cells were infected with vaccinia VTF7-3 and transfected with 6 ug DNA of plasmid SP72-Cea10 or control plasmid SP72. Fourteen hours later, the cells were lysed with 300 ul buffer. To detect the protein, fifteen ul of cell lysate or 10 ug of BALB/c liver extract were loaded on to the 8% gel, electrophoresed and blotted with MAb-CC1 (1:80), anti-rat ectoATPase antibody 669 (1:200), or anti-MHVR antibody 655 (1:250). An approximately 37 kDa protein was detected in samples transfected with pSP72-Cea10 by both polyclonal antibodies, but not by MAb-CC1. Two weaker bands (approximately 35 kDa and 39kDa respectively) were also detected.

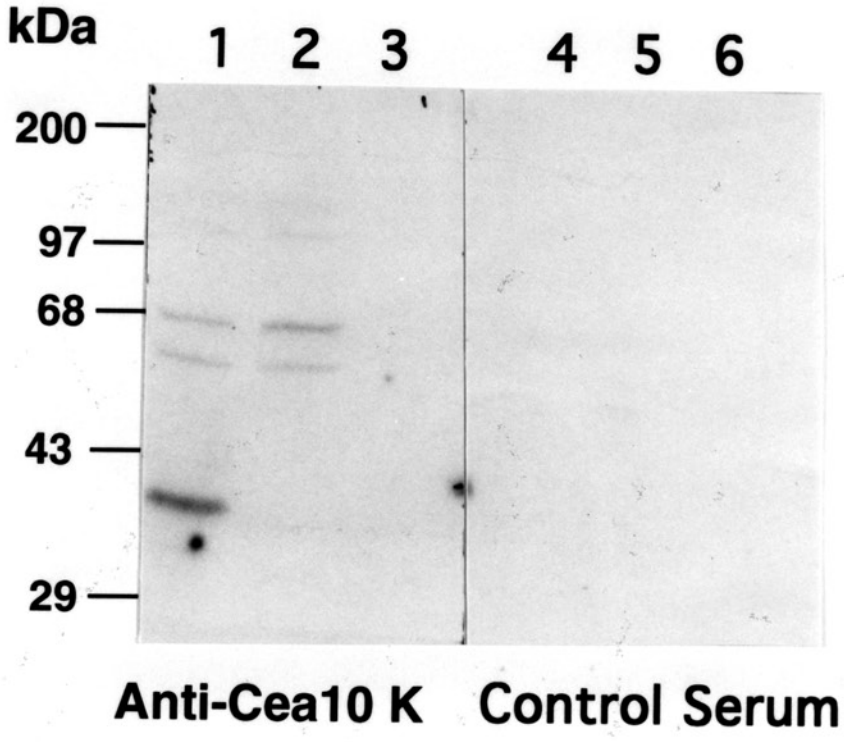

A
SP72-Cea10
SP72
Balb/c Liver
kDa
200
97
68
43
29
MAb-CC1
B
SP72-Cea10
SP72
Balb/c Liver
200
97
68
43
29
Anti-rat ectoATPase
C
SP72
SP72-Cea10
Balb/c Liver
200
97
68
43
29
MHVR
Cea10
Anti-MHVR

37 kDa protein that was present in samples transfected with Cea10 cDNA plasmid, but not in samples with the control empty plasmid. The anti-serum did not detect MHVR in a BALB/c liver extract nor did it detect other cellular proteins in this extract. The 37 kDa protein was not detected by rabbit pre-immunization serum (Fig. 16), although both the pre-immune serum and anti-peptide serum detected several proteins in samples with or without the Cea10 cDNA plasmid. These results indicated that the anti-Cea10 K anti—peptide antibody detected the Cea10 protein. The apparent molecular size (37 kDa) of the Cea10 protein is larger than that predicted by its amino acid sequence (29.4 kDa for precursor protein, 25.8 kDa for mature protein), suggesting that Cea10 maybe a glycosylated protein. When the complete Cea10 protein was expressed in BHK cells using the vaccinia VTF7-3 expression system and BHK cells were challenged with MHV-A59, the cells did not become infected. This result confirms the results from the Cea10-MHVR chimeric protein, showing that the Cea10 protein is not a receptor for MHV-A59.

Detection of Cea10 in Supernatant Medium

Immunoblotting analysis also showed that Cea10 protein expressed using vaccinia VTF7-3 virus was a secreted protein. MHVR, anchored by its transmembrane segment, was abundant in MHVR plasmid transfected BHK cells, but only a

Fig. 16. Detection of Cea10 protein by anti-Cea10 peptide antibody anti-Cea10 K: The protein samples were prepared as described in Fig. 15. and run on 10% gels in duplicate and blotted with either rabbit anti-Cea10 K anti-serum (1:1000) or preimmune anti-serum (control serum, 1:1000). Lanes 1 and 4: Twenty ul BHK cells transfected with SP72-Cea10 plasmid. Lanes 2 and 5: 20 ul BHK cells transfected with SP72 cells. Lanes 3 and 6: 10 ug Balb/c liver extract.


kDa
1 2 3 4 5 6
200
97
68
43
29
Anti-Cea10 K
Control Serum

small amount was found in the cell supernatant as detected by antibody 669 (Fig. 17A, lane 3 and 4). In contrast immunoblotting with either antibody 669 or with the anti-Cea10 K showed Cea10 protein in both the cell supernatant and the cell lysate (Fig. 17A and B. lane 1 and 2).

Detection of Cea10 Protein and mRNA in the Cl 1D Cells

CEA-related genes undergo extensive alternative splicing that generates multiple mRNA species which encode multiple protein isoforms (Barnett, et al., 1988). Anti-Cea10 antibody detected a 35 kDa Cea10 proteins in both the Cl 1 D cell lysate and the supernatant medium (Fig. 18, lane 1, 2, 4, 5, 6). This protein is not present in Opti-MEM medium (Fig. 18, lane 3, 7, 8).

An RT-PCR assay was used to test if Cea10 in Cl 1 D cells has the same 3' end as that from the mouse colon. A primer (primer 980) was synthesized according to the sequence of Cea10 near the polyadenylation signal (see Fig. 14 for position of primer). RT-PCR was performed with RNA from Cl 1 D cells using another primer (primer 253) corresponding to a sequence within the N-terminal domain of Cea10. Hybridization with the Cea10 specific probe (Cea10 oligomer) detected only one band at approximately 720 bp (Fig. 19), the same size as the Cea10 cDNA from mouse colon. Several restriction enzyme sites in the full-length Cea10 cDNA clone from CD-1 mouse colon were also present in the

Fig. 17. Cea10 protein expressed from cDNA plasmids is a secreted protein. Cea10 and MHVR proteins were expressed by transfecting plasmid pSP72-Cea10 or pSK-MHVR into vaccinia VTF7-3 infected BHK cells. Sixteen hours later, supernatant medium was collected and cells were lysed. The supernatant was depleted of cells by centrifugation and then concentrated approximately 20 fold with a Centricon-10. Lane 1, Cea10 transfected cells. Lane 2, Concentrated supernatant medium from Cea10 transfected cells. Lane 3, Concentrated supernatant medium from MHVR-transfected cells. Lane 4, MHVR-transfected cells. Twenty ul of each sample was loaded onto each lane of panel A, and 5 ul onto each lane of panel B.

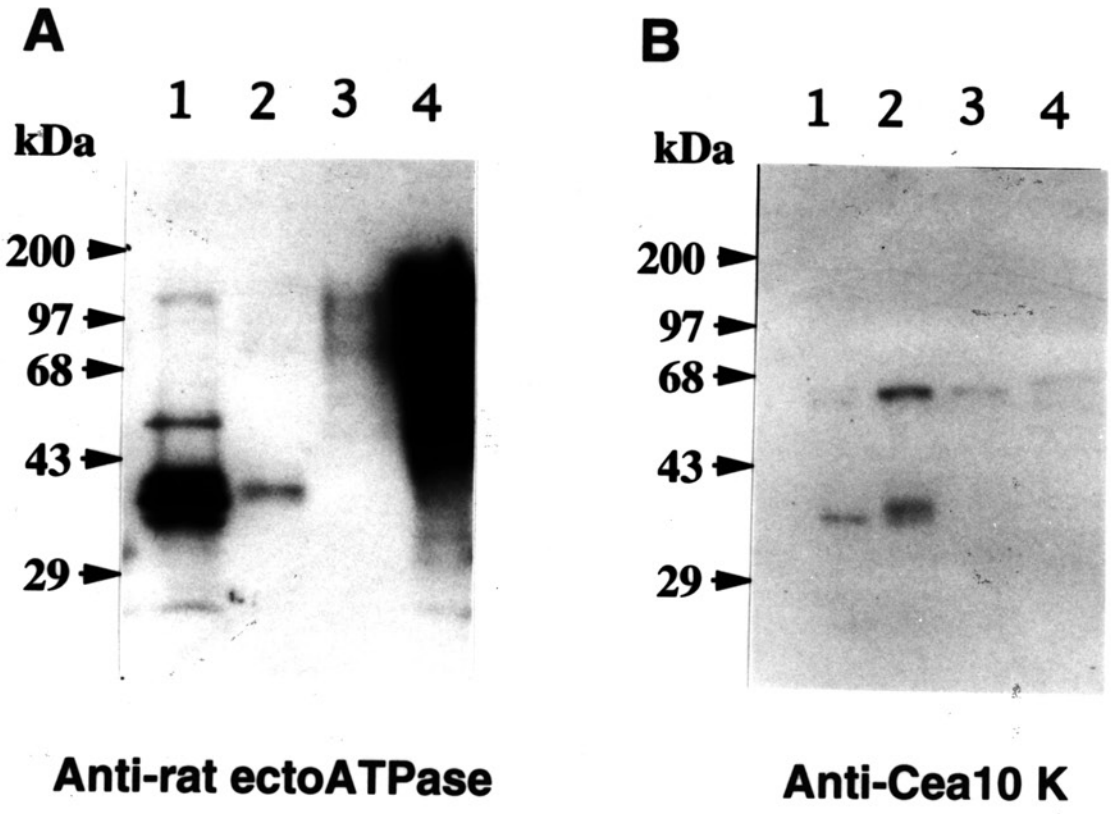

A
1 2 3 4
kDa
200
97
68
43
29
Anti-rat ectoATPase
B
1 2 3 4
kDa
200
97
68
43
29
Anti-Cea10 K

Fig. 18. Detection of the Cea10 protein in Cl 1 D cells or supernatant medium by anti-Cea10. The supernatant from Cl 1 D cells and Opti-MEM medium were concentrated and the proteins were separated on 10% SDS acrylamide gel and blotted with rabbit anti-Cea10 peptide anti—serum (1:1000). Lane 1 and 2: two different batchs of concentrated Cl 1 D supernatant, 10 ul on each lane. Lane 3: 10 ul Opti-MEM medium concentrated by centricon-10. Lane 4. 10 ul of BHK-21 cells transfected with SP72-Cea10 plasmid. Lane 5. 10 ul of Cl 1 D cell lysate. Lane 6. 25 ug concentrated Cl 1D cell supernatant. Lane 7. 25 ug (same amounts protein as lane 6) concentrated Opti-MEM medium. Lane 8. 7.5 ul concentrated Opti-MEM medium (same concentrated fold as lane 6)

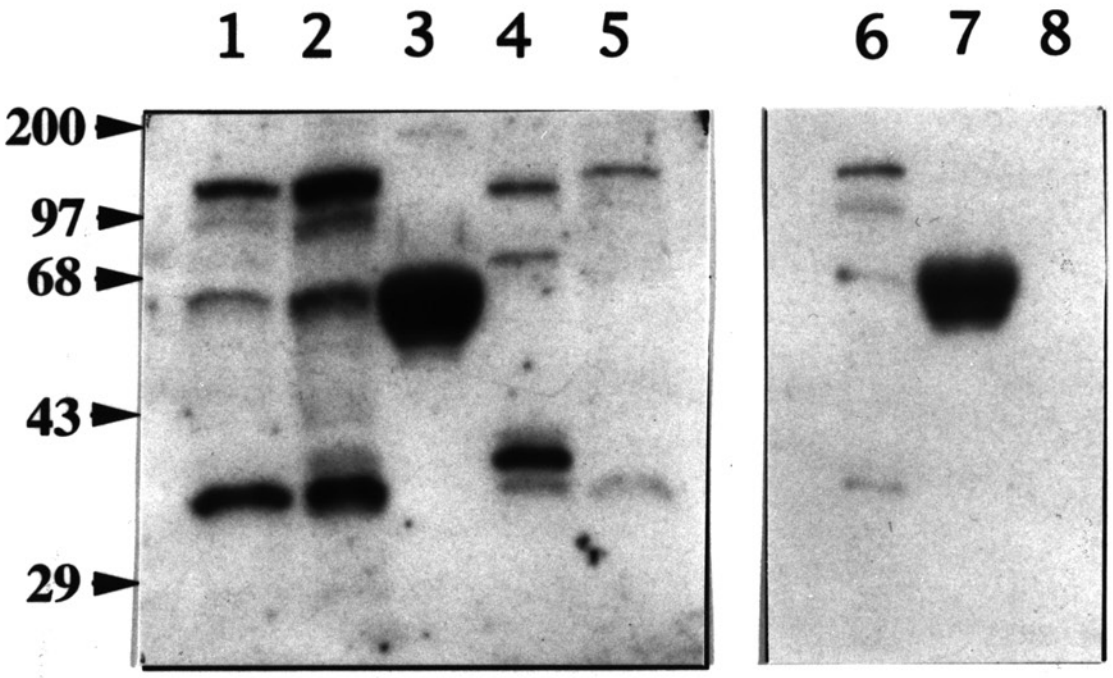
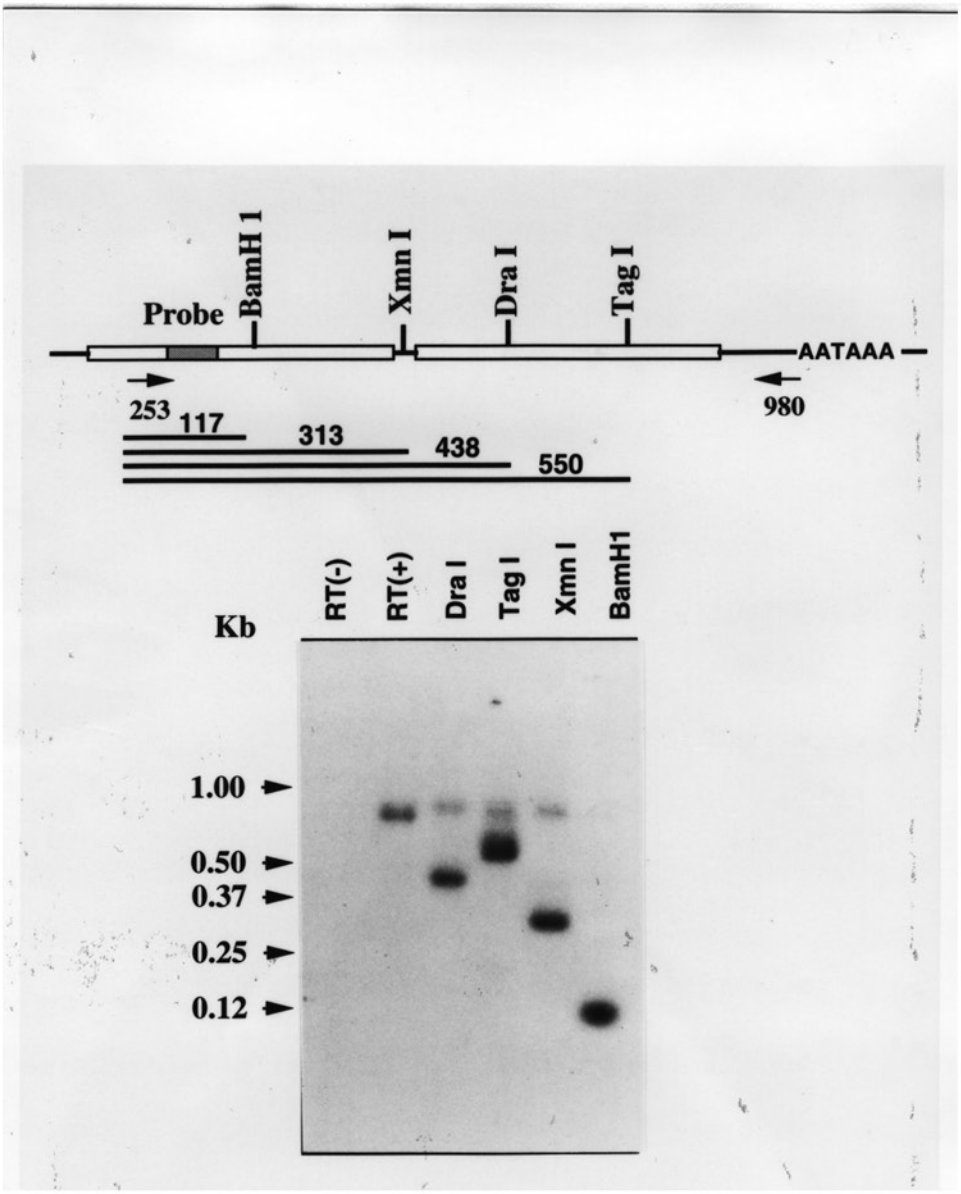

1 2 3 4 5
6 7 8
200
97
68
43
29

Fig. 19. Detection of the 3'-end of Cea10 mRNA in Cl 1D cells. Four ug total RNA was reverse transcribed into cDNA using oligo(dT) as a primer. The Cea10 cDNA was amplified using primers 253 and 980. The PCR conditions were 94°C, 45 S, 57°, 30 S and 72°C, 3 min for 30 cycles. Four ul PCR products were digested with each restriction enzyme and run on 2% agarose gel. After transferring to membrane, the blot was hybridized with end-labelled Cea10 oligomer. The size of each band predicted from cDNA sequence was: uncut PCR product, 746bp; PCR product cut with Dra I, 420 bp; cut with Tag I, 550 bp; cut with Xmn I, 300bp and cut with BamH I, 110bp. The lane RT(-) is same as lane RT(+) except that no reverse transcriptase was added during cDNA synthesis.


Probe
BamH 1
Xmn I
Dra I
Tag I
AATAAA
253
980
117
313
438
550
Kb
RT(-)
RT(+)
Dra I
Tag I
Xmn I
BamH1
1.00
0.50
0.37
0.25
0.12

PCR product obtained with primer 253-980 from Cl 1 D RNA (Fig. 19). The results were consistent with the immunoblot blot results, indicating that the Cea10 protein in Cl 1 D cell is a secreted protein.

Discussion

A new murine CEA-related mRNA, Cea10, was discovered by RT-PCR in murine Cl 1 D and F40 cells. The following data show that the Cea10 protein was not a MHV receptor: 1. Cea10 and MHVR were co-expressed in Cl 1 D and F40 cells as shown at both the mRNA and protein levels. 2. MHVR-specific MAb-CC1 protected both cell lines from MHV infection. 3. The expression of an anchored, chimeric Cea10-MHVR protein in which the N-terminal domain of MHVR was replaced by that of Cea10, did not lead to MHV infection in virus resistant BHK cells. 4. The Cea10 was a secreted protein. This is the first report that two different murine CEA-related proteins from different genes can be co-expressed in cells from an inbred mouse. This is also the first report of a murine CEA-related protein that lacks MHV receptor activity.

