Persistent Infection Promotes Cross-Species Transmissibility of Mouse Hepatitis Virus

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Persistent infection with mouse hepatitis virus (MHV) strain A59 in murine DBT (delayed brain tumor) cells resulted in the emergence of host range variants, designated V51A and V51B, at 210 days postinfection. These host range mutants replicated efficiently in normally nonpermissive Chinese hamster ovary (CHO), in human hepatocarcinoma (HepG2), and to a lesser extent in human breast carcinoma (MCF7) cell lines. Little if any replication was noted in baby hamster kidney (BHK), green African monkey kidney (COS-7), feline kidney (CRFK), and swine testicular (ST) cell lines. By fluorescent antibody (FA) staining, persistent viruses V10B and V30B, isolated at days 38 and 119 days postinfection, also demonstrated very low levels of replication in human HepG2 cells. These data suggest that persistence may rapidly select for host range expansion of animal viruses. Pretreatment of HepG2 cells with a polyclonal antibody directed against human carcinoembryonic antigens (CEA) or with some monoclonal antibodies (Col-1, Col-4, Col-12, and Col-14) that bind human CEA significantly inhibited V51B infection. Under identical conditions, little or no blockade was evident with other monoclonal antibodies (kat4c or Col-6) which also bind the human CEA glycoproteins. In addition, an antibody (EDDA) directed against irrelevant antigens did not block V51B replication. Pretreatment with the Col-4 and Col-14 antibodies did not block Sindbis virus replication in HepG2 cells or MHV infection in DBT cells, suggesting that one or more CEA glycoproteins likely functioned as receptors for V51B entry into human cell lines. To test this hypothesis, the human biliary glycoprotein (Bgp) and CEA genes were cloned and expressed in normally nonpermissive BHK cell lines by using noncytopathic Sindbis virus replicons (pSinRep19). By growth curves and FA staining, human CEA and to a much lesser extent human Bgp functioned as receptors for V51B entry. Furthermore, V51B replication was blocked with polyclonal antisera directed against human CEA and Bgp. Under identical conditions, the parental MHV strain A59 failed to replicate in BHK cells expressing human Bgp or CEA. These data suggest that MHV persistence may promote virus cross-species transmissibility by selecting for virus variants that recognize phylogenetic homologues of the normal receptor.

Animal virus host range specificity and the evolution of new viral diseases are complex phenomena involving interactions between the virus, the host, and the environment (2). Although some new diseases may have resulted from mutations that altered virus tissue tropism, virulence and pathogenesis in the normal host, many new human viruses probably arose by cross-species transmissibility from animal reservoirs (21, 22, 41). Recent examples of emerging viruses include equine morbillivirus, human immunodeficiency viruses (HIVs), hantavirus, hemorrhagic fever viruses, arboviruses, and influenza viruses (27, 45, 47). While these emerging viruses are highly heterogeneous in their structures and replication strategies, they probably have bridged the species barrier by evolving the capacity to interact with specific cellular factors which regulate virus entry, replication, or transmissibility in the new host species. Unfortunately, few studies have attempted to link specific conditions of environmental change with the molecular targets and evolutionary mechanisms that promote the emergence and cross-species transmissibility of animal viruses.

Coronaviridae include a diverse group of highly species specific avian and mammalian viruses and are excellent models to study the fundamental principles governing virus cross-species transmissibility and xenotropicism (4, 21, 22, 41). For mouse hepatitis virus (MHV), host range specificity is almost exclusively mediated at entry since the genomic RNA is infectious in nonpermissive cell lines and expression of the MHV receptor (MHVR), a biliary glycoprotein (Bgp1), converts nonpermissive hamster, human, and primate cell lines into susceptible hosts for virus replication (21, 22, 38). In addition to MHVR, a codominant Bgp1 allele (Bgp1P), a second biliary glycoprotein (Bgp2), and a brain pregnancy-specific carcoembryonic antigen (CEA) glycoprotein may also function as weak receptors for MHV entry in the mouse (11, 51, 70). Although MHV is highly species specific, intracranial inoculation of MHV-JHM into the primate central nervous system (CNS) has resulted in the rapid emergence of primate-adapted strains of MHV that induced encephalitis and demyelination in the new host (50). Using an in vitro model that may reflect conditions present in heavily immunosuppressed xenograph recipients, we have shown that MHV rapidly evolves the capacity to replicate efficiently in many different species (4).