Recently, another murine CEA-related protein, Bgp2, was found to be an MHV receptor (Nédellec, et al., 1994). The sequence and structure of Bgp2 were different from those of Cea10 (see Fig. 7); therefore, they are not alleles of the same gene as suggested for MHVR and mmCGM2 (Dveksler, et al., 1993b). The sequence of the N-domain of Cea10 is more

like that of a BGP-related protein than of a PSG-protein (data not shown). Sequence comparison at the promoter region also indicated that Cea10 is more closly related to BGP than to PSG (Keck, et al., 1995). However, the structure of the Cea10 protein is more like a PSG instead of BGP in that Cea10 protein contains two IgV-like domains according to the predicted amino acid sequence, and Cea10 is secreted into the culture medium. These structural features are consistent with the characteristics of PSG proteins. Therefore, Cea10 appears to represent a link between the mouse PSG and Bgp genes (Nédellic, et al., 1995). Some glycosyl phosphatidylinositol (GPI) linked CEA proteins can be released into the medium. However, the detection of the Cea10 protein in the culture medium is unlikely to be the result from the release of GPI-linked Cea10 protein. Although no single common sequence has been found, a hydrophobic region at the C-terminal of these proteins is required for GPI linkage processing (Doering, et al., 1990). The lack of such a hydrophobic region at the C-terminal end of Cea10 made it unlikely that the Cea10 protein was attached to the membrane through a GPI linkage.

One of the major reasons for studying Cea10 was to see if this secreted protein somehow affected MHV infection in cells expressing it. One possibility was that it could serve as a "decoy" or soluble receptor for virus. Our results with the MHVR-Cea10 chimeric protein suggest that is

an unlikely possibility. Virus infectivity did not drop after treatment of virus stocks with Cl 1 D supernatant (data not shown), also supporting this notion. Another possibility was that the secreted Cea10 protein binds to a ligand on the cell surface and masks the virus receptor. Although we did not rule out the possibility that Cea10 binds to the cell surface, it apparently does not affect virus susceptibility. Treatment of F40 cells with Cl 1 D conditioned medium before MHV-A59 inoculation did not inhibit the MHV infection in treated cells (data not shown).

It is interesting to note that the level of Cea10 expression in liver and intestinal cells is very low compared to MHVR expression. In contrast, in Cl 1 D and F40 cells, MHVR expression was much lower than that of Cea10 based on our PCR experiments. Whether this opposite pattern for the expression of MHVR and Cea10 is true in other cell lines or tissues is an unresolved but interesting question. The expression of PSG genes in human and rodent placenta has been shown to be co-regulated (Thompson, et al., 1991), but recent reports from several groups suggest that CEA-related genes in different subfamilies differed in their patterns of expression. For example, the expression of human CEA protein is usually high in malignant tumor cells, but the expression of Bgp genes in tumor cells is down-regulated compared to normal tissue (Boucher, et al. 1989; Neumaier, et al. 1993). The MHVR is a mouse Bgp-related protein,

while the Cea10 is not a typical Bgp-related protein as discussed above. It is important to know if the expression of these two genes are positively or negatively co-regulated, as they may affect the MHV susceptibility of different cells and tissues. Further study on the mRNA levels of Cea10 in the different tissues may offer more insight into the regulation of expression of these two murine genes.

MHV can use more than one Bgp-related molecule as its receptor. These alternative receptors have different amino acid sequences in their N terminal domains, the virus binding domain (Dveksler, et al., 1993; Nédellic, et al., 1994). This fact has made it difficult to identify the amino acid residues in the virus- binding domain which is actually in contact with the virus spike proteins. The identification of Cea10, a closely related murine CEA-related protein that lacks MHV receptor activity, may help to identify these amino acid residues. For example, the chimeric molecule between MHVR and Cea10 glycoprotein will be useful in identifying the virus binding sequences in the N-terminal domain of the MHVR glycoprotein. Considering the fact that the N-terminal domain of Cea10 is more closely related to MHVR than to Bgp2, it may be feasible to convert Cea10 into an MHV receptor by point mutation.

IV. THE ROLE OF MHVR IN MHV INFECTION OF MURINE CELL LINES

Introduction

As described in the general introduction, inbred mouse strains differ markedly in their susceptibility to MHV infection. For instance, MHV-A59 infection in BALB/c mice causes mice to die of hepatitis and related diseases. In contrast, adult SJL mice are highly resistant to MHV infection (Stohlman and Frelinger, 1978; Barthold and Smith, 1987). In vivo studies showed that the immune response may be an important factor for the MHV-resistance of the SJL/J mouse. At the cellular level, MHV receptor activity or the spreading efficiency of MHV have been suggested as factors affecting the MHV resistance in SJL/J mice (Stohlman, et al., 1980; Boyle, et al., 1987; Wilson and Dales, 1988). In vitro studies showed that cell lines differed dramatically in their susceptibility to MHV infection (Kooi,et al., 1988). Some cell lines, like L2 cells, showed extensive virus-induced cell fusion, while other cell lines, such as the mouse fibroblast cell line LM, showed very little cytopathology. Different cell lines may also yield different amounts of MHV virus.

Several factors that affect the process of coronavirus-induced cell fusion have been identified. Host-dependent protease cleavage of the viral S glycoprotein can affect the extent of cell-cell fusion (Storz, et al., 1981; Frana, et

al., 1985). The lipid composition of the cell membrane (Daya, et al., 1988; Roos, et al., 1990), an unknown membrane component (Daya, et al., 1989) may also affect coronavirus-induced cell-cell fusion. The structure of MHV spike glycoprotein has important effects on MHV-induced cell fusion. Small plaque mutants of MHV-A59 virus have mutations in the predicted protease cleavage site (Sawicki, 1987). Fusion-defective MHV-A59 mutants selected from persistent infection of glial cells also have mutations in the cleavage site of the S protein (Gombold, et al., 1993).

Persistent infection of MHV in cell lines is relatively easy to establish (Lucas, et al., 1977; Holmes and Behnke, 1981; Gombold, et al., 1993). Several hypotheses have been put forward to explain this phenomenon, including mutation of virus structure protein, cleavage of virus spike proteins, and fusion resistance of host cells (Stohlman, et al., 1979; Mizzen, et al., 1983; Hingley, et al., 1994).

The role of the MHV receptor in determining cellular susceptibility to MHV infection, MHV-induced cell fusion, or MHV persistent infection was not clear when I began my work. In BALB/c mice, MHVR expression in target tissues of MHV infection is high (Williams, et al., 1991), suggesting that the expression of MHVR may play a role in MHV tissue tropism in vivo.

This study was undertaken to address whether the level of MHVR expression in different cell lines affected their

susceptibility to MHV infection. Three cell lines, L2, Cl 1 D and F40 cells, were used in this study. All three cell lines were derived from the L929 line of C3H fibroblast cells, and therefore were genetically closely related. Table 3 summarizes some characteristics of Cl 1 D and F40 cells. F40 cells were derived from Cl 1 D cells by treating the Cl 1 D cells with polyethylene glycol(PEG) just before trypsinization for 40 cycles (Roos, 1984). The F40 cells were more resistant to PEG-induced cell fusion than were Cl 1 D cells. In contrast, F40 cells showed in general, more cell fusion than Cl 1 D cells when infected with several enveloped viruses such as MHV or vesicular stomatitis virus (VSV)(Roos, et al., 1990). In this study, I found that the level of MHVR expression was a major determinant of cellular susceptibility to MHV-A59 infection, and that it also affected the levels of MHV-induced cell fusion and virus yield. The importance of the level of MHVR expression in determining cellular susceptibility to MHV infection was further confirmed using normal and transformed murine cell lines and cell lines persistently infected with MHV.

Results

MHV-A59 Infection of L2, Cl 1 D and F40 Cells

MHV infection was compared in L2, Cl 1 D and F40 cells (Fig. 20). L2 cells were highly susceptible to MHV-A59 infection as shown by their high virus yield (> 10^7 PFU/ml).

Table 3. Comparison of Cl 1 D and F40 Cells

	Cl 1 D	F40
Sensitivity to PEG Fusion	++	-
Lipid (S/P)*	0.9	2.9
Growth rate	+	++
Tumorigenicity	+/-	+++
Endocytosis	++	+

*S/P: Ratio of saturated fatty acid to polyunsaturated fatty acid

(Based on Roos,1984; Li, et al, 1986)

In contrast, the virus yield from Cl 1 D cells was at least 100-fold lower than that of L2 cells inoculated at the same MOI. F40 cells, derived from Cl 1 D cells by resistance to polyethylene glycol treatment, gave an intermediate yield of virus. While the initial period of virus release was intermediate between L2 and Cl 1 D cells, the yield from F40 cells continued to rise between 12 and 24 hours until it was at least 50-fold higher than that from Cl 1 D cells. The level of cell fusion induced by MHV infection was higher in L2 and F40 cells than in Cl 1 D cells (Fig. 21)(Roos, et al., 1990).

To determine in which stage of MHV infection these three cell lines differed, cellular susceptibility to MHV-A59 was assessed by infectious center assay, FACS analysis and plaque assay. In the infectious center assay, after virus adsorption and internalization, L2, Cl 1 D and F40 cells were plated onto L2 monolayers. The plaque number was proportional to the number of infected cells plated onto L2 monolayers. At an MOI of 2 PFU/ml, L2 cells were about 100-fold more susceptible to MV-A59 infection than either Cl 1 D or F40 cells, which were susceptible at about the same low level (Table 4). This result was confirmed by FACS analysis of the expression of MHV viral N antigen in infected cells at 4 hours p.i. (Fig. 22). MHV release from the cells has a latent period of about 6-7 hours. At 4 hours p.i., no virus-induced cell fusion was observed even in highly

Fig. 20. MHV-A59 growth in L2, Cl 1 D and F40 cells. Confluent cultures in 24 well plates were inoculated with MHV-A59 at a MOI of 3 PFU/cell. After 1 hour of adsorption at 37°C, the virus inoculum was washed away, and cells were refed with 1 ml fresh medium. The virus released into the culture medium was harvested at the indicated time points and the yield of virus was determined by plaque assay in L2 cells. The virus titer given is the mean and standard deviation of plaque number from 3 dishes. The experiment was repeated 3 times with similar results.

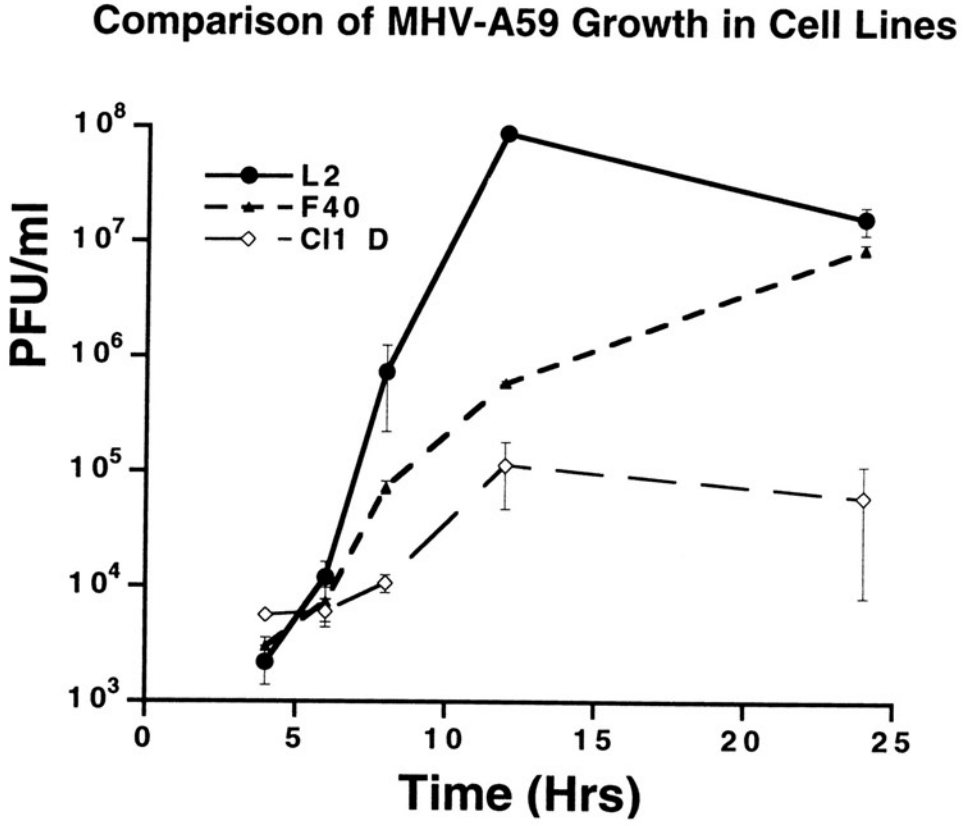

Comparison of MHV-A59 Growth in Cell Lines
PFU/ml
10^8
10^7
10^6
10^5
10^4
10^3
L2
F40
Cl1 D
0
5
10
15
20
25
Time (Hrs)

Fig. 21. Comparison of MHV-A59 induced cell-cell fusion in Cl 1 D and F40 cells. Cl 1 D and F40 cells were infected at an MOI of approximately 3-5 PFU/cell. The picture was taken at 24 hours p.i.. Cl 1 D cells (200X), F40 cells (100X).

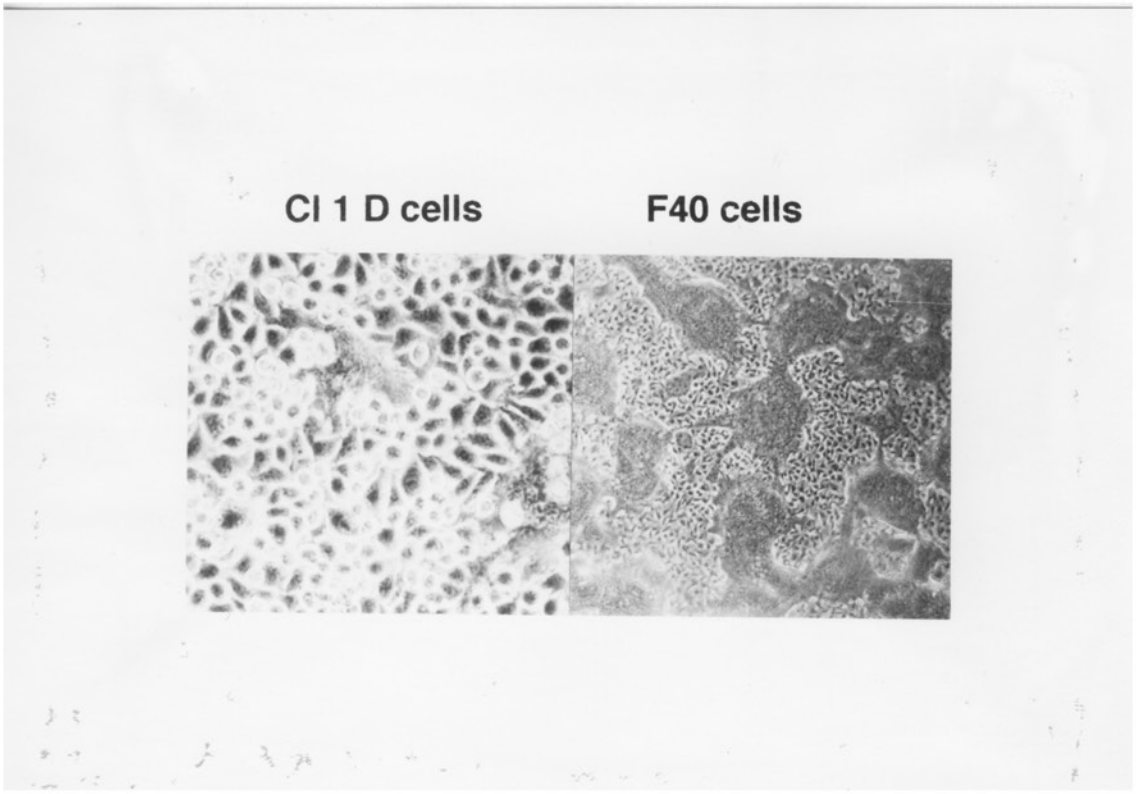

Cl 1 D cells
F40 cells

Table 4 : Susceptibility of Murine Cell Lines to MHV-A59

Cells infected	Number of cells plated onto L2 monolayers			
	10	100	1000	10000
L2	2 ±2	10 ±1	82 ±5	N.D
F40	N.D	0	2 ±1	13 ±1
Cl 1 D	N.D	0	1 ± 0	11 ±3

N.D: not done

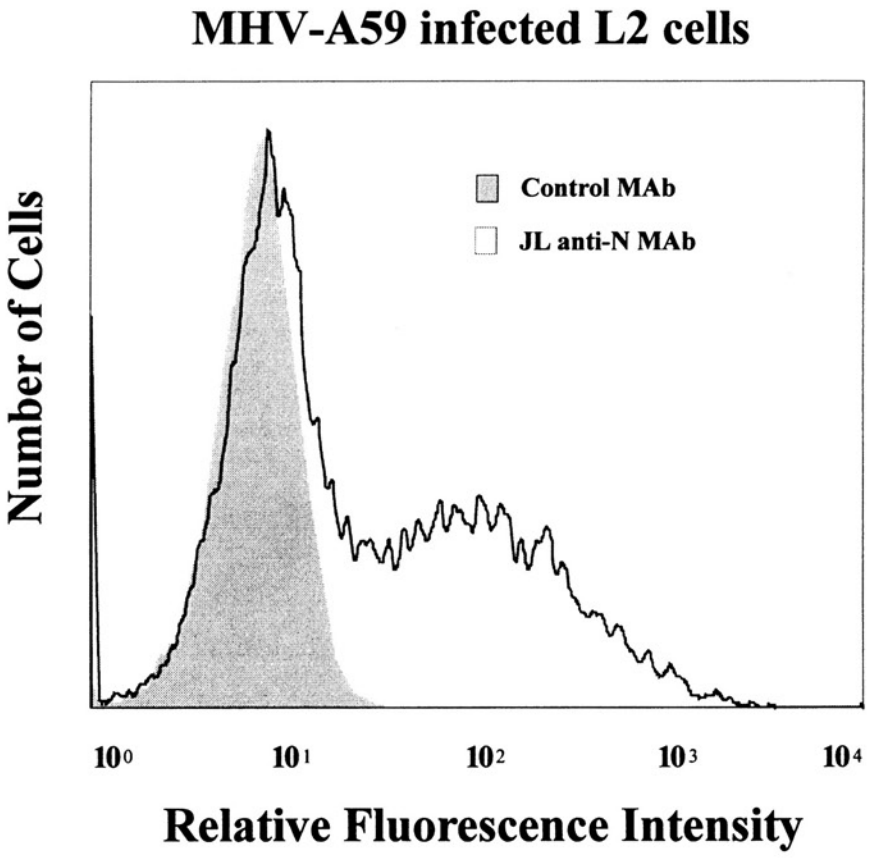
Cells were infected at MOI of 2 PFU/cell. After incubation with virus at 37°C for 1 hour, cells were washed twice and seeded onto L2 monolayers at the indicated dilutions. Plaque number is the mean of triple samples.

susceptible L2 cells. Therefore, the number of cells expressing viral antigen at this time should be a good indicator of the level of cellular susceptibility to MHV. FACS analysis of MHV infected L2 cells at 4 hours p.i. using a control monoclonal antibody yielded only one cell population in MHV-infected L2 cells with the fluorescence intensity defined as background level, while MAb JL anti-N revealed two cell populations. One had the same immunofluorescence intensity as the control antibody, and the other clearly showed labeling with anti-N antibody (Fig. 22). Quantitation of the proportion of the cell population expressing virus antigen at this time point showed that about 45% of L2 cells were virus-antigen positive cells, but both F40 and Cl 1 D cells had only approximately 1% cells expressing virus antigen (data not shown). These assays showed that the three related murine cell lines had different initial levels of susceptibility to MHV-A59 infection, which may result in differences in MHV yield and MHV-induced cell fusion.

Correlation of the Level of Expression of MHV Receptor with Cellular Susceptibility to MHV Infection

Multiple interactions between MHV and the host cell are needed for productive infection of MHV. The fact that all three cell lines produced infectious virions suggested that the low susceptibility of Cl 1 D and F40 cells might be

Fig. 22. FACS analysis of MHV-A59 infection in L2 cells. L2 cells were inoculated with MHV-A59 at MOI of 2 PFU/cell and then were harvested at 4 hours p.i. The infected cells were then permeabilized and labelled with monoclonal antibody JL anti-N or a control monoclonal antibody followed by phycoerythrin-coupled goat anti-mouse IgG_1 antibody. The FACS profiles from the MHV-infected L2 cells is shown.


MHV-A59 infected L2 cells
Number of Cells
Control MAb
JL anti-N MAb
10⁰
10¹
10²
10³
10⁴
Relative Fluorescence Intensity

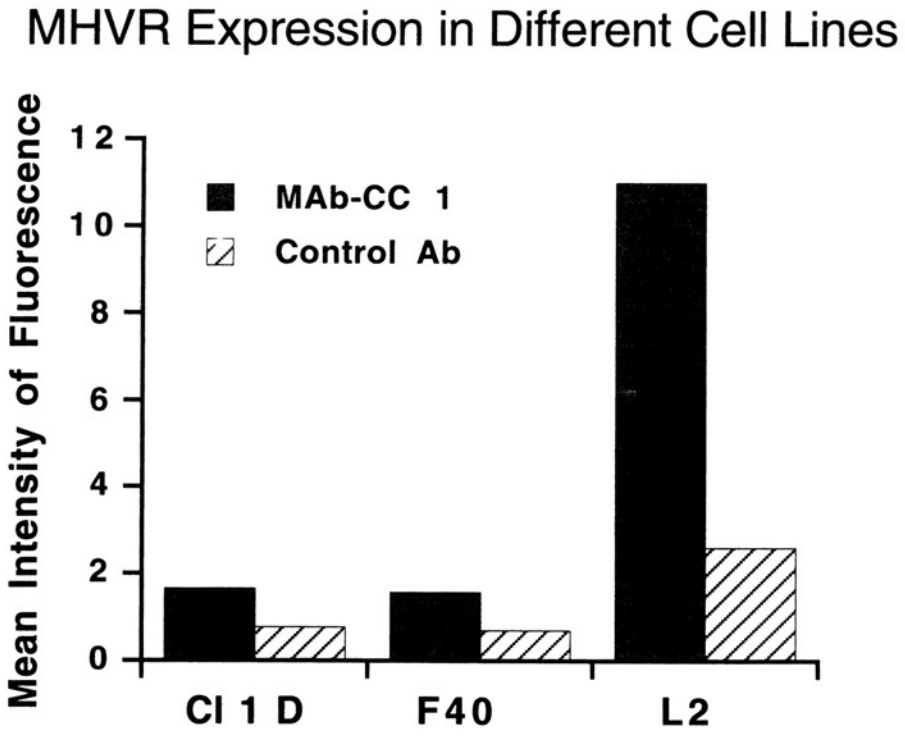
controlled at an early stage of virus infection. Before MHV viral antigens can be expressed in the cells, a virus must first bind to its cellular receptor, fuse with the cell membrane, and uncoat its infectious RNA genome. Different host cell mechanisms at each of these stages may protect cells from MHV infection as suggested by studies from Beushausen, et al., (1987); Boyle, et al. (1987); and Kooi, et al., (1991) and discussed in the introduction of this dissertation. The three cell lines that I studied allow dissection of the host-cell interaction at each of these steps in virus replication. For example, Cl 1 D and F40 cells differ in the lipid composition of their cell membranes. Possibly these two cell lines may differ in cellular susceptibility to MHV infection due to the differences in susceptibility to fusion between these cells. Also, the level of non-receptor mediated endocytosis in Cl 1 D cells is higher than that in F40 cells. This may also affect cellular susceptibility to MHV infection, if endocytosis is important in the early steps of MHV infection (Mallucci and Edward, 1982; Kooi, et al., 1991).

I was interested in learning whether the MHV receptor discovered in our laboratory played a major role in determining the cellular susceptibility to MHV infection. In the course of this work, it became known that several additional murine gene products can serve as functional receptors for MHV-A59 (Yokomori and Lai, 1992b; Dveksler, et

al., 1993; Nédellic, et al., 1994). Different cells may use receptor molecules that have different affinities for MHV spike proteins, and this may affect the MHV entry process. However, as shown in the previous chapter, we know that MHV-A59 infection of all the cell lines that I used, i.e., L2, Cl 1 D and F40 cells, can be blocked by pretreating the cells with anti-MHVR antibody MAb-CC1 which recognizes only MHVR. This indicates that MHVR is the only functional receptor for MHV-A59 in these cells.

A related hypothesis was tested, i.e., the level of MHV receptor on the cell surface may be an important determinant of cellular susceptibility to MHV infection. The relative levels of expression of MHVR in the three cell lines were determined by FACS analysis using anti-MHVR MAb-CC1. An isotype-matched monoclonal antibody to an irrelevant antigen was used as the control. Fig. 23 shows the mean intensity of fluorescence of antibody labelling of the MHVR glycoprotein in three cell lines. L2 cells expressed a much higher level of MHVR glycoprotein on the cell surface than did Cl 1 D or F40 cells, which expressed approximately the same low level of MHVR. Combined with results from infection data, these results indicate that the level of MHVR expression on the cell surface correlated with the degree of cellular susceptibility to MHV-A59 infection, despite the differences in cell fusion, endocytosis, and growth phenotype observed between Cl 1 D and F40 cells.

Fig. 23. Relative levels of MHVR expression on the plasma membranes of the L2, Cl 1 D and F40 cell lines. L2, Cl 1 D and F40 cells were harvested and counted. One million cells were then transferred to FACS staining buffer and labeled with MAb-CC1 or an isotype-matched control monoclonal antibody (40 ug/ml) followed by phycoerythrin-coupled goat anti-mouse IgG_1 antibody. The mean intensity of antibody labeling from the three cell lines is shown here. The data is the representative of three experiments.


MHVR Expression in Different Cell Lines
Mean Intensity of Fluorescence
12
10
8
6
4
2
0
MAb-CC 1
Control Ab
Cl 1 D
F40
L2

This result suggested that the level of MHV receptor glycoprotein on cell surface was a major factor determining the cellular susceptibility to MHV-A59 infection.

Effect of Transfection of MHVR Plasmid on the Susceptibility of Cl 1 D Cells to MHV-A59 Infection

To learn whether the low level of expression of MHVR in Cl 1 D cells had a causal relationship with the low susceptibility of these cells to MHV-A59 infection, I made a stable cell line named 9B1 by transfecting Cl 1 D cells with the pCMVneoMHVR plasmid. As shown in Fig. 24, the transfected cell line 9B1 expressed a high level of MHVR on the surface as determined by FACS analysis with MAb-CC1 in comparison with another clone, 9A7 derived from the same transfection which gained G418 resistance, but did not show an increased level of MHVR expression. When Cl 1 D cells were challenged with MHV-A59 at an MOI of 2 PFU/cell, after 12 hours p.i., <5% cells were infected and most infected cells were not fused (Fig 25). In contrast, more than 95% of 9B1 cells were infected and formed syncytia (Fig. 25).

Relationship of the Level of MHVR Expression and Susceptibility to MHV-induced Cell Fusion and Virus Yield

Many factors can regulate the level of cell fusion induced by MHV-A59. However, how these factors regulate this process and whether MHV receptor also affects this

Fig. 24. The expression of MHVR in Cl 1 D cells after transfection with the plasmid containing MHVR and neo gene. The Cl 1 D cells were transfected with plasmid pCMVneoMHVR using lipofectamine. The plasmid can express MHVR and neo genes in the cells using two independent promoters. Following Neo selection, two cell clones were selected for analysis. The expression of MHVR in the cells was detected by FACS analysis using MAb-CC1 as described in Fig. 23.

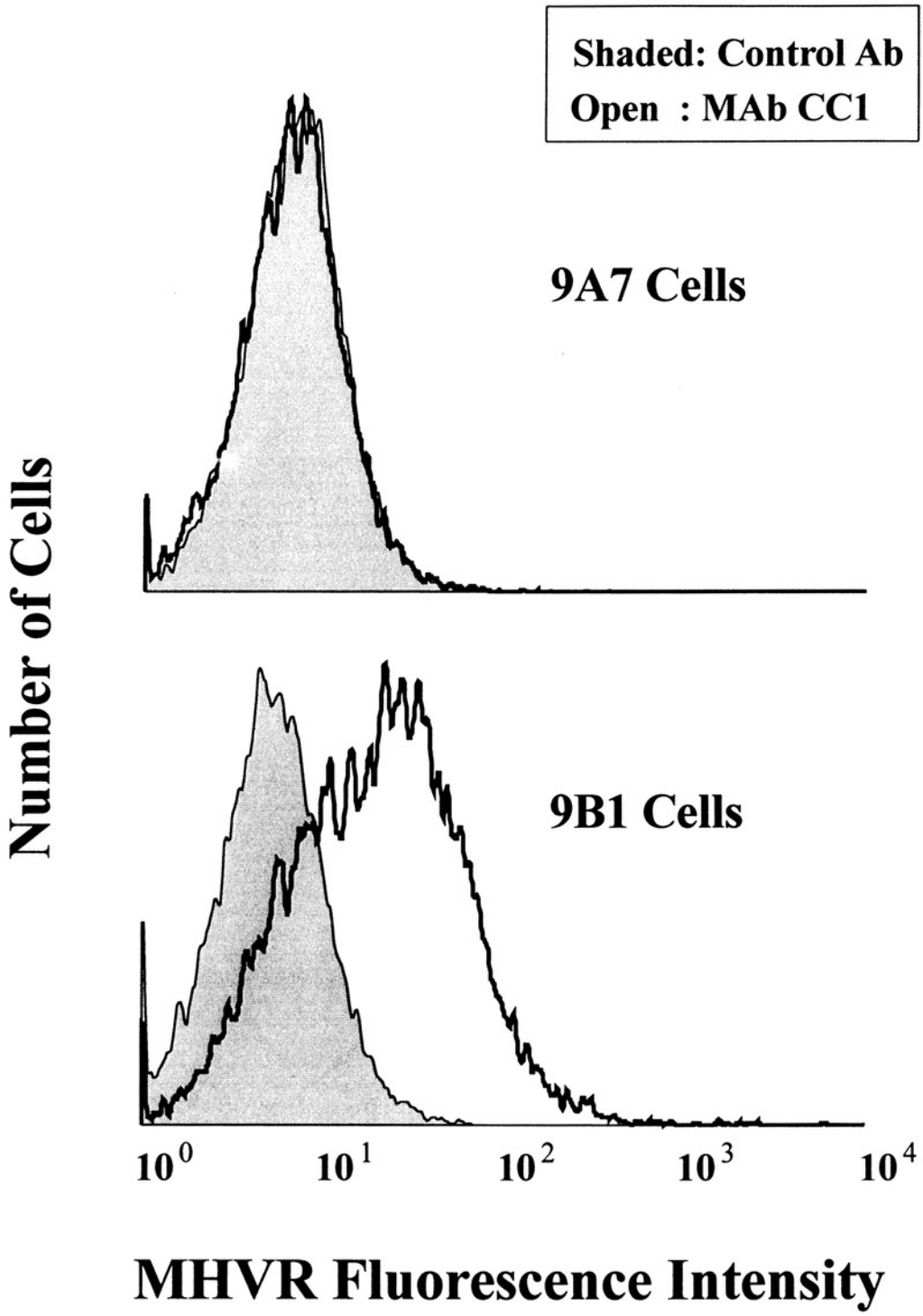
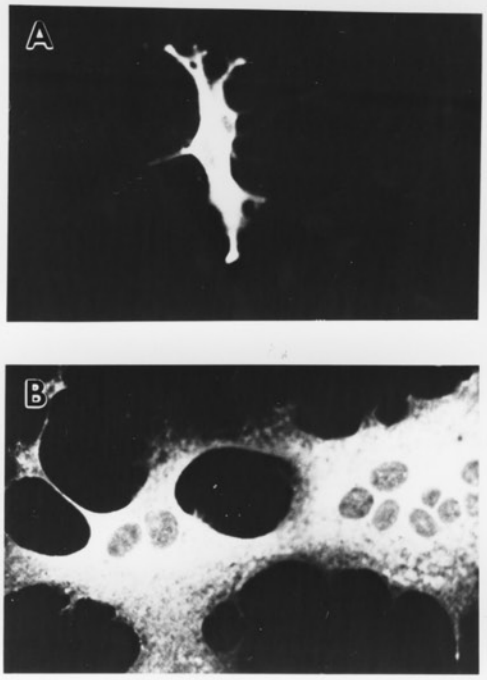

Shaded: Control Ab
Open : MAb CC1
9A7 Cells
9B1 Cells
Number of Cells
10^0
10^1
10^2
10^3
10^4
MHVR Fluorescence Intensity

Fig. 25. Comparison of the susceptibility to MHV-A59 infection of Cl 1 D cells and MHVR-transfected Cl 1 D cells. The Cl 1 D (A) or MHVR-transfected Cl 1 D cells, 9B1 (B) grown on coverslips were inoculated with MHV ($4x10^6$ PFU in 0.8 ml medium) and fixed with cold acetone at 12 hours p.i. The cells were then labeled with mouse anti-MHV-A59 antiserum followed by rhodamine-coupled goat anti-mouse antibody. A rare, fused Cl 1 D giant cell was selected to show (A), while extensive fusion was common throughout the monolayer of 9B1 cells. (Magnification: 630 X).



A: Cl 1 D cells

B: MHVR-transfected Cl 1 D cells

process were not known. The virus-induced cell fusion was compared in Cl 1 D and MHVR-transfected Cl 1 D cells. Cl 1 D cells became fully susceptible to virus-induced cell fusion after transfection with the MHVR plasmid (Fig. 26). Although the level of MHVR expression in 9B1 cells was comparable to that in L2 cells, the MHV-induced fusion of 9B1 cells was delayed for about 2 hours compared to L2 cells (Fig. 26). These results showed that the low level of MHVR expression in Cl 1 D cells, along with the lipid composition of the cell membranes (Roos, et al., 1990), affected the level of MHV-induced cell fusion in Cl 1 D cells. The level of expression of MHVR also affected the yield of MHV in the medium over Cl 1 D cells as shown in Fig. 27. Thus, the low level of expression of MHVR in Cl 1 D cells was the major factor that determined their low level of fusion and their low level of susceptibility to MHV-A59 infection.

The Regulation of MHVR and its Effect on MHV-A59 Infection

After establishing that the level of MHVR expression is the major determinant of cellular susceptibility to MHV-A59 infection in L2, Cl 1 D and F40 cells, I wondered if this was also true for other cell lines and how changes in expression of MHVR may affect MHV infection. This set of experiments was used to answer some of these questions.