MHV normally causes an acute, self-limited cytopathic infection, but persistent infections can be established in neonatal or immunosuppressed mice and in cultured cells in vitro (12, 26, 53, 55). Following infection, MHV may also persist for a year or more in the CNS of susceptible mice (26). Although the molecular mechanisms of MHV persistence in vivo are unknown, persistent infection in DBT (delayed brain tumor) cells...
is likely mediated by virus selection for host cells that resist infection by downregulating expression of MHVR (12, 55). In response to emerging host cell resistance, persistent viruses evolve increased virulence and display increased affinity for MHVR and perhaps other Bgp receptors for entry (12). In this report, we demonstrate that persistent MHV infection rapidly promotes the emergence of host range mutants of MHV which replicate efficiently in normally nonpermissive Chinese hamster ovary (CHO) and human cell lines. These variants replicated poorly in baby hamster kidney (BHK) and swine testicular (ST) cells. Using antibody blockade and transfection studies, we showed that persistent viruses use human CEAs (hCEAs) as receptors for entry into nonpermissive hosts. Persistent infection may promote MHV cross-species transmissibility by selecting for viruses that recognize phylogenetic homologues of the normal receptor.

MATERIALS AND METHODS

Virus and cell lines. DBT cells were originally established from a delayed brain tumor in a CDF1 mouse inoculated intracerebrally with the Schmidt-Ruppin strain of Rous sarcoma virus (12). DBT cells were maintained in Eagle’s minimum essential medium (MEM) containing 8% fetal clone II supplemented with 5% tryptose phosphate broth (TPB), gentamicin (0.05 \( \mu \)g/ml), and kanamycin (0.25 \( \mu \)g/ml). BHK cells were kindly provided by Robert E. Johnston (University of North Carolina at Chapel Hill) and maintained within 12 passages of the original stock in alpha MEM supplemented with 7% fetal calf serum (FCS), 10% TPB, and 1% penicillin-streptomycin. CHO cells were maintained in MEM containing 10% FCS, 5% TPB, gentamicin (0.05 \( \mu \)g/ml), and kanamycin (0.25 \( \mu \)g/ml).
Virus stocks were propagated in DBT-9 cells grown in 75-cm² and 400 m² cultures. Virus was removed, the cultures washed twice with PBS to remove residual virus, and the cells were inoculated with virus at an MOI of 5 for 1 h at room temperature. The antibodies were then removed, and the cells were washed twice with PBS to remove residual virus. At 18 h postinfection, infected cells were fixed with acetone-methanol at 94°C and counted by plaque assay in DBT-9 cells. As a control, cells were pretreated with an equivalent amount of a monoclonal antibody (EDDA or R501) directed against an irrelevant antigen.

**Cloning and transfection studies.** Human Bgp (hBgp) was cloned from HepG2 cells by using reverse transcription-PCR and was subcloned into TA cloning vectors as instructed by the manufacturer (Promega). Briefly, total intracellular RNA was isolated from HepG2 cells by using RNA STAT-60 reagents as instructed by the manufacturer (Tel-Test “B”, Inc.). To clone the hBgp gene, reverse transcription was performed with Superscript 2 reverse transcriptase and an oligodeoxynucleotide primer located just downstream from the C-terminal of the Bgp1 open reading frame (ORF) (5'-ACAGAGAAATCTAGGAGC-3') (5). Following cDNA synthesis at 42°C for 1 h, the cDNA was denatured for 5 min at 94°C and amplified by PCR with Taq polymerase (28 cycles of denaturation at 94°C for 30 s, 58°C for 40 s, and 72°C for 105 s). The forward (5'-CAGGGGAGACAGGAGGAC-3') and reverse primer pairs derived from the reported sequences were used to amplify the cDNA.