Fig. 26. Effect of the level of expression of MHVR on MHV-A59-induced cell-cell fusion. L2, Cl 1 D, F40, and MHVR-transfected Cl 1 D cells (9B1) were infected with $1x10^6$ PFU of MHV-A59 in 0.1 ml medium for 1 hour at 37°C. After washing off the virus inoculum, the cells were refed with fresh medium. The pictures were taken at the indicated times post inoculation (200 X)

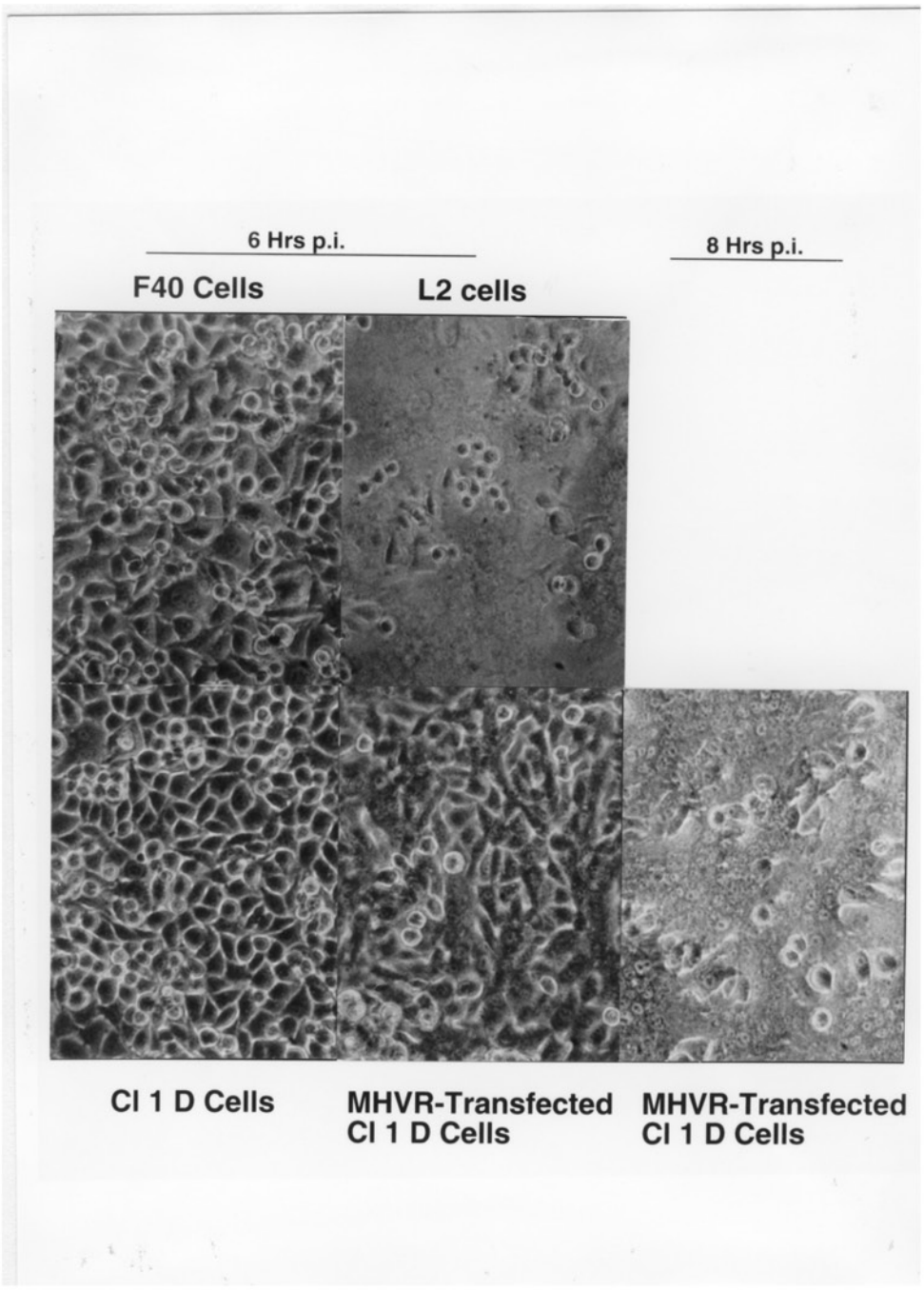
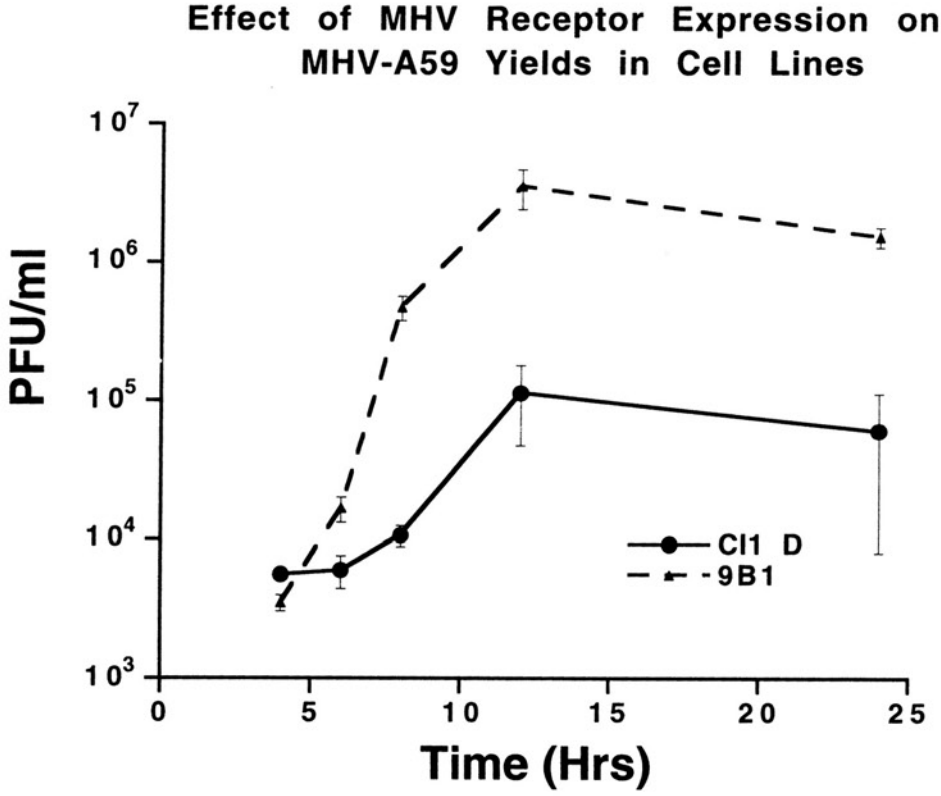

6 Hrs p.i.
8 Hrs p.i.
F40 Cells
L2 cells
Cl 1 D Cells
MHVR-Transfected Cl 1 D Cells
MHVR-Transfected Cl 1 D Cells

Fig. 27. The level of expression of MHVR affects the yield of MHV-A59 virus. Cl 1 D and MHVR-transfected Cl 1 D cells (9B1) were infected at an MOI of 3 PFU/cell. At the indicated time, the virus in medium was collected and titered on L2 cells. For comparison, this experiment was done at the same time as Fig. 20. The numbers shown were the means and standard deviation of three replicate dishes.


Effect of MHV Receptor Expression on
MHV-A59 Yields in Cell Lines
PFU/ml
10^7
10^6
10^5
10^4
10^3
0
5
10
15
20
25
Time (Hrs)
Cl1 D
9B1

Alteration of MHVR expression in L2 cells that survived MHV-A59 infection

Many cellular factors have been implicated in the control of MHV-induced cell fusion and cellular susceptibility to infection as discussed in the Introduction. In theory, cells which survive MHV infection may have changes in expression of either MHVR or postulated host factors. I conducted experiments to test this hypothesis.

After infection at a MOI of 5 PFU/cell, about 3% of L2 cells avoided MHV-induced fusion by 20 hours p.i.. These L2 cells were collected and their levels of MHVR expression were analyzed by FACS. Interestingly, these cells showed a sharp decrease of MHVR expression (Fig. 28), again indicating the role of the level of MHVR expression in MHV-induced cell-cell fusion. The level of MHVR expression in this cell population increased slightly after several cell passages (Fig. 28 and data not shown). Furthermore, the number of viral antigen-positive cells was increased in surviving cells after two passages compared to that in cells 20 hours after MHV-A59 infection, but was markedly lower than that in L2 cells acutely infected with MHV-A59 (Fig. 29). Thus, these observation demonstrated the relationship between the level of MHVR expression and cellular susceptibility to MHV infection. The majority of infected cells did not undergo cell fusion.

Fig. 28. Comparison of the expression of MHVR in L2 cells before and after acute MHV-A59 infection. The expression of MHVR was determined by labeling with MAb-CC1. The L2 cells infected with MHV-A59 at MOI of 5 PFU/cell. At 20 hours p.i., the cells were washed with PBS and harvested. This cell population was designated as surviving L2 cells 20 hours after MHV-A59 infection. Some of these cells were passaged 3 days later, incubated for 3 days, then analyzed for MHVR expression. This new cell population was designated as surviving L2 cells after 2 passages.

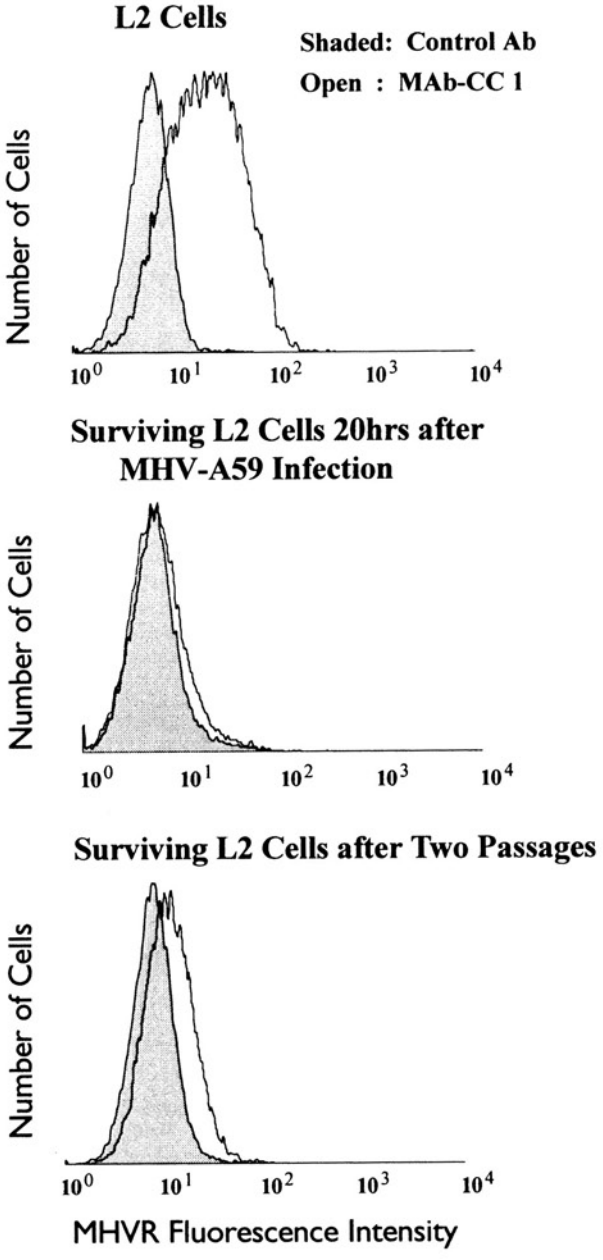
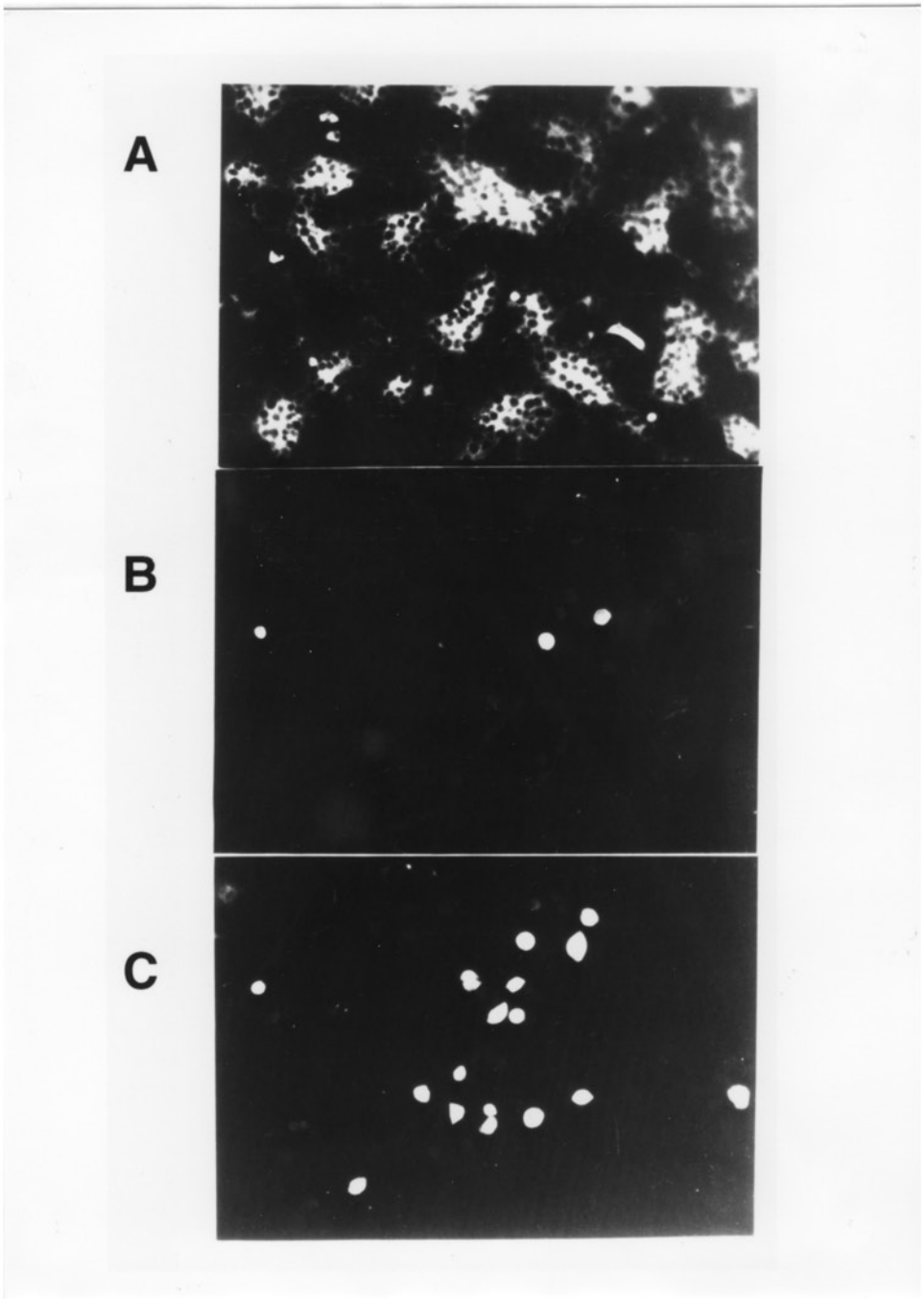

L2 Cells
Shaded: Control Ab
Open : MAb-CC 1
Number of Cells
10^0 10^1 10^2 10^3 10^4
Surviving L2 Cells 20hrs after MHV-A59 Infection
Number of Cells
10^0 10^1 10^2 10^3 10^4
Surviving L2 Cells after Two Passages
Number of Cells
10^0 10^1 10^2 10^3 10^4
MHVR Fluorescence Intensity

Fig. 29. MHV-A59 infection of L2 cells surviving MHV-A59 infection. A. L2 cells were infected at MOI of 2 PFU/cell, incubated 8 hours, and then fixed with acetone for immunofluorescent labeling with anti-MHV-A59 antiserum. By 20 to 24 hours, these giant syncytia would have peeled away leaving about 3% of the original cell population. B. The surviving L2 cells 20 hours after MHV-A59 infection were harvested and plated on coverslips in 60 mm dishes for 10 hours and then processed for detection of MHV antigen. C. After two cell passages, the surviving L2 cells were processed as described above. The secondary antibody was rhodamine-coupled goat anti-mouse antibody. (Magnification 200 X)


A
B
C

Effect of dibutyryl cyclic AMP or 5-azacytidine on susceptibility of Cl 1 D cells to MHV-A59 infection

To better understand how the cellular susceptibility to MHV-A59 infection is determined in cultured cells, several drugs which may affect the susceptibility of Cl 1 D cells to MHV infection were used. Treatment of Cl 1 D cells with dibutyryl cyclic AMP, which was suggested to decrease the susceptibility of rat oligodendrocytes to MHV infection (Beushausen, et al., 1987), did not change the susceptibility of Cl 1 D cells to MHV infection significantly (Fig. 30), probably because the drug has different effects in different types of cells.

Five-azacytidine, a DNA demethylation reagent, can activate transcription of many genes (Jones and Taylor,1980; Mohandas, et al., 1981; Venolia, et al., 1982). When Cl 1 D cells were treated with this compound for 50-60 hours, their MHV susceptibility was enhanced in a dose-dependent manner (Fig. 31). The drug had to be added into the cells at an early time after cell plating to show this effect on MHV infection (data not shown), probably because 5-azacytidine needs to be integrated into DNA to activate genes. The same treatment of L2 cells did not change their susceptibility to MHV infection.

To determine whether demethylation of DNA activated the expression of an alternative MHV receptor, the receptor blockade assay with MAb-CC1 was performed on 5-azacytidine-

Fig. 30. Effect of dibutyryl cyclic AMP (dbcAMP) on susceptibility of Cl 1 D cells to MHV-A59 infection. Cells grown on coverslips were treated with dbcAMP at the indicated concentrations for 48 hours before virus infection. The cells were then inoculated with MHV-A59 at 37°C and refed with fresh medium without the drug. At 8 hours p.i., the cells were fixed with acetone and the viral antigen was detected with mouse anti-MHV antiserum followed by rhodamine-coupled goat anti-mouse IgG antibody. To quantify the level of susceptibility to MHV-A59 infection, the number of positive cells in each field was counted. Thirty to fifty randomly selected fields were examined for each sample.

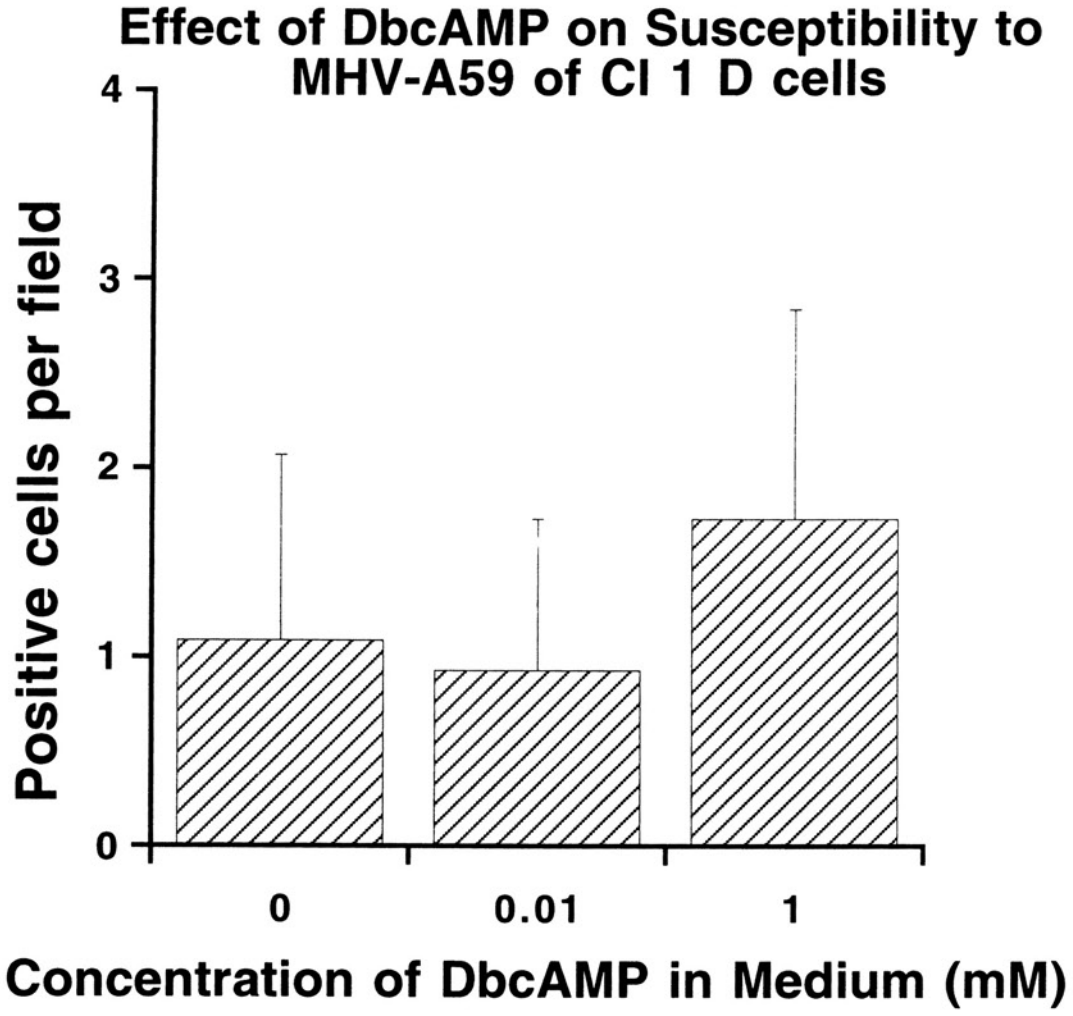
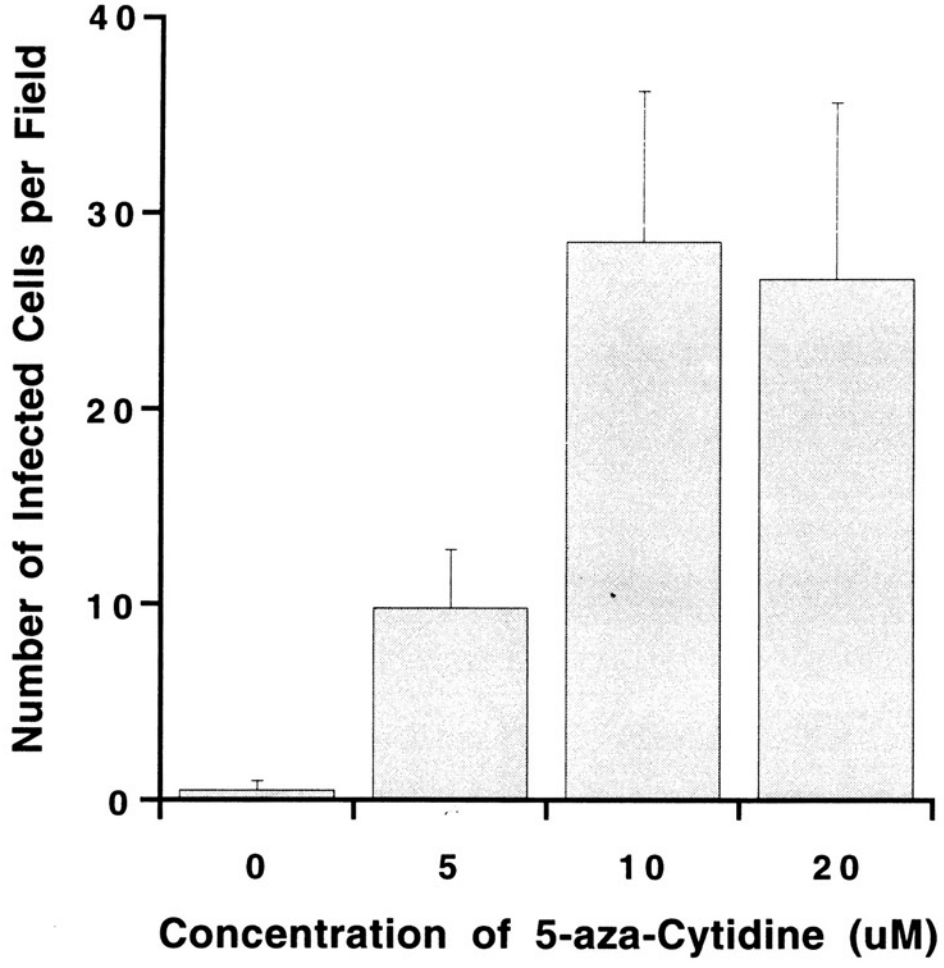

Effect of DbcAMP on Susceptibility to MHV-A59 of Cl 1 D cells
Positive cells per field
4
3
2
1
0
0
0.01
1
Concentration of DbcAMP in Medium (mM)

Fig. 31. Enhancement of MHV-A59 infection by 5-azacytidine treatment of Cl 1 D cells. Cl 1 D cells were seeded into 60 mm dishes. Three hours later, 5-azacytidine was added into medium at the concentrations shown and cells were incubated for 60 hours. The cells were then inoculated with MHV-A59 at MOI of 3 PFU/cell. At 7 hours p.i., the cells were processed for detection of MHV antigen by immunofluorescence with mouse anti-MHV-A59 antiserum. To quantify the level of susceptibility of cells to MHV infection, ten randomly selected fields for each sample were surveyed by microscopy. The data are the representive of 4 replicate experiments. Treatment of Cl 1 D cells with 20 uM 5-azacytidine showed some cytotoxicity.

Enhancement of Cellular Susceptibility to MHV-A59 Infection by 5-aza-Cytidine



treated Cl 1 D cells. As shown in Fig. 32, MHV infection of 5-azacytidine treated Cl 1 D cells was still blocked by MAb-CC1, suggesting that no alternative receptor was induced. However, this experiment did not rule out the possibility that some molecule other than MHVR was activated by demethylation and could have enhanced the cellular susceptibility to MHV infection. FACS analysis was used to measure the expression of MHVR in Cl 1 D cells after 5-azacytidine treatment. Fig. 33 shows that MHVR expression was significantly increased in 5-azacytidine treated cells compared to untreated cells, although the level of expression was significantly lower than that of L2 cells. Our results with 5-azacytidine suggest that a major factor limiting the susceptibility of Cl 1 D cells to MHV-A59 is their low level of MHVR expression, and that methylation of DNA may be involved in the regulation of MHVR expression in Cl 1 D cells.

Correlation of MHVR expression in transformed cells and cellular susceptibility to MHV infection

More than 20 years ago, it was observed that transformed cells yielded higher levels of MHV than non-transformed cells (Sturman and Takemoto, 1972). The mechanism of this enhancement is not clear. One report suggested that the cell cycle may affect the mouse hepatitis virus yield in infected cells (Talbot and Daniel,1987).

Fig. 32. MAb-CC1 protection of MHV-A59 infection in 5-azacytidine-treated Cl 1 D cells. The treatment of cells with 5-azacytidine and virus infection were the same as those described in Fig. 33. For receptor blockade using MAb-CC1, the cells were treated with MAb-CC1 hybridoma supernatant(1:5) for 1 hour before and during MHV-A59 inoculation. Cl 1 D cells with control medium (A), or treated with 5 uM (B), or 10 uM 5-azacytidine (C and D). Cells in D were also treated with MAb-CC1 before MHV-A59 inoculation.

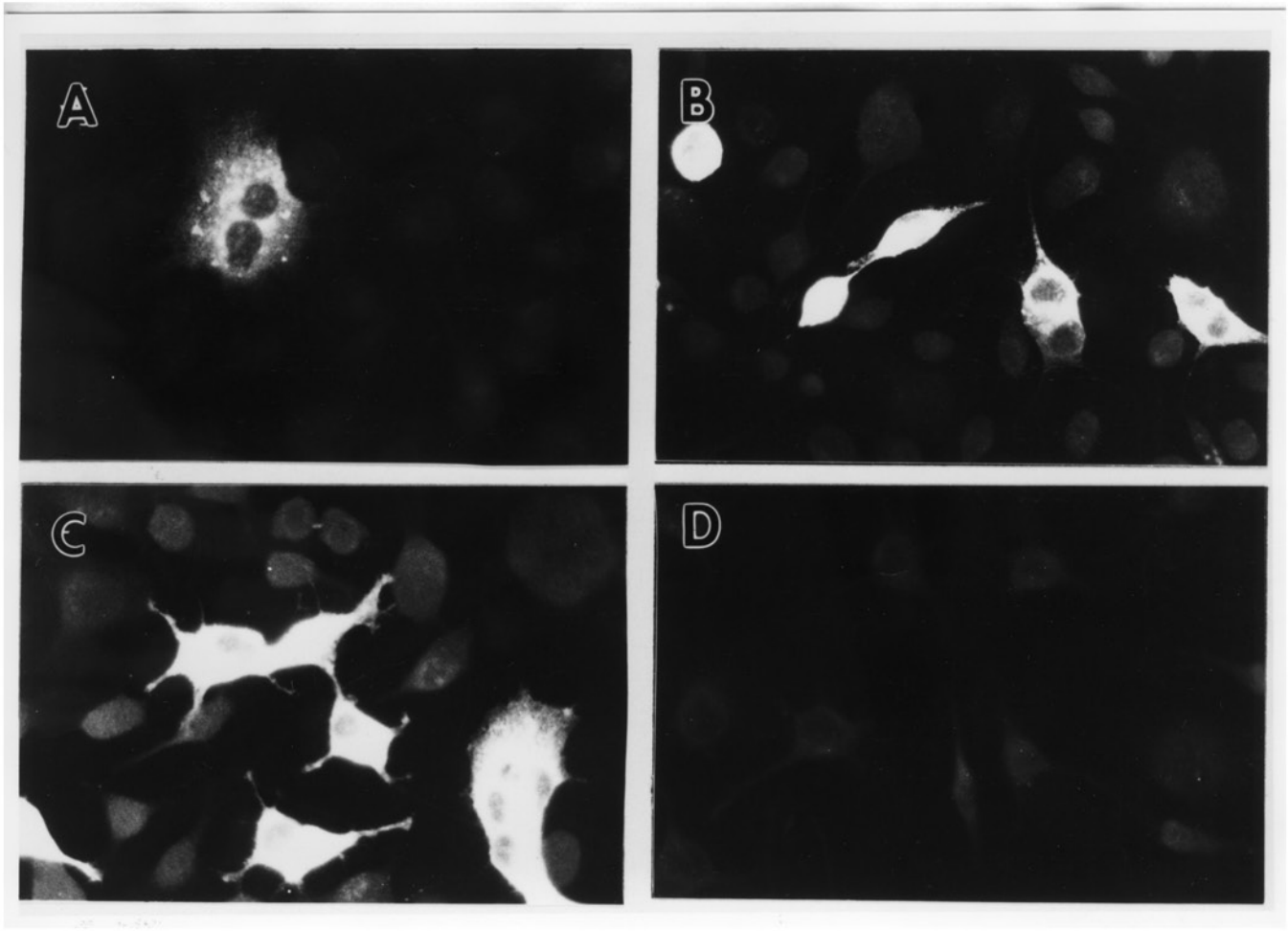
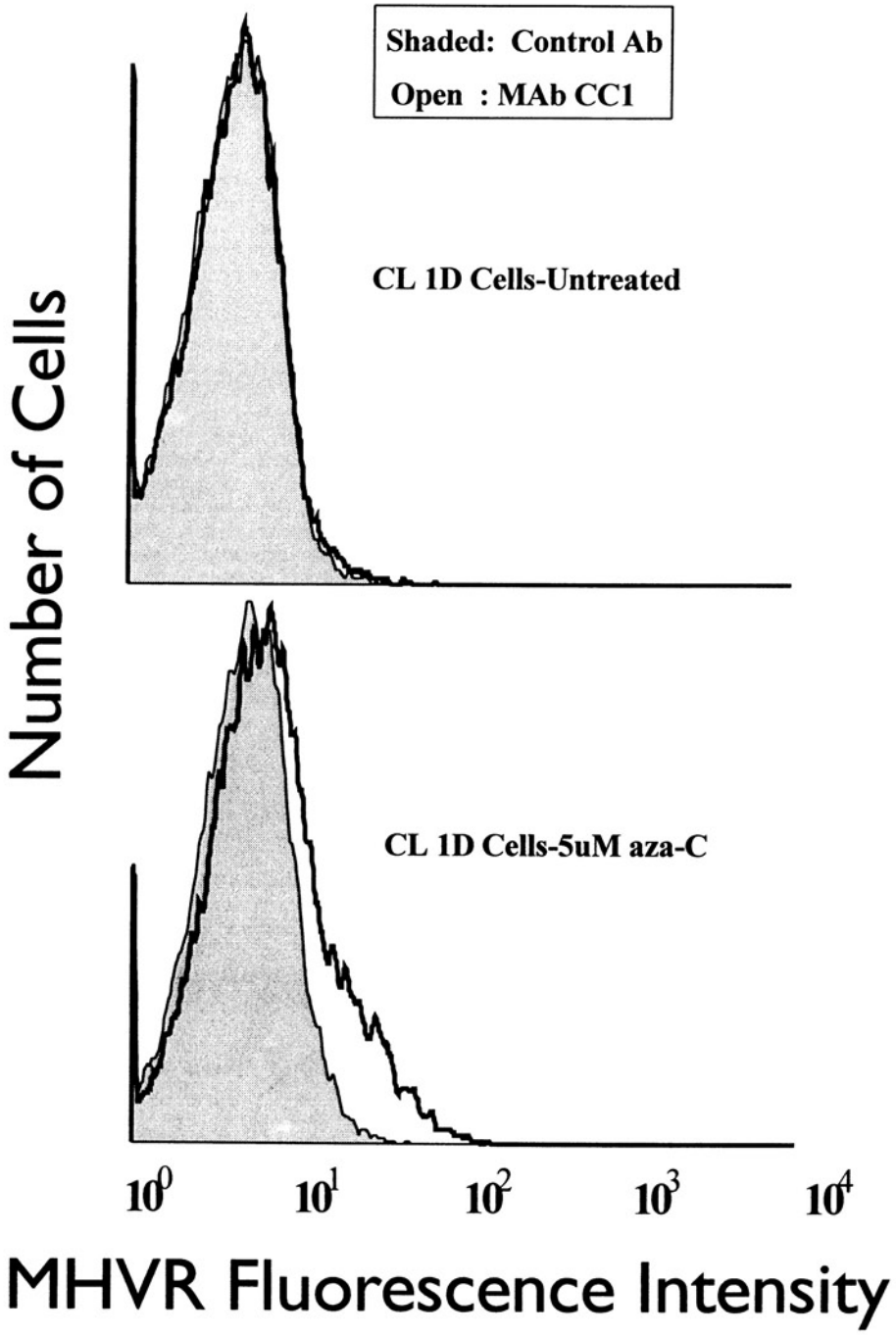

A
B
C
D

Fig. 33. The expression of MHVR in Cl 1 D cells after 5-azacytidine treatment. Cl 1 D cells were treated with 5 uM 5-azacytidine (aza-C) for 60 hours. The cells were then harvested and the expression of MHVR was determined by FACS using anti-MHVR MAb-CC1. The experiments was representative of two similar experiments.


Shaded: Control Ab
Open : MAb CC1
CL 1D Cells-Untreated
CL 1D Cells-5uM aza-C
Number of Cells
10^0
10^1
10^2
10^3
10^4
MHVR Fluorescence Intensity

These authors observed increased MHV-A59 titers from actively growing DBT cells compared to resting cultures. Infection of cells at early stages of the S phase of the cell cycle yielded 6 to 12-fold higher titers of MHV than infection of cells at the G2/M phase of the cell cycle. Similarly, growth of Sendai virus (HVJ strain) in mouse 3T3 cells was inhibited in resting cells (Ogura, et al., 1984). Respiratory syncytial virus growth was stimulated 20 to 60-fold in actively growing Hep-2 cells (Pons, et al., 1983). Since I found that the level of MHVR expression in Cl 1 D cells also affected the yield of MHV, I investigated whether the level of MHVR expression was changed in transformed cells, contributing to their increased MHV production. We measured the MHVR levels by FACS in the control and transformed cell lines studied by Sturman and Takemoto (1972). The results are shown in Table 5. The N-BALB-3T3 cells were non-transformed 3T3 cells. SV-BALB-3T3 were SV40 virus-transformed N-BALB-3T3 cells, and ST-BALB-3T3 (17 Cl 1) cells were spontaneously transformed N-BALB-3T3 cells. Receptor blockade assay with MAb-CC1 followed by challenge with MHV confirmed that these cells used MHVR as their only functional receptor. Both DNA virus-transformed and spontaneously transformed cells expressed higher levels of MHVR than non-transformed cells. The cellular susceptibility to MHV-A59 infection was directly correlated to the level of MHVR expression. The level of MHVR in these

Table 5. Comparison of MHV suscepbility and MHV receptor expression in normal and transformed cells

Cell Line	Receptor Level[a]		Susceptibility[b]	Virus Yield[c]
	Peak Position	Mean		
N-BALB-3T3	0.75	1.2	+/-	1
SV-BALB-3T3	4.1	3.7	++	3x
ST-BALB-3T3 (17 Cl 1)	8.3	6.1	++++	3x

a. MHV receptor level was detected on FACS analysis using anti-MHVR MAb CC1.

b. Cells were inoculated with MHV-A59 at MOI of 3 PFU/cell and fixed with acetone at 7 hrs p.i.for indirect immunofluorescence labelling. +/-: < 1% cells were infected. ++: about 10%-40% cells were infected. ++++: >50% cells were infected.

c. Cells were inoculated with MHV-A59 at MOI 70 PFU/cell The cumulative yield of released virus from N-BALB-3T3 cells at 12 hours p.i. was used as 1. (Data from Sturman and Takemoto,1972)

cells also affected MHV yields as shown in Table 5. This result confirmed and extended our finding of the relationship between the level of MHVR expression and cellular susceptibility to MHV-A59 infection in L2, Cl 1 D and F40 cells. Similar observations were made with normal vs transformed cell lines from mouse embryo of the AL/N strain (data not shown).

The Expression of MHVR in cells selected by persistent infection with MHV-A59

Persistent infection with MHV *in vitro* has been reported by several laboratories (Lucas, *et al.*, 1977; Holmes and Behnke, 1981; Gombold, *et al.*, 1993). In general, these persistently infected cultures showed long term, continuous MHV production, little or no cytopathic effects and the selection of small plaque mutants. In collaboration with Dr. Sawicki (Medical College of Ohio), we examined the role of the expression of MHVR in persistent MHV infection *in vitro*. 17 Cl 1 cells were infected with MHV-A59 and persistent infection was established. This persistently infected culture was resistant to superinfection with wild type MHV-A59, but was susceptible to infection with other enveloped RNA viruses such as VSV virus. #97 cells were derived by single-cell cloning from the 97th passage of a persistently infected culture. These cells are free of MHV-A59 as no antigen expressing cells

were detected by immunofluorescent labelling of MHV antigen. When #97 cells and 17 Cl 1 cells were inoculated with MHV-A59, less than 5% of #97 cells became infected while almost 100% of the 17 Cl 1 cell was infected (Fig. 34). MHV infection of 17 Cl 1 and #97 cells was blocked by pretreatment of the cells with anti-MHVR MAb-CC1, indicating that no alternative receptor for MHV was expressed in these cells (Fig. 34). The MHVR level in #97 cells was markedly lower than that in parental 17 Cl 1 cells (Fig. 35). Interestingly, the MHV infection of #97 cells selectively eliminated the MHVR expressing population from the culture. These results, combined with the results from cells surviving MHV-A59 infection, suggested that the level of MHVR expression plays an important role in the establishment and maintenance of persistent MHV infection, and that MHV-A59 infection might alter the expression of MHV receptor in the cells.

Discussion

1. The Level of MHVR Expression as a Determining Factor of Cellular Susceptibility to MHV-A59 Infection

A productive viral infection depends on virus successfully overcoming host restrictions at the multiple steps of the virus life cycle. These restrictions can be qualitative or quantitative in their nature. Virus receptors are important determinants of both the host range

Fig. 34. MHV-A59 infection of 17 Cl 1 and #97 cells. 17 Cl 1 and #97 cells that were selected from a 17 Cl 1 culture persistently infected with MHV-A59 were inoculated with MHV-A59 at MOI of 5 PFU/cell. The cells were fixed at 8 hours p.i. and labelled with anti-MHV-A59 antiserum to detect of virus antigens. For MAb-CC1 protection, the cells were treated with MAb-CC1 supernatant (1:2 dilution) for 1 hour before and during virus inoculation.