**TABLE 1. hCEA antibodies**

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<th>Antibody</th>
<th>V51B blockade in HepG2 cells</th>
<th>hCEA antigen cross-reactivity</th>
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<tr>
<td>Bgp1</td>
<td>+</td>
<td>NCA CEA</td>
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<tr>
<td>CGM6</td>
<td>+</td>
<td>CEA CEGM7</td>
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**Detection of viral antigens by immunofluorescence.** Different cell clones grown in LabTek chamber slides were infected with MHV-A59 or persistent virus isolates at an MOI of 5 h at room temperature. The inocula were removed, and the cells were washed twice with PBS to remove residual virus. At different times postinfection, infected cells were fixed with acetone-methanol (1:1) and stored at 4°C. Fixed cells were rehydrated by several washes in PBS and then incubated with a 1:20 dilution of the appropriate monoclonal or polyclonal (pCEA) antibody for 1 h at 37°C. The cultures were overlaid with a 1:4 dilution of a monoclonal (Kat4c) or polyclonal (pCEA) antibody for 1 h at 37°C. These antibodies will bind with different times postinfection and stored at −70°C for plaque assay in DBT-9 cells. As a control, cells were pretreated with an equivalent amount of a monoclonal antibody (EDDA or R501) directed against an irrelevant antigen.
sequence of the hBgp1 gene (GenBank accession no. J03858) (5). The 1.6-kb products were separated in 1.0% agarose gels and isolated by using Qiagen reagents (Qiagen Inc., Chatsworth, Calif.) prior to subcloning into the TA cloning vector. Positive clones were identified by restriction digestion profiles and sequence analysis. The hBgp1 ORF was reamplified by using primers that contained a 5' XbaI site (5'-CAGTCATCTAGAAGACACCATGGGGCACCTCTC-3') and a 3' SphI site (5'-CAGTCAGCATGCCAGGACAGGTTTCATTACGTGCT-3') that spanned the CEA ORF were used for PCR amplification of the approximate 1.5-kb insert. Following restriction analysis, these products were inserted into the pSinRep19 expression vector downstream of a 26S promoter element. The pSinRep19 replicons encoding the T7 RNA polymerase (pSinRep19/T7pol) and green fluorescent protein (GFP) (pSinRep19/GFP) were kindly provided by C. Rice, Washington University.

Selection for stable cell lines expressing human Bgp or CEA. T7 transcripts were synthesized as instructed by the manufacturer (Stratagene) from XhoI- or NotI-linearized plasmid pSinRep-Bgp1, pSinRep-CEA, pSinRep19/T7pol, or pSinRep19/GFP and electroporated into BHK cells. Briefly, 5 × 10^6 BHK cells were mixed with GFP, Bgp, or CEA transcripts and electroporated with three pulses at a setting of 850 kV and a capacitor setting of 25 mF, with an approximate time constant after the pulse of 0.6 ms in a 0.2-cm cuvette. Following a 10-min incubation at room temperature, the cells were transferred into a 60-mm^2 dish in complete medium. Transfection efficiencies averaged 60 to 80% as determined with the pSinRep19/GFP control. After 12 h, medium containing 5 μg of puromycin per ml was added to the cultures, and cell lines expressing each gene were isolated over the following week.

Cell lines stably expressing similar amounts of human Bgp or CEA were isolated by cell sorting. Briefly, cultures of cells were trypsinized from 75-cm^2 flasks, washed twice in PBS, and incubated with monoclonal antibody Kat4c for 30 min at room temperature. The cells were precipitated by centrifugation at 2,500 rpm in an Eppendorf centrifuge for 2 min and washed three times with 1 ml of PBS. The cells were then incubated with FITC-conjugated rabbit anti-mouse IgG antiserum was added for 30 min at room temperature. Following additional washing, the cells were analyzed by FACS techniques. (A) Col-1; (B) Col-12; (C) Col-14; (D) Col-6; (E) Kat4c.

RESULTS

Persistence selects MHV host range mutants. MHV infection in DBT cells rapidly established a carrier-state culture in which only a portion of the cells expressed viral antigen (5–20%) and virus titers persisted at around 10^6 PFU/ml throughout the first 210 days of culture (12). As the CEA gene family is highly homologous in mammals (5, 6, 10, 22, 51, 54) and we have previously demonstrated that adaptation of MHV to BHK cells resulted in the emergence of mutants with broad host range specificity (4), we determined if persistent infections selected for host range mutants of MHV. Persistent viruses V8A and V10A, V19A, and -B, V30A and -B, and V51A and -B were isolated on days 28, 38, 78, 119, and 210 postinfection, respectively. All persistent viruses replicated efficiently in BHK cell lines, approaching titers of 10^7 to 10^8 PFU/ml within about 24 to 30 h postinfection. None of the persistent viruses replicated efficiently in DBT cell lines, approaching titers of 10^7 to 10^8 PFU/ml within about 24 to 30 h postinfection. None of the persistent viruses replicated efficiently in BHK cell lines, although a rare V51A- or V51B-infected BHK cell could be
detected by fluorescent antibody (FA) staining at frequencies of less than 0.01%. Presumably too few cells were productively infected to increase virus titers above background levels ($\sim 10^3$) associated with the inoculating dose (see Fig. 7). All persistent viruses infected more than 95% of the DBT cells in culture, as evidenced by FA staining (data not shown).