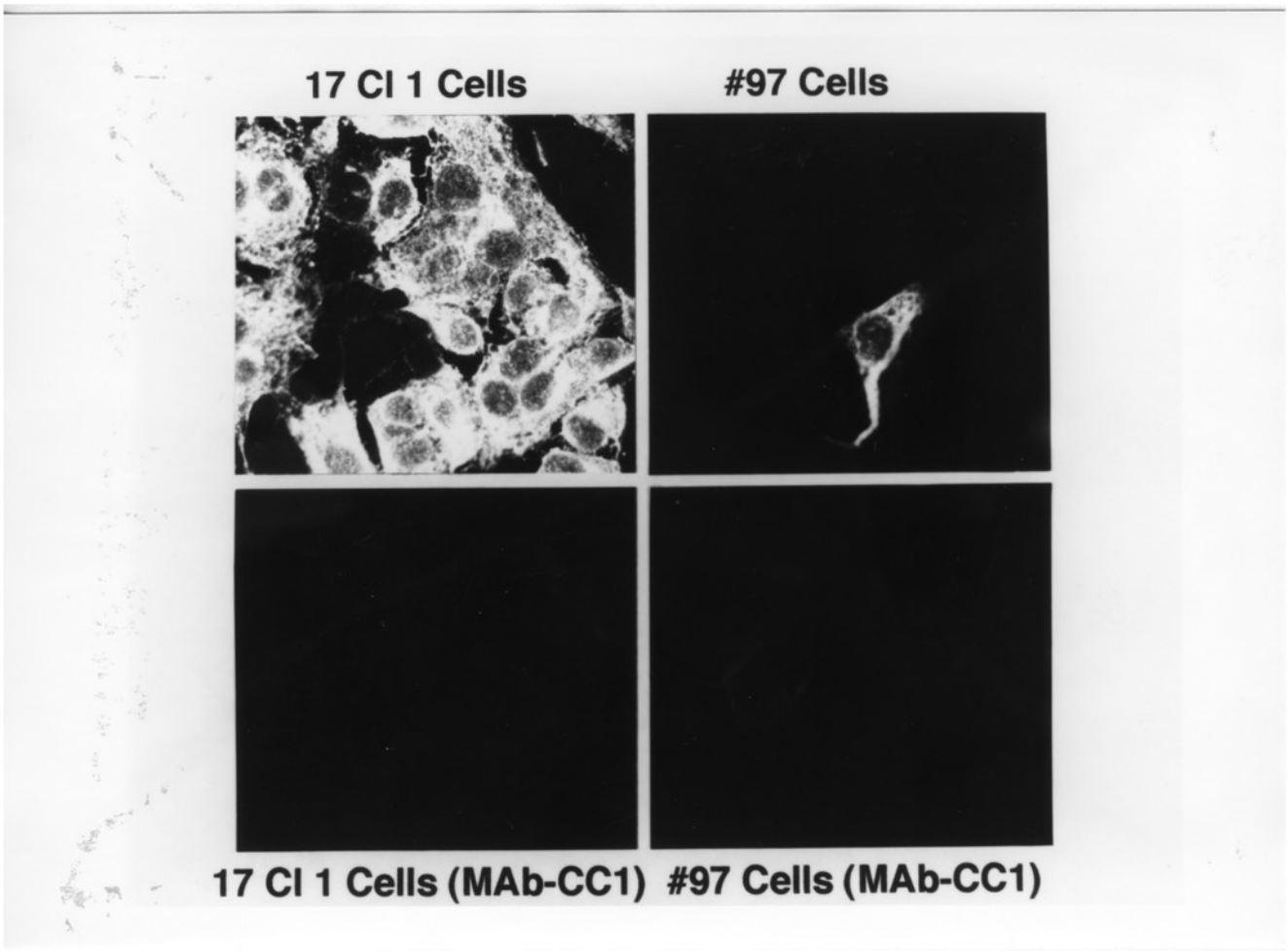
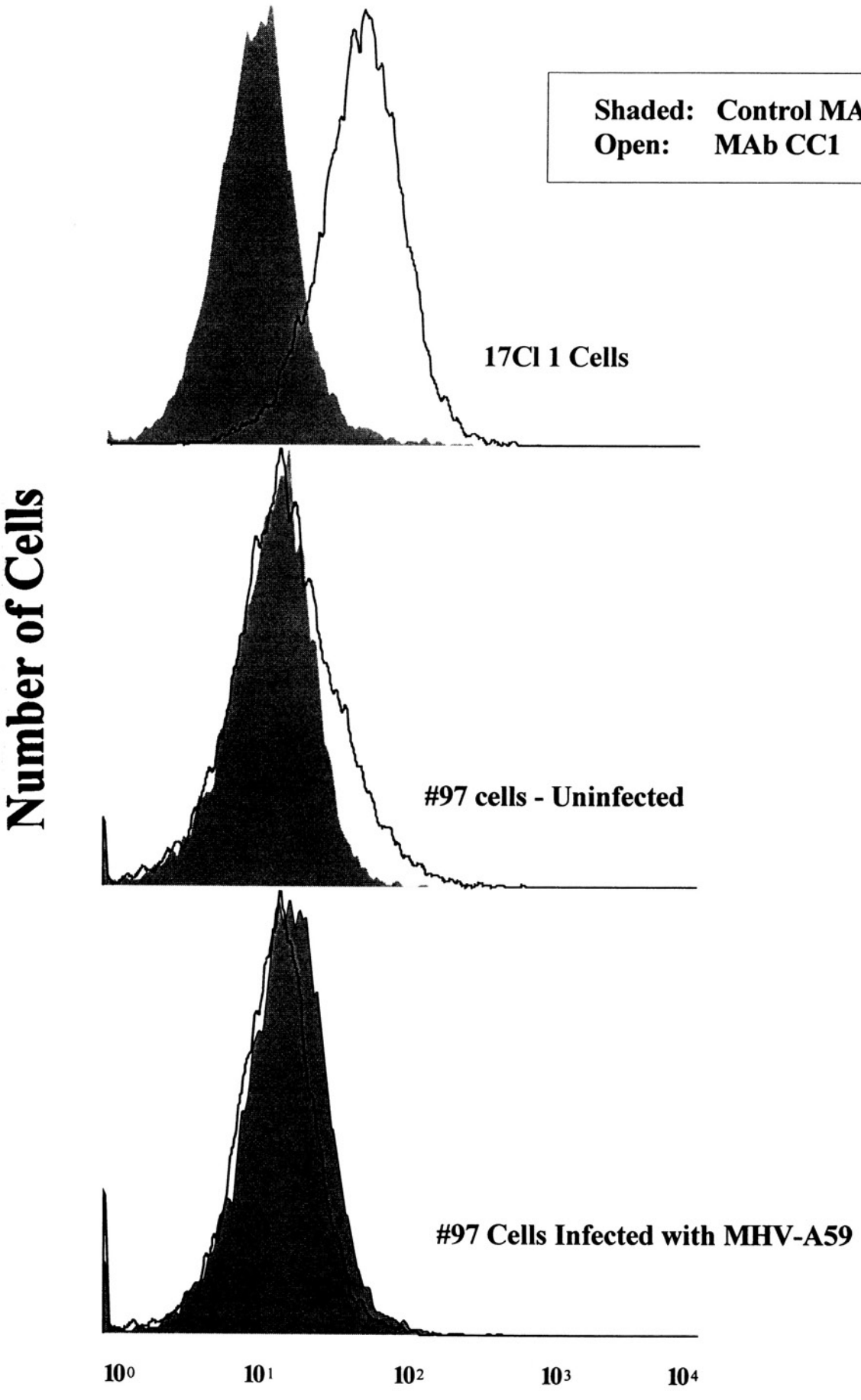

17 Cl 1 Cells
#97 Cells
17 Cl 1 Cells (MAb-CC1)
#97 Cells (MAb-CC1)

Fig. 35. The expression of MHVR in 17 Cl 1 cells, #97 cells, and MHV-infected #97 cells. The expression of MHVR was determined by FACS using MAb-CC1. An isotype matched monoclonal antibody was used as the control. Approximately 18% of #97 cells expressed MHVR by this assay, compared with more than 95% of 17 Cl 1 cells. The expression of MHVR in #97 cells infected with MHV-A59 for about one week was not detectable by this assay.


Shaded: Control MAb
Open: MAb CC1
17Cl 1 Cells
#97 cells - Uninfected
#97 Cells Infected with MHV-A59
Number of Cells
10⁰
10¹
10²
10³
10⁴
Relative Fluorescence of Cells

and tissue tropism of many viruses (Ren, et al., 1990; Koike, et al., 1991; Compton, et al., 1992). However, there are many exceptions (Maddon, et al., 1986; Mendelsohn, et al., 1989). One hypothesis is that several cell surface proteins are necessary to mediate virus entry either as a complex or in a series of sequential steps (reviewed by White, 1993). Other hypotheses focus on the fusion process of the virus membrane with the plasma membrane or endosomal membrane. Membrane phospholipids and pH have been implicated as parameters affecting this process (Helenius, et al., 1985; Herrmann, et al., 1990). The uncoating process of the viral genome has also been suggested as a potential step limiting cellular susceptibility (Yokomori, et al., 1993). In this study, we addressed the role of the MHV receptor, MHVR, in cellular susceptibility to MHV-A59 infection and in MHV-A59-induced fusion of different murine cell lines. We found that cellular susceptibility to MHV was mainly determined by the level of MHVR expression in cells even though these cells showed different composition of membrane lipid and different endocytic activities (Roos, 1984; Li, et al., 1986). The level of MHV-A59-induced cell fusion was also affected by the level of MHVR expression.

These results were consistent with other findings in our laboratory. For example, fibroblasts from MHV-A59-resistant SJL/J mice were resistant to MHV-A59 infection, but became susceptible to MHV infection after transfection

with MHVR or mmCGM2 (Dveksler, et al., 1993a), indicating the importance of the quantitative aspect of MHV-cell receptor interaction in determining cellular susceptibility to MHV infection. Theoretical model of virus-cell receptor interaction also supports the importance of receptor density on the cell surface in determining cellular susceptibility to infection. For example, in some virus-cell interaction systems, the model predicts that a 3-fold reduction of receptor number or receptor affinity results in a nearly 50-fold decrease in virus affinity for the cells (Wickham, et al., 1990).

These results are not necessarily in contradiction with the findings of other studies in which a cellular accessary factor for MHVR has been suggested to be necessary for MHV entry (Yokomori, et al., 1993). This putative secondary factor affecting MHV entry was believed to be able to discriminate S proteins of different MHV strains since only MHV-JHM infection was limited in the cells studied. Our observation that 5-azacytidine treatment of Cl 1 D cells induced the expression of MHVR and increased the MHV susceptibility of Cl 1 D cells argues that the presence of such factors as a limiting factor for MHV-A59 in Cl 1 D cells is unlikely. This experiment does not rule out the possibility that MHVR molecules may associate with other factors to become a functional receptor.

Our study supports the hypothesis that more than one

factor affects the outcome of MHV infection in different cell lines such as virus yield and virus induced cell fusion. For example, earlier studies showed the importance of cellular protease activity in controlling the levels of MHV-induced cell fusion (Frana, et al., 1985). We showed here that the level of expression of MHVR can also affect the degree of MHV-induced cell fusion.

2. Changes in the Level of MHVR Expression and Persistent MHV Infection in Cell Lines

It is interesting that the expression of MHVR in cell lines decreased quickly after MHV infection. There are other examples of cellular changes after virus infection, but the molecular nature of these changes is not clear. For instance, BHK-21 cells cured of acute foot-and-mouth disease virus (FMDV) infection by treatment with the cytostatic nucleoside analogue ribavirin (1-β-D-ribofuranosyl-1-H-1,2,4-triazole-3-carboxamide) at 12 hours p.i. or several days p.i. were several fold more resistant to FMDV infection than were mock-infected BHK-21 cells. This increased resistance was specific to FMDV virus, but not to other RNA viruses. The FMDV virus was not attenuated (Martin, et al., 1994). It would therefore be interesting to learn whether the cells have changed expression of the receptor for FMDV virus, similar to our findings in the MHV system.

In persistent viral infections, there is usually co-

evolution of viruses and cells (Younger and Preble, 1980; Holland, et al., 1980). While changes in viruses have been studied extensively, the changes induced in the host cells are usually not understood. Cellular mutations affecting reovirus entry steps have been suggested for persistent infection of mammalian reoviruses in L2 cells, but the molecular basis is not understood (Dermody, et al., 1993). Our studies showed that the #97 cell line derived from persistent MHV infection expressed a low level of MHVR in a small portion of the cells. In line with our finding that the level of MHVR expression also affected cell fusion, we predicted that a low level of MHVR expression is the explanation for low virus susceptibility and high resistance to fusion, two factors originally proposed for MHV persistence in vitro (Mizzen, et al., 1983). The rapid change in the level of expression of the MHV receptor which results from virus infection is important in both the establishment and maintenance of persistent MHV infection. Whether this change selected the fusion-defective MHV mutants that develop in persistently infected cultures is not yet clear.

The role of the level of expression of a virus receptor in persistent virus infection may not be limited to MHV. For example, HeLa cells resistant to poliovirus infection were selected after long term infection with poliovirus. The expression of poliovirus receptor was

significantly decreased in these cells (Kaplan and Racaniello, 1991).

3. Regulation of MHVR Expression and Implications for MHV Pathogenesis

There are few reports about the regulation of expression of murine CEA-related genes (Rosenberg, et al., 1993; Nédellec, et al., submitted). We found that 5-azacytidine enhanced cellular susceptibility to MHV infection in Cl 1 D cells possibly through activation of expression of MHVR. This result was surprising for two reasons. First, MHV can use glycoproteins encoded by more than one gene in the same family as receptors, yet the activation of gene expression by demethylation did not generate an alternative functional receptor in cells containing multiple CEA-related genes since infection can be blocked by anti-MHVR MAb-CC1. Second, in mouse hepatoma cells, demethylation at the possible promoter region of murine Bgp genes decreased the expression of the corresponding genes (Rosenberg, et al., 1993), in contrast to the up-regulation of expression of many other genes by demethylation (Razin and Cedar, 1991). It is possible that different cell types may utilize different mechanisms to control the expression of MHVR. Whether 5-azacytidine activated the expression of MHVR through directly demethylating the CpG in its promoter region or indirectly

through the activation of an activator of MHVR or inactivation of a putative repressor of MHVR is unknown. A better understanding of the control of MHVR expression will have great impact on our understanding of MHV pathogenesis such as persistent infection, age-dependent susceptibility to MHV infection, and virus tissue tropism. In some of the HeLa cells that are resistant to poliovirus infection, the gene encoding the poliovirus receptor (PVR) had a changed pattern of methylation (Kaplan and Racaniello, 1991). The MHVR gene in cells selected from persistently infected culture such as #97 cells may also show an altered methylation patterns that decrease the expression of MHVR. The rodent brain as well as other tissues have shown changes of methylation of genome in the aging process (Rath and Kanungo, 1989). Whether this change has any effect on MHVR expression needs to be examined, since young and adult mice show a marked difference in susceptibility to MHV infection and tissue tropism of MHV infection.

V. MHV INFECTION OF CL 1 D AND F40 CELLS AND THE EFFECT OF FATTY ACIDS ON MHV INFECTION

Introduction

As shown in the previous chapter, F40 cell monolayers showed higher levels of both MHV-induced cell fusion and virus yield than Cl 1 D cell monolayers. The level of fusion induced by MHV or several other enveloped viruses in both of these cell lines can be regulated by addition of saturated or unsaturated fatty acids to the culture medium (Roos, et al., 1990). For example, addition of linolenic (18:3) acid into Cl 1 D culture medium, not only decreases the ratio of saturated fatty acid (S) to polyunsaturated fatty acid (P) from 0.9 to 0.3, but also strongly inhibits the level of MHV-induced cell fusion. In contrast, nonadecanoic acid (19:0) increases the extent of MHV-induced cell fusion in Cl 1 D cells. In F40 cells, which have a S/P ratio of 2.9, addition of 18:3 fatty acid decreases and 19:0 fatty acid increases cell fusion (Roos, et al., 1990). However, the effect of fatty acids on cell fusion is not limited to 18:3 or 19:0 fatty acids. In general, unsaturated fatty acids (polyunsaturated fatty acids between 18:2 and 22:6) decrease virus-induced fusion of F40 cells and saturated fatty acids (between 14 and 24 carbons in length) increase the fusion of Cl 1 D cells (Roos, 1984). The mechanism underlying the differences between MHV

infection of F40 and Cl 1 D cells and the regulation of MHV induced cell fusion by fatty acids is not clear. Since once inside the cells, MHV replicated in Cl 1 D cells as readily as detected by the production of virus antigen, it was postulated that the inhibition of the MHV replicative cycle in Cl 1 D cell occurred before the synthesis of MHV antigen or at the virus maturation stage (Roos, et al., 1990).

Study of the difference between Cl 1 D and F40 cells and the mechanism of fatty acid modulation of cell fusion may help us to understand the control of cellular susceptibility to MHV infection and MHV spread between cells. We hypothesized that F40 and Cl 1 D cells differed in the expression of the MHV receptor and that fatty acids might regulate MHV-induced cell fusion in these cells by affecting the expression or function of MHV receptor. I describe here experiments to test this hypothesis. Based on the results from these experiments, a new hypothesis to explain the different effects of MHV infection in these cells is presented.

Results

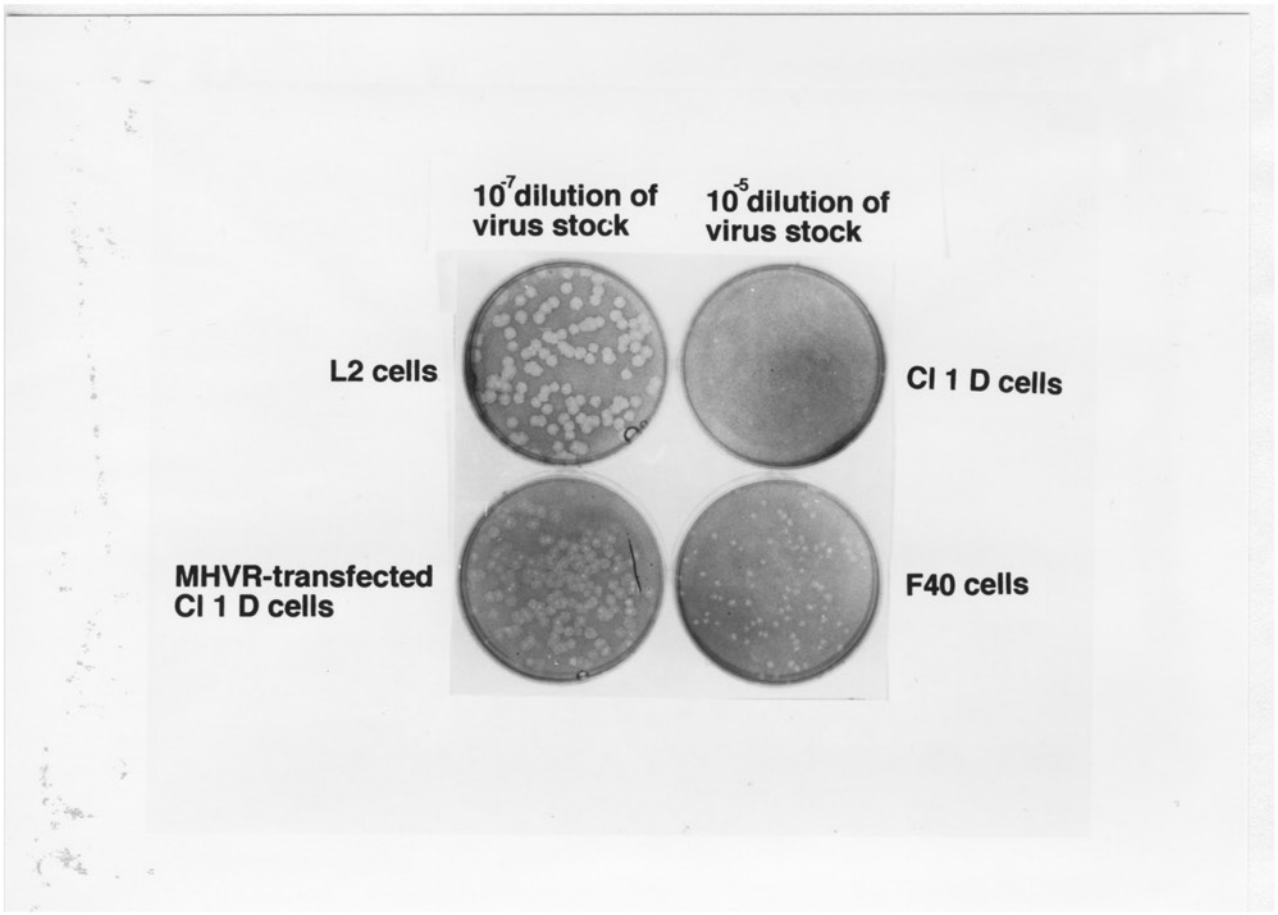
Effects of Fatty Acids on MHV Infection in Cl 1 D and F40 Cells

A possible mechanism for the difference of MHV infection between Cl 1 D and F40 cells could be that the expression/function of MHVR in these two cell lines differs

because of the difference of fatty acid composition in the cell membrane. The fatty acids may regulate cell fusion by regulating the expression or function of MHVR. If this is true, one would expect that Cl 1 D and F40 cells differ in their susceptibility to MHV-A59 infection and perhaps in their expression of MHV receptor. I showed in sections III and IV that each of these cell lines used MHVR glycoproteins as their functional MHV receptor, that they both expressed the same low level of MHVR, and that they were equal in their susceptibility to initial MHV-A59 infection. These findings made the above hypothesis unlikely. The following results were also consistent with this notion. L2 cells produced large plaques with high efficiency. Cl 1 D cells could not form visible plaques. Transfection of the MHVR plasmid into Cl 1 D cells increased cellular susceptibility to MHV-A59 infection, and the cells produced large plaques like those seen in L2 cells. Interestingly, F40 cells produced plaques, although they showed a much lower susceptibility to MHV-A59 infection than L2 cells (Fig. 36). Therefore one of the differences between F40 and Cl 1 D cells, the formation of plaques, is not linked to the expression of MHVR in these cells.

If fatty acids function through the regulation of MHVR expression, we would expect that unsaturated fatty acid (18:3) would inhibit and saturated fatty acids (19:0) would increase cell susceptibility to MHV infection in Cl 1 D and

Fig. 36. Comparison of plaque formation and MHV susceptibility in L2, Cl 1 D, F40 and MHVR-transfected Cl 1 D cells. Cells were inoculated with MHV-A59 at 37°C for 1 hour. The virus inoculum was removed and the cells were washed and then overlaid with medium containing 0.9% agar. Two days later, the monolayers were stained with 0.02% neutral red and plaques were counted.


10^{-7} dilution of virus stock
10^{-5} dilution of virus stock
L2 cells
Cl 1 D cells
MHVR-transfected Cl 1 D cells
F40 cells

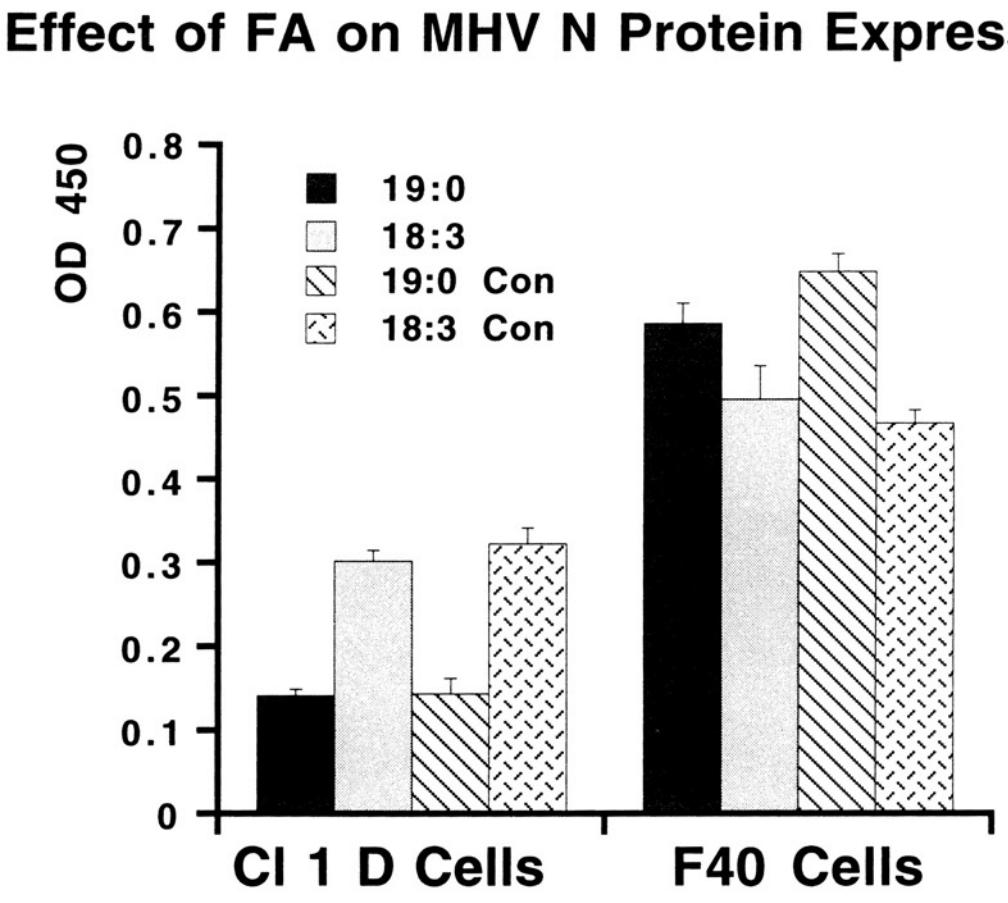
F40 cells, since we showed that the level of cell fusion and cell susceptibility are correlated to the level of expression of MHVR. The infectious center assay was used to test this hypothesis in F40 cells. Table 6 shows that after pre-treatment with fatty acids (19:0 or 18:3) for 24 hours, F40 cells did not show any significant difference in their susceptibility to MHV-A59. The same conclusion was reached using plaque assays of infectious virions from treated F40 cells. When untreated or fatty acids-treated F40 cells were inoculated with the same MHV stock, the number of plaques formed in untreated or treated cells did not change, although unsaturated fatty acids caused a reduction of the size of plaques (data not shown). When the levels of N antigen of MHV in infected cells were compared in fatty acid-treated Cl 1 D and F40 cells, it was found that fatty acids had different effects on the production of N antigen in Cl 1 D and F40 cells (Fig. 37). F40 cells treated with unsaturated fatty acid (18:3) expressed a lower level of N protein than did F40 cells treated with saturated fatty acid (19:0). If the fatty acids were added back after MHV inoculation, the difference of the levels of N antigen in different fatty acids-treated cells was augmented, suggesting that fatty acids may also affect cellular events other than MHV entry (Fig. 37). In contrast to F40 cells, treatment with unsaturated fatty acid (18:3) increased the level of MHV N antigen in Cl 1 D cells, treated before or

Table 6: Effect of Fatty Acid Treatment of F40 cells Upon Infectious Centers

MOI	Treatment		
	19:0	18:3	None
10	25 ±5	17 ±5	31 ± 4
1	6 ±3	3 ±1	8 ± 2

Note: The cells were treated with fatty acid (100 uM) for 24 hours and infected by MHV at the MOI indicated. $1X10^6$ cells were diluted and seeded on L2 monolayers. The number of infectious centers was the average number from 3 (for untreated sample) or 5 dishes (fatty acid treated sample) plated with 1000 cells.

Fig. 37. Alteration of production of MHV N protein in Cl 1 D and F40 cells after treatment with fatty acids. The Cl 1 D and F40 cell lines were grown in 96 well plates and treated with 100 uM of the fatty acids (FA) nonadecanoic acid (19:0) or linolenic acid (18:3) for 24 hours. The cells were then inoculated with 10^6 PFU of MHV-A59 in 100 ul of medium at 37°C for 1 hour. After removing the virus inoculum, the cells were incubated continuously for another 14 hours with fresh medium without fatty acids (19:0 and 18:3) or with fatty acids (19:0 con and 18:3 con). The cells were fixed with cold methanol, and the N protein in infected cells was detected by ELISA using a monoclonal antibody against MHV nucleocapsid protein.


Effect of FA on MHV N Protein Expression
OD 450
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
19:0
18:3
19:0 Con
18:3 Con
Cl 1 D Cells
F40 Cells

before/after MHV inoculation (Fig. 37). If fatty acids only affected the expression or function of MHVR, we would have expected that unsaturated fatty acids would inhibit and saturated fatty acids would increase the levels of N antigen similarly in both Cl 1 D and F40 cells, since both types of fatty acids have similar effects on MHV-induced cell fusion in Cl 1 D and F40 cells. Therefore, our finding of different effects of fatty acids on the production of MHV N antigen in the two cell lines suggested that fatty acids may regulate cellular factors other than MHVR to affect the MHV-induced cell fusion in Cl 1 D and F40 cells.

The Expression of MHV Antigen in Cl 1 D and F40 Cells

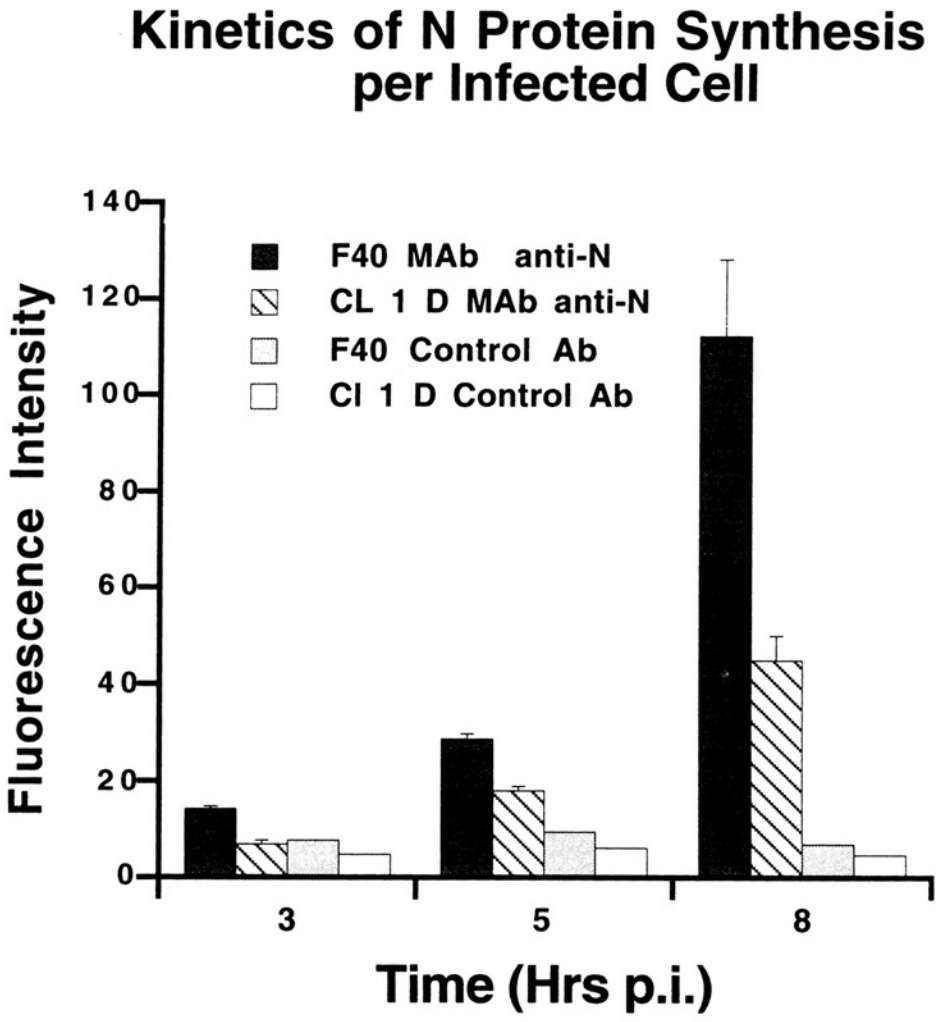
At 13 hours p.i., the level of N antigen expressed in F40 cells was much higher than in Cl 1 D cells as detected by ELISA assay, although both cell lines were infected at approximately the same level at the initial stage (Fig. 10). Since at 13 hours p.i., the level of cell fusion in F40 cells was much higher than in Cl 1 D cells, it was possible that the increased level of N protein was due to the recruitment of additional uninfected cells by fusion rather than production of higher amounts of N antigen by F40 cells than by Cl 1 D cells. Treatment of Cl 1 D cells with unsaturated fatty acid inhibited cell fusion, yet the level of N antigen in the culture increased, suggesting that the level of fusion may not be the only reason for the

difference of level of N protein in Cl 1 D and F40 cells. A FACS analysis was used to address this question. The infected F40 and Cl 1 D cells were permeabilized and labelled with monoclonal antibody against nucleocapsid protein (N), and the level of N protein expressed in single infected cells was examined by FACS. As shown in Fig. 38, even at 3 hours post inoculation, the amount of N protein per individual infected F40 cell was significantly higher than that detected in Cl 1 D cells. This difference was maintained and increased, with longer incubation. The same experiment showed that the production of N protein in L2 cells was also higher than that in Cl 1 D cells (data not shown). Thus, different cell lines showed quantitative differences in the intracellular production of virus N antigen.

The Production of MHV Virions in Cl 1 D and F40 Cells

The finding that single infected F40 cells expressed higher levels of N antigen than Cl 1 D cells, could help to explain the differences of MHV susceptibility between F40 and Cl 1 D. First, like N antigen, the levels of other MHV antigens including the spike protein which are required for cell fusion may also be higher in F40 cells than in Cl 1 D cells. Higher levels of spike proteins on the membrane of F40 cells than on Cl 1 D cells could increase the level of cell fusion in F40 cells. Second, the level of N protein in

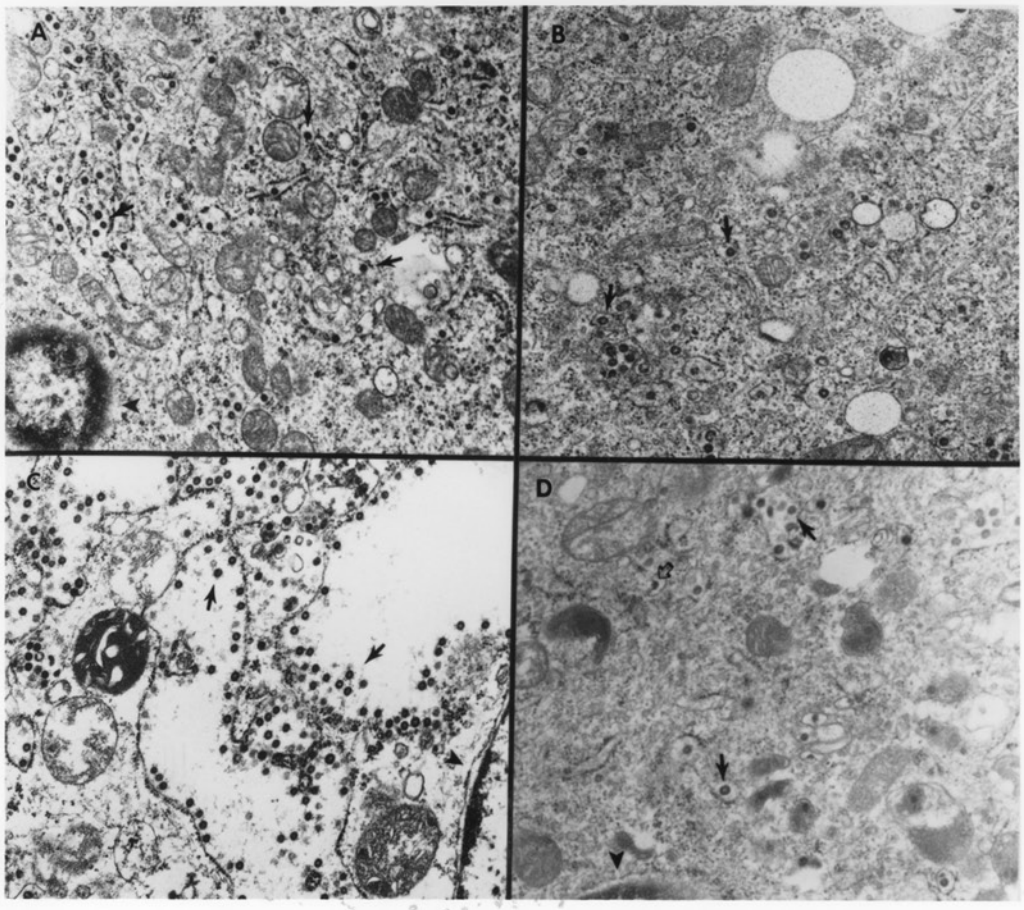
Fig. 38. Comparison of MHV N protein synthesis in MHV infected Cl 1 D and F40 cells. Cl 1 D and F40 cells were infected with MHV-A59 at a MOI of approximately 10. After virus inoculation, MAb-CC1 (1:5 dilution) was added to the cells to prevent cell fusion. The cells were harvested at 3, 5, 8 hours p.i.. The cells were fixed with 2% paraformaldehyde at 4°C. To determine the level of N protein by FACS, the cells were permeabilized with 0.2% Triton X-100 and stained with monoclonal antibody against nucleocapsid protein followed by phycoerythrin-coupled goat anti-mouse IgG_1. The level of N protein in single infected cells was expressed as the mean intensity of immunofluorescence.