To determine if persistent infection selected for the emergence of viruses that replicated more efficiently in other mammalian hosts, virus replication was examined in hamster (CHO), primate (COS-7), human (HepG2 and MCF7), feline (CRFK), and swine (ST) cell lines. These cell lines were chosen for their susceptibility (CHO, HepG2, COS-7, and MCF7) or resistance (ST and CRFK) to infection with the hamster-adapted isolate, MHV-H2 (4). The V51A and V51B persistent isolates replicated to titers approaching $10^6$ PFU/ml in CHO cells (data not shown). Both viruses also plaqued efficiently in CHO cells (data not shown). In addition, the V51A and V51B isolates replicated to titers that approached $5 \times 10^6$ PFU/ml in HepG2 cells within 36 h postinfection (Fig. 1). Under identical conditions, neither the MHV-A59 parent nor any of the persistent viruses isolated at or before 119 days postinfection replicated efficiently in these cells (Fig. 1). While low levels of V51B replication were noted in human breast carcinoma MCF7 cells with virus titers approaching $10^4$ PFU/ml, no replication was evident in ST or CRFK cells (data not shown).

Viral titers directly correlated with the percentage of infected cells noted in culture. By FA staining techniques, V51A and V51B replication was clearly evident in HepG2 cell lines, with about 10 to 20% of the cells, respectively, productively expressing viral antigens, at 18 h postinfection (Fig. 2). At later times, more than 70% of the cells expressed viral antigens (data not shown). Importantly, an occasional (at frequencies of less than 0.1%) antigen-positive HepG2 cell was noted in V10B- or V30B-infected cultures, suggesting that limited MHV host range expansion into human cells may rapidly evolve within the first 38 days of persistence. However, the rare productively infected cell likely failed to produce sufficient progeny virions to increase titers above background levels of the residual inoculum (Fig. 1). These data demonstrated that persistent viruses between 119 and 210 days postinfection had evolved mutations that conferred efficient cross-species transmissibility to some human and hamster cell lines.

**hCEA antiserum blocks persistent virus entry into human cell lines.** In humans, at least 22 CEA-related genes have been demonstrated to cluster on chromosome 19 (5, 6, 10). These genes have been subdivided into the CEA, Bgp, nonspecific cross-reacting antigen, and pregnancy-specific glycoprotein subgroups based on expression patterns, gene structure, and sequence homologies (6). Sequence comparisons revealed that the human CEA and Bgp glycoproteins are well conserved with MHVR and to a slightly lesser extent with Bgp2 (10) (data not shown). Since previous studies have demonstrated that abundantly expressed hCEA and Bgp may serve as receptors for MHV entry into nonpermissive cells (10), persistent viruses may recognize phylogenetic homologues of the normal receptor to gain entry into human cells. To address this question, we analyzed whether different monoclonal and polyclonal antisera that bind hCEA glycoproteins could block V51B infection in HepG2 cells (16). Incubation of HepG2 cells with pCEA, with monoclonal antibody Kat4c, or with the noncytopathic pSinRep19 replicons block MHV replication, cultures of BHK-MHVR cells were transfected with the pSinRep19/T7pol transcripts and selected with puromycin (5 $\mu$g/ml) for several days. Cultures of BHK-MHVR/ Sindbis virus replication in HepG2 cells or block MHV replication in DBT cells (data not shown). FA staining revealed significant reductions in the percentages of infected cells also in cultures pretreated with Col-4 and pCEA (>95% reduction) but not with the EDDA or Kat4c antibody, suggesting that inhibition of virus replication occurred early in infection (data not shown). By FACS analysis, the monoclonal antibodies used in these studies were shown to recognize epitopes in one or more hCEA glycoproteins expressed on HepG2 cells (Fig. 4).
Comparisons between the antibody cross-reactivities among the different hCEA glycoproteins and their capacity to block V51B infection in HepG2 cells suggested that one or more hCEA glycoproteins, possibly CEA itself, were likely candidate receptors for V51B entry (Table 1).