Kinetics of N Protein Synthesis
per Infected Cell
Fluorescence Intensity
140
120
100
80
60
40
20
0
F40 MAb anti-N
CL 1 D MAb anti-N
F40 Control Ab
Cl 1 D Control Ab
3
5
8
Time (Hrs p.i.)

the cells may affect the replication or transcription of the MHV genome, thereby, affecting either the production of other MHV antigens or assembly of virions in infected cells.

Cultures of F40 cells yielded higher amounts of infectious virions than did Cl 1 D cells. The virion production in these two cell lines was also compared directly by electron microscopy. The results are shown in Fig. 39. Mock infected L2, Cl 1 D and F40 cells contain a low level of retrovirus which is known to be in L cells. These retroviruses were readily distinguished from MHV virions by electron microscopy (data not shown). In MHV-A59 infected L2 cells, at 6 hours p.i., many MHV virions had already formed (Fig. 39A). MHV virions were also present in infected MHVR-transfected Cl 1 D cells at 6 hours (Fig. 39B). Infected F40 cells at 24 hours p.i. contained many virions in membrane vesicles (Fig. 39C), and also in the lumen of the nuclear membrane (data not shown). The spikes typical of coronaviruses can be seen clearly on the virions (arrow). In contrast to infected F40 cells, infected Cl 1 D cells contained few MHV virions at 24 hrs p.i. When virus was found, only a few virions were present in each field (Fig. 39D). To compare the MHV production in infected Cl 1 D, F40 and MHVR-transfected Cl 1 D cells inoculated at an MOI of 25 PFU/cell and collected at 24 hours p.i., ten fields were randomly selected for Cl 1 D and F40 cells at 15,000X magnification. Examination of these fields showed

Fig. 39. MHV-A59 production in infected L2, Cl 1 D, F40 and 9B1 cells. The cells were infected with MHV-A59 at MOI of 25 PFU/cell. The samples were fixed at 6 hours or 24 hours p.i., embedded in Epon and sectioned for electron microscopy. A. Infected L2 cells, 6 hours p.i. B. Infected 9B1 cells, 6 hours p.i. C. Infected F40 cells, 24 hours p.i. D. Infected Cl 1 D cells, 24 hours p.i. Arrow: the MHV virions. Arrow head: the cell nucleus. A budding MHV virion in Cl 1 D cells was indicated with open arrow. (Magnification 30,000X).


A
B
C
D

that MHV virions were present in 10 fields from F40 samples. The number of virions in each field varied greatly. In 5 fields counted, two had less than 50 virions, two had more than 800 virions, and one had about 250 virions. In Cl 1 D samples, only two among 10 fields showed MHV virions. In MHVR-transfected Cl 1 D cells, seven among 10 fields showed MHV virions. Four fields had fewer than 20 virions and two had about 250 virions. Virions in membrane vesicles from one field were compared in infected L2, 9B1 and F40 cells harvested at 24 hours p.i., I found that L2 cells had an average of about 16 virions/vesicles, at a magnification of 30,000, compared to 3 and 25 for 9B1 and F40 cells respectively. Therefore, infected F40 cells may produce more MHV virions than infected Cl 1 D cells.

Comparison of MHV Growth in Cl 1 D and F40 Cells Transfected with MHVR.

In Cl 1 D and F40 cells, only a small portion of cells became infected after inoculation at an MOI of 5 PFU/cell). Results from these cells may not be representative of the whole cell population. We therefore compared the virus yields from MHVR-transfected Cl 1 D and F40 cells. F40 cell clones that stably express high levels of MHVR were selected and the levels of MHVR expression in these cells were examined by FACS analysis. Two clones (F9C1 and F9C4) that expressed comparable levels of MHVR to the MHVR-transfected

Cl 1 D cells were used for comparison of virus infection (Fig. 40). These cells and 9B1 and F40 cell were infected at an MOI of 20-25 PFU/cell. Fig. 40 (A+B) shows that at an early time point, the virus yield from MHVR-transfected F40 cells was higher than that from untransfected F40 cells, and did not exceed the yield from MHVR-transfected Cl 1 D cells. However, at a later stage of MHV-A59 infection, the virus yield from F40 cells was the highest. Thus, the level of expression of receptor is an important determinant of initial susceptibility to infection with MHV-A59, while later spread of infection from cell to cell may be less dependent upon the level of receptor expression.

Discussion

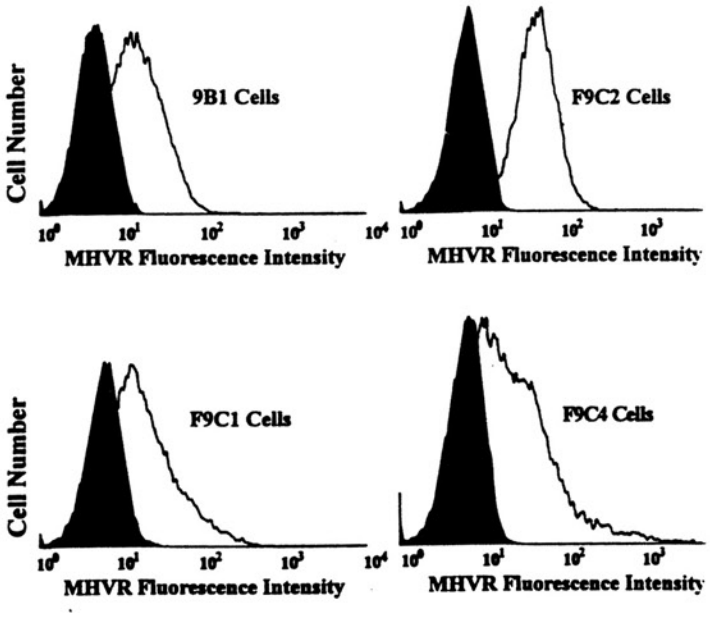
In this chapter, I described the experiments designed to analyze mechanisms by which Cl 1 D and F40 cells yielded different amounts of MHV and different levels of MHV-induced cell fusion, and the mechanisms through which fatty acids regulated MHV-induced cell fusion.

Fatty acids on the cell membranes have many functions (Nunez, 1993). Detailed discussion of these functions is beyond the scope of this thesis. However, several functions are pertinent to our studies.

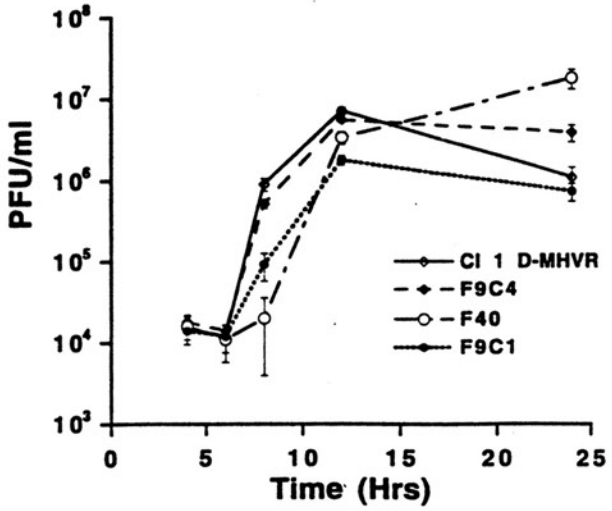
1. Fatty acids in the membrane can affect the function of some receptor molecules. For examples, the number and the affinity of insulin-binding sites on insulin receptors

Fig. 40. MHV-A59 growth in F40 cells and MHVR-transfected cells. F9C4 and F9C1 were two MHVR-transfected F40 cell clones which had levels of expression of MHVR comparable with MHVR-transfected Cl 1 D cells (9B1) as determined by FACS (A). Confluent cells grown in 24 well plates were infected at MOI of 20 to 25 PFU/cell. Virus in the medium was collected and the virus yield was determined by plaque assay on L2 cells (B).

A



B Comparison of Virus Yield in Different Cells



are affected by the degree of unsaturation of the membrane phospholipids that surround the insulin receptor molecules. An increase in the degree of unsaturation of membrane phospholipids is associated with an increase in the number of insulin binding sites and a decrease in the affinity of these sites. It was postulated that the insulin receptors may exist in two forms. The low affinity receptor form may self-associate to form the high affinity receptor form. In an unsaturated lipid environment, the high affinity receptor tends to dissociate and form the low affinity receptor (Gould, et al., 1982).

2. Fatty acids can participate in the pathway for signal transduction, which may in turn affect the expression of some genes. For example, diacylglycerol (DAG) can stimulate the activity of protein kinase C. Interestingly, an unsaturated fatty acid at position 1 or 2 or both in DAG is needed for DAG to exert this function (Hirata, 1984).

Based on these observations, we postulated that the degree of unsaturation of fatty acids may regulate the expression or function of MHVR in Cl 1 D and F40 cells, and thereby alter MHV-induced cell fusion through the interaction of MHVR with MHV spike protein in these cells. Our results did not favor this hypothesis. The difference of virus yield and fusion in Cl 1 D and F40 cells probably resulted from cellular factors other than MHVR. Our results suggested that the virus yield was not controlled only at

virus entry stages.

Instead of MHVR, we found that the level of N protein of MHV was different in infected Cl 1 D and F40 cells, and that fatty acids affected the production of N protein. The N protein of MHV apparently regulates the *in vitro* synthesis of MHV RNA (Compton, *et al.*, 1987). For vesicular stomatitis virus, the level of N protein in a cell has been postulated to regulate the switch of synthesis of mRNA to genomic RNA (Blumberg, *et al.*, 1981). A similar function has been postulated for MHV N antigen in infected cells (Lai, 1990). If this hypothesis is true, it may explain why F40 cells have a higher virus yield than Cl 1 D cells, particularly at the later stages of MHV infection. Both saturated and unsaturated fatty acids affected N protein expression in F40 cell and Cl 1 D cells, but in both cell lines, the virus yield at 14 hours p.i. was not changed significantly by fatty acid treatment (result of at least 5 experiments, data not shown). These results suggest that fatty acids may also affect other cellular processes required for successful virus production.

Why infected F40 cells have a higher level of N protein than infected Cl 1 D cells is unknown. F40 cells grow faster than Cl 1 D cells, and MHV infection does not shut down the host protein synthesis machinery very efficiently. These two factors may affect the production of viral antigens and possibly of virions in F40 or Cl 1 D cells. The production

of MHV is affected by the cell cycle as discussed in Section IV (Talbot and Daniel, 1987). In line with this consideration, the effect of 18:3 on the different levels of N proteins in Cl 1 D and F40 cells may be explained as follows: Lysophospholipids with unsaturated fatty acids were recently shown to stimulate the cell growth of quiescent fibroblast cells, but to inhibit the growth of tumor cells (Moolenaar, 1994). It is possible therefore that 18:3 stimulated the growth of Cl 1 D cells which grow slowly and were only weakly tumorigenic (Table 3), but inhibited the growth of F40 cells which were highly tumorigenic, and therefore affected the production of N protein differently in these two cells.

The difference of the MHV yields between F40 and Cl 1 D cells can not be explained simply by the difference of cell fusion observed in these two cell lines. The difference of MHV-induced cell fusion between Cl 1 D and F40 cells is less than 5-fold, but the difference of virus yield is more than 50 fold. Using the electron microscope, we observed that infected F40 cells produced more viruses than infected Cl 1 D cells. This result was consistent with the yields of infectious virus from MAb-CC1 treated samples. These results demonstrated that single infected F40 cells produced more MHV virions than Cl 1 D cells. Combined with the levels of fusion in cells, this result may explain why F40 cells have higher virus yields than Cl 1 D cells.

MHVR-transfected F40 cells did not show higher levels of virus yield than MHVR-transfected Cl 1 D cells. This is probably not because of the particular MHVR-transfected F40 cell clones selected. F40 cells were relatively uniformly resistant to PEG-induced cell fusion. In our experiments, two clones of MHVR-transfected F40 cells gave us basically the same result, i.e., they did not yield more virus than MHVR-transfected Cl 1 D cells. It was more likely that the kinetics of the fusion process altered the virus yield (Sawicki and Sawicki, 1986). The fact that F40 cells have a higher virus yield in these experiments (Fig. 40) also supports this idea. Extensive cell fusion may cause cell to die earlier so that less infectious virus can be produced. In the other hand, moderate cell fusion may facilitate the spread of viruses to uninfected cells, increasing the virus yield.

Since the MHV yield and MHV-induced cell fusion are interrelated, in the experiments described, we tried to separate two processes by examining the MHV infection not only at the level of cell population, but also on a single cell basis. The fusion and virus yield observed in F40 cells and Cl 1 D cells can be partially explained by our findings that the two cell lines differ in the production of MHV antigens and virus in single cells.

I therefore postulate that the difference between F40 cells and Cl 1 D cells with regard to MHV infection probably

occurs after MHV entry. The level of MHV N protein and possibly of other virus antigen as well were higher in F40 cells than in Cl 1 D cells. Virus antigen production, particularly of the spike protein, may contribute to the higher level of MHV-induced cell fusion in F40 cells through the interaction with MHVR on the cell membrane. Cl 1 D cells had low level of expression of MHVR and low level of MHV antigens including spike proteins. Therefore, these cells yield low level of virions and show little virus-induced cell fusion. The synthesis of viral RNA and other stages of MHV replication cycle need to be examined in these cells to identify factors that may control the levels of MHV antigens expressed in infected cells.

VI. SIGNIFICANCE

Ever since Holland and coworkers showed that the host range of polio viruses may be determined by the restriction occurring at the cell surface mediated by binding of viral capsid proteins to species-specific receptors (Holland, et al., 1959; McClaren, et al., 1959), the idea that specific virus receptors serve as determinants for both host-range and tissue tropism has been vigorously pursued (Crowell, et al., 1981; Lentz, 1990; Nomoto, 1992; Haywood, 1994). While many examples supported the hypothesis that specific receptor molecules serve as host-range determinants (Racaniello and Baltimore, 1981; Ahearn, et al., 1988; Dveksler, et al., 1991), increasing evidence has accumulated to suggest that other factors in addition to virus receptors determine the tropism of virus infection. Several new themes have emerged with regard to the virus-host cell interaction:

1. The diversity of the nature of virus receptor molecules. While some viruses use specific proteins with limited tissue expression as their receptors, many viruses choose entities such as proteins, sugars, or lipid molecules as their receptors that are distributed everywhere. The expression of the receptor for Epstein-Barr virus (EBV), the type 2 complement receptor (CR2 or CD21), is limited to B lymphocytes and some epithelial cells, which are the precise

targets of virus infection (Cooper, et al., 1988). However, the receptor for influenza, sialic acid, is present on many cell surfaces, yet the main target of influenza virus is the upper respiratory tract.

2. The divergence and convergence of virus receptor utilization. Viruses in the same family or same genus may use different molecules as their receptors. Among the picornaviridae, for examples, the poliovirus receptor is an Ig-like protein, while major group of rhinoviruses utilizes ICAM-1 and the minor group of rhinoviruses uses an LDL receptor-related protein as their respective receptors (Greve, et al., 1989; Hofer, et al., 1994). Furthermore, even one virus such as MHV and HIV can use multiple molecules as its receptors (Bhat, et al., 1991; Dveksler, et al., 1993a). Conversely, different viruses may use similar molecules as their receptors, although the diseases resulting from these virus infections may be totally different. Recent studies, for example, have shown that Herpesvirus 7 also used CD4 molecules, which is the receptor for HIV, as its receptor (Lusso, et al, 1994).

3. Dependence of successful virus entry upon the interaction of the virus with more than one molecule on the cell surface. The interaction of a virus with its receptor may merely facilitate the further interactions between the virus and a host cell. Therefore, the critical steps limiting the infection of cells may lie down-stream of the

virus-virus receptor interaction. Several studies suggested the requirement of accessory factors for virus entry (Yokomori and Lai, 1993; McKnight, et al., 1994). Even for EBV, the best sample so far to show the determination of tissue tropism by the virus receptor, there are data which showed EBV can only achieve a very low level of infection in some cells that express a high level of expression of CR2, suggesting the requirement of other factors for virus entry (Carel, et al., 1989). For Sendai virus, proteolytic cleavage of the viral envelope glycoprotein F (fusion protein) is required for viral infectivity, while the virus can bind to sialoglycoproteins on many cells (Schied, et al., 1974).

In light of this general complexity with regard to virus-host cell interactions, the results of MHV-A59 infection in different cell lines are somewhat of a surprise. The seemly simple result that the cellular susceptibility of different cell lines to MHV-A59 infection is directly correlated with the level of expression of MHV receptor among these cell lines, may well reflect some unique features of the interaction between MHV-A59 and host cells:

1. Binding and fusion functions are carried out by the same spike-glycoprotein. Since MHV contains a positive-sense RNA genome, the fusion process may be a critical step in determining the level of viral infection in the cells.

Because the binding and fusion functions are carried out by the same protein, it is, therefore, conceivable that the level of fusion is directly proportional to the binding capacity of the cells which is in turn determined by the levels of expression of the virus receptor.

2. MHV-A59 can enter cells through direct fusion on the cell surface. This characteristic of virus entry bypasses the regulation of the vesicle acidification step which may vary in different cells.

3. The end point of MHV infection in cells that I used for my experiments bypassed the effects of proteolytic cleavage of MHV spike proteins. It is well established that the cleavage of MHV spike proteins affects the capability of the spike glycoproteins to induce membrane fusion (Frana, et al., 1985). The results after the first infectious cycle may be affected by the amount and property of virus progeny produced in different cell lines other than cellular differences themselves.

With these constraints in mind, it may be expected that different strains of MHV may behave differently in terms of how the infection of cells is affected by the MHV receptor. For example, MHV-JHM contains two glycoproteins, HE and S, which can interact with different ligands on the cell surface. How the interactions of these two attachment proteins with cell surface affect MHV-JHM fusion which is mediated by spike-receptor interaction needs to be

addressed.

While the role of virus receptor molecules in virus entry has been appreciated since the realization of the presence of specific receptor for virus, little attention has been paid to the contribution of the virus receptor to cell injury induced by virus infection. For example, the fusion induced by some viruses on adjacent cells, the so called "fusion from without" process, has long been recognized to be different from fusion induced by viral spike proteins expressed on the infected cell surfaces, the "fusion from within" process (Bratt, et al., 1969). Do these two processes have fundamentally different requirements for the involvement of virus receptor? In many in vitro systems for virus persistence, cell injury is decreased. Interestingly, few studies have addressed the role of virus receptors in this process. The underlying assumption may be that if a virus infection is persistent, the cells must express the virus receptor. However, as shown in this study as well as in some recently published studies of parainfluenza virus (Moscona and Peluso, 1993), the virus receptor plays an important role in determining the level of cell injury.

Further understanding of the molecular mechanisms of the interaction between MHV and its receptor is needed. Does the interaction of MHV with its receptor result in a change in conformation of the viral spike proteins? Do

different MHV strains use the receptor differently? Does the low level of expression of MHV receptor on cells pose a pressure for selection of some virus mutants? The answers to these questions will not only benefit the understanding of pathogenesis of MHV infection, but also help to understand viral entry in general and to design drugs aimed at interrupting early stages of virus-receptor interaction to combat viral infections.

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