Persistent viruses recognize hCEA glycoproteins as receptors. To determine if hCEA glycoproteins could function as receptors for persistent virus entry into human cells, the hBgp gene was cloned from HepG2 cells by using primer pairs obtained from the published sequence (5). The cDNA to the hBgp mRNA was then subcloned downstream of a 26S promoter element in the pSinRep19 noncytopathic Sindbis virus replicons kindly provided by C. Rice (29, 30, 64). In contrast to other Sindbis virus replicons lacking the structural genes (30), these replicons are noncytopathic in BHK cells due to adaptive mutations in the nonstructural protein nsP2 of Sindbis virus (67). Consequently, they establish persistent infections in BHK and other hamster cell lines without apparent deleterious effects. These replicons also encode puromycin resistance from a second downstream 26S promoter, allowing rapid selection of puromycin-resistant cells (67). To determine if these vectors display any toxicity to MHV replication, BHK-MHVR cells were transfected with the pSinRep19/T7pol replicons and puromycin-resistant cells were isolated and designated BHK-MHVR/SinT7pol cells. MHV-A59 replication was equally efficient in both BHK-MHVR and BHK-MHVR/SinT7pol cells, with peak virus titers approaching $10^9$ PFU/ml by 30 h postinfection, respectively (Fig. 5). These data demonstrate that the Sindbis virus replicons were not inhibitory or antagonistic to efficient MHV infection. Similar findings were noted with several other MHV strains (data not shown).

Transcripts were synthesized from pSinRep-CEA and pSinRep-Bgp and electroporated into nonpermissive BHK cells. Following selection with puromycin, cell lines expressing levels of human CEA and Bgp comparable to that noted in HepG2 cells were isolated with monoclonal antibody Kat4c by FACS. We reasoned that by isolating cell lines that expressed levels of CEA and Bgp comparable to those seen in susceptible cells, we would prevent possible receptor-mediated overexpression blockade of MHV replication and allow direct comparisons of receptor usage under normal levels of receptor bioavailability (13). A single cell line expressing hCEA (BHK-hCEA) and two different cell lines expressing either low (BHK-hBgp1) or high (BHK-hBgp1+) levels of hBgp were isolated (Fig. 6). FACS analysis with monoclonal antibody Kat4c demonstrated that hCEA and hBgp were expressed in BHK cell lines at levels comparable with that detected in HepG2 cells (Fig. 4 and 6). Stable hCEA and hBgp expression has been noted for about 2 months in culture.

Cultures of BHK, BHK-hBgp1, BHK-hBgp1, and BHK-hCEA cell lines were infected with MHV-A59 and V51B at an MOI of 5 for 1 h at room temperature. The cultures were washed extensively to remove unabsorbed viruses, and growth curves were analyzed over the next 30 h. Efficient V51B virus replication was noted in the BHK-hCEA cell line, with virus titers exceeding $10^5$ PFU/ml—an approximate 2-log increase above background levels (Fig. 7). By FA staining, significant numbers of BHK-hCEA cells expressed viral antigens compared to the controls (Fig. 8). A lower level of V51B replication ($\sim 1$ log) was noted in the BHK-hBgp1+ cell line, suggesting that hBgp may also function as a receptor for entry although with much less efficiency than hCEA (Fig. 7). Similar results were seen following V51B infection in BHK-Bgp1 cells (data not shown). Little if any replication of V51B was noted in BHK cells (Fig. 7). In contrast to previous reports suggesting that hBgp and CEA may function as receptors for MHV-A59 entry (10), no evidence for MHV-A59 replication was detected in the BHK, BHK-hCEA, BHK-hBgp1, and BHK-hBgp1+ cell lines.
lines (Fig. 7 and 8). To determine if other V51 isolates or persistent viruses could replicate in the BHK-hCEA cell lines, cultures were infected with V51A, V51B, V30B, V10B, and MHV-A59. Under conditions in which the V51A and V51B isolates replicated to titers approaching $10^5$ PFU/ml, little if any replication was noted with V30B, V10B, or MHV-A59 (Fig. 9).

To provide additional evidence that hCEA and hBgp were functioning as receptors for V51B entry, blockade experiments were performed with rabbit polyclonal antisera directed against the hCEA glycoprotein family. Pretreatment of cells with pCEA significantly blocked V51B replication in BHK-hCEA and BHK-hBgp1+ cell lines by 1 to 2 logs of titer (Fig. 7). A decrease in the number of V51B-infected cells as detected by FA staining was also noted, consistent with the hypothesis that both hCEA and hBgp could function as receptors for entry into BHK cells (data not shown). These data suggest that persistence may promote MHV cross-species transmissibility by selecting for variants that recognize human homologues of the normal murine Bgp receptor for entry into human cell lines.

**DISCUSSION**

Virus persistence and cross-species transmissibility. MHV is highly species specific, yet host range mutants have been isolated from mixed cell cultures in vitro under conditions that may reflect mechanisms of exogenous virus cross-species transmissibility in human xenograft recipients, following intracranial inoculation and persistence in the primate CNS, and following persistent infection in cell lines derived from outbred and inbred mouse strains in vitro (4, 50, 56). As MHV species specificity is likely mediated at entry, these model systems are uniquely positioned to identify the virus-receptor interactions that mediate virus cross-species transmissibility and host range expansion. These models may also allow comparison of the molecular mechanisms that allow viral cross-species transmissibility under a variety of different environmental conditions and in the setting of persistent viral infections. In contrast to the picornavirus, myxovirus, and paramyxovirus host range mutants (14, 32, 48, 57, 61, 69), the MHV models are uniquely positioned for the examination of the molecular mechanisms regulating cross-species transmissibility of zoonotic viruses to human, primate, and other mammalian hosts.

The emergence of new viral diseases in humans is usually attributable to environmental, cultural, or behavior changes that provide new opportunities for virus replication and transmissibility between hosts (45). A prediction of high mutation frequency suggest that RNA viruses evolve rapidly in the face of changing environmental conditions (3, 4, 37, 42). It is less clear, however, whether such changes select for the emergence of viruses from preexisting pools of host range mutants or select for advantageous mutations in a virus which permit cross-species transmissibility into the new host. In this report, we demonstrate that persistent infections rapidly result in a ready source of host range mutants of animal viruses in the absence of any alternate host species. In persistently infected murine 17CII cell lines, similar findings have been reported by Schickli et al. (56) with the isolation of MHV host range variants at passage 600 (3 to 4 years postsinfection) that replicate efficiently ($\sim 10^5$ PFU/ml) in some hamster cell lines. In an extension of these studies, we demonstrate that MHV host range expansion evolves within the first 38 days postinfection (passage 10), although efficient infection and replication of human cell lines was evident only with persistent viruses that emerged sometime between 119 and 210 days postinfection (passages 30 to 51). Thus, persistence in DBT cells, which were derived from outbred CD1 mice, rapidly selects for host range mutants of animal viruses which can replicate efficiently in some human, primate, and hamster cell lines. Since many other RNA and DNA viruses persist by selecting for increased host cell resistance and viruses that replicate more efficiently in these resistant cells (12, 17, 19, 28, 36), it will be of interest to determine if cross-species transmissibility represents a common consequence of persistence. Such a hypothesis may not be unprecedented, as substitutions in the capsid of persistent poxviruses also confer a neurovirulent phenotype on the Mahoney type 1 strain in mice (15). As many emerging viruses, such as retroviruses, morbilliviruses, arenaviruses, and many arthropod-borne viruses, rapidly establish persistent infections in their natural hosts, persistent infection in vivo may produce preexisting pools of host range mutants that are rapidly selected following a change in the natural ecology of the host-parasite interaction.

MHV rapidly results in a carrier persistent culture in vitro in which only a small percentage of cells are infected and actively producing progeny virions. Virus infection selects for host cell populations that express little if any MHVR and are resistant to infection (12, 55). Over time, more virulent viruses with altered receptor specificities rapidly emerge, subsequently in-

![Image](http://jvi.asm.org/Downloaded from http://jvl.asf.org, on August 28, 2017 by guest)
fecting and replicating in these highly resistant host cell populations (12). Consequently, MHV persistence represents a model for identifying sites of virus-receptor interaction at the cellular level that regulate the coevolution of virus entry, virulence, and host cell resistance. Two general mechanisms may account for host range expansion during persistent MHV infection in vitro and in vivo. During infection, cells expressing little MHVR may select for variants with altered receptor specificities driving cross-species transmissibility (12). While such a mechanism may account for the emergence of host range mutants during persistent infection in vitro, MHV infection is normally acute and self-limiting in vivo, suggesting that host range mutants would have little opportunity to evolve before immunologic clearance. In some cases, however, MHV may reach immunologically privileged sites like the CNS and persist in tissues where MHVR or other alternative Bgp or CEA receptors are expressed at low levels (26, 31, 38, 53). In the murine CNS, MHV sequences may persist for over 1 year, although little if any infectious virus has been demonstrated under these conditions (26). Persistent coronavirus infections in the CNS of humans and primates have also been described (49, 50, 60). Long-term persistence in the CNS may also downregulate receptor expression and effectively select for virus variants with altered receptor specificities and tissue tropisms that fortuitously extend host range specificity. Organ-specific selection of viral variants following chronic infection in mice has been described, and virus infection may downregulate expression of the receptor (1, 36).

As an alternative to this hypothesis, MHV persistence in DBT cells may also be analogous to natural conditions where outbred mice encode multiple Bgp receptor alleles that confer increased host cell susceptibility or resistance to MHV infection (12, 51, 70). For example, the Bgp1b allele is not as efficient a receptor as MHVR, and it confers resistance to MHV-A59 infection in SJL mice (12, 52). In DBT cells which encode the Bgp1a and Bgp1b alleles, persistent viruses rapidly evolve the capacity to efficiently utilize the Bgp1b glycoprotein as a receptor for entry into cells (12, 70). MHV infection in outbred mice that encode several different polymorphic Bgp receptor alleles might also coselect for virus receptor mutants during either an acute or a persistent infection. Over time, such variants may fortuitously extend host range and be amplified following exposure in a new host ecology. Although speculative, both hypotheses provide ample opportunity for the selection of virus variants with altered receptor specificities resulting in host range expansion of MHV. As receptor/entry mutants of many DNA and RNA viruses have been isolated from persistently infected cells (12, 19, 20, 28), persistence may represent an important pathway for host range expansion of many animal and plant viruses. The detection of persistent MHV-like sequences in the CNS of multiple sclerosis patients also supports
the disturbing hypothesis that MHV host range expansion may also occur in vivo (8, 49).

**Molecular mechanisms of MHV cross-species transmissibility.** We and others have shown that MHV persistence in vitro is maintained by the epigenetic expression of MHVR and by the emergence of persistent viruses which display increased affinity for MHVR and other polymorphic Bgp alleles as receptors for entry (12, 55). All of the persistent viruses use MHVR as a receptor (4a). Consequently, persistent viruses may have an increased affinity for highly conserved domains within all murine Bgp genes, may tolerate increased residue heterogeneity within a common virus binding domain in different Bgps, or may bind to completely different receptor residues in different Bgps. Since persistent viruses also evolve efficient host range expansion between 119 and 210 days postinfection, virus scanning for murine Bgp receptor homologues within a common virus binding domain in different Bgps, or may bind to completely different receptor residues in different Bgps. Since persistent viruses also evolve efficient host range expansion between 119 and 210 days postinfection, virus scanning for murine Bgp receptor homologues may have inadvertently expanded the MHV host range by increasing virus affinity for highly homologous domains in the CEA glycoproteins from different species. Since the N-terminal domain of S likely contains the MHVR binding residues, it seems likely that S glycoprotein gene mutations may be responsible for expanded host range (62).

The murine and human CEA genes are well conserved in nature. MHV-A59 but not MHV-JHM can utilize the human Bgp and CEA glycoproteins as receptors for entry when expressed at extremely high levels in nonpermissive cells (10). Using blockade experiments with hCEA antiserum and expression of human CEA and Bgp in nonpermissive BHK cells, V51B entry into human cell lines likely occurs by high-affinity interactions with one or more phylogenetic homologues of the normal viral receptor. Although blockade experiments suggest that the V51 variants may use one or more CEA or Bgp glycoproteins as receptors, we have not identified the actual receptor for persistent virus entry into human cell lines. The hCEA glycoprotein is a more likely choice than Bgp, since it functions much more efficiently as a receptor for V51B entry into BHK cells. It should be noted, however, that not all CEA- or Bgp-expressing cells became productively infected, suggesting that other CEA glycoproteins or that combinations of hCEA glycoproteins may function in persistent virus entry into human cells. Such findings are not unprecedented, as different human chemokine coreceptors function as receptors for HIV entry into different host cell populations (9, 23, 25, 43, 58). The presence of a conserved consensus motif which may function as a common binding domain in murine Bgp, hBgp, and hCEA glycoproteins further supports the possibility that several CEA family members function as receptors for V51B entry (10). Since human and porcine coronaviruses recognize entirely unique portions of their aminopeptidase receptors for entry, considerable latitude might exist in defining the CEA glycoprotein receptor residues that bind V51B and mediate host range expansion into alternative species (40). Detailed analysis of receptor binding residues in the N-terminal domain of the MHV-A59 S glycoprotein gene coupled with identification of virus binding residues in murine and human CEA glycoproteins should reveal fundamental mechanisms regulating the emergence of new viral diseases and adaptation of zoonotic viruses to the human host.

In contrast to previous reports following transient expression of human Bgp and CEA in COS cells (10), we have shown
that these glycoproteins do not serve as receptors for MHV-A59 entry when expressed at normal levels of bioavailability. Although speculative, it seems likely that the different results in the two systems may be related to actual levels of human Bgp or CEA receptor bioavailability in the different nonpermissive host backgrounds. Our results, however, are consistent with the inability of MHV to infect any known human cell lines in culture. Additional studies are clearly needed to identify the exact CEA receptor that functions as a receptor for V51 entry into human cell lines and to elucidate the exact conditions of receptor bioavailability required for efficient MHV-A59 infection.

Molecular mechanisms of virus cross-species transmissibility. The cellular receptor is an essential component for virus entry and a major determinant of host range specificity, tissue tropism, and pathogenesis (35, 65, 66). Since little information is available regarding receptor utilization among phylogenetically related viruses that replicate in distinct species (65), the molecular and evolutionary mechanisms that regulate virus cross-species transmissibility are obscure. Focusing at the level of entry, we propose that new viral diseases may emerge by either a homologue scanning or a receptor switching mechanism. The homologue scanning model predicts that phylogenetic homologues of the normal receptor function as natural conduits for cross-species transmissibility and entry into alternative host species. For example, HIV likely evolved from simian immunodeficiency virus (27). Both viruses utilize CD4 and various related chemokine glycoproteins as receptors and coreceptors for entry into cells (9, 58). Among the group I coronaviruses, transmissible gastroenteritis virus, feline infectious peritonitis virus, and human coronavirus 229E use the aminopeptidase N glycoprotein as a receptor, and they cause similar enteric or upper respiratory tract infections in their hosts (18, 40, 63, 68). Moreover, the feline aminopeptidase N glycoprotein serves as a receptor for entry of all three coronaviruses into cells (63). In these virus families, it seems likely that host range expansion evolved along phylogenetically related receptor molecules.

Receptor switching suggests that host range expansion may occur by virus recognition of entirely new receptor molecules. Murine-adapted strains of poliovirus do not recognize the murine homologue of the poliovirus receptor; rather, these host range mutants recognize some alternative receptor for entry into murine cells (7, 44). Concomitant with virus recognition of unique receptor moieties for entry into murine cells, these viruses cause highly variable disease syndromes (65, 66). Although the speculation is controversial, HIV may also expand its cell tropism in vivo by recognizing galactosyl ceramide as a receptor in neuronal cells and intestinal epithelium (34). Tissue culture adaptation of foot-and-mouth disease virus also results in the emergence of virus variants that recognize heparan sulfate as a receptor for virus entry (39). Clearly, mechanisms regulating virus cross-species transmissibility may be extremely plastic and heavily influenced by the prevailing environmental conditions, selective pressures, and sites of virus-host interaction which regulate species specificity. The same theoretical mechanisms which allow viruses to circumvent the natural barriers of cross-species transmissibility at the level of virus-receptor interaction may also affect the ability of a virus to cross the species barrier by overcoming blocks at the level of transcription, replication, assembly, or release (59, 69). For example, Vif (virus infectivity factor) function is somehow host cell restricted and may represent a critical determinant in the ability of HIV to switch host species (24). Understanding the fundamental molecular mechanisms for coronavirus host range expansion may shed considerable insight into the molecular and evolutionary mechanisms of virus cross-species transmission and the emergence of new diseases in humans and animals.

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