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The N-Terminal Region of the Murine Coronavirus Spike Glycoprotein Is Associated with the Extended Host Range of Viruses from Persistently Infected Murine Cells

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Although murine coronaviruses naturally infect only mice, several virus variants derived from persistently infected murine cell cultures have an extended host range. The mouse hepatitis virus (MHV) variant MHV/BHK can infect hamster, rat, cat, dog, monkey, and human cell lines but not the swine testis (ST) porcine cell line (J. H. Schickli, B. D. Zelus, D. E. Wentworth, S. G. Sawicki, and K. V. Holmes, J. Virol. 71:9499–9507, 1997). The spike (S) gene of MHV/BHK had 63 point mutations and a 21-bp insert that encoded 56 amino acid substitutions and a 7-amino-acid insert compared to the parental MHV strain A59. Recombinant viruses between MHV-A59 and MHV/BHK were selected in hamster cells. All of the recombinants retained 21 amino acid substitutions and a 7-amino-acid insert found in the N-terminal region of S of MHV/BHK, suggesting that these residues were responsible for the extended host range of MHV/BHK. Flow cytometry showed that MHV-A59 bound only to cells that expressed the murine glycoprotein receptor CEACAM1a. In contrast, MHV/BHK and a recombinant virus, k6c, with the 21 amino acid substitutions and 7-amino-acid insert in S bound to hamster (BHK) and ST cells as well as murine cells. Thus, 21 amino acid substitutions and a 7-amino-acid insert in the N-terminal region of the S glycoprotein of MHV/BHK confer the ability to bind and in some cases infect cells of nonmurine species.

Mouse hepatitis virus (MHV) causes inapparent infection or diseases in mice, including diarrhea, hepatitis, splenolysis, immunological dysfunction, and acute and chronic neurological disorders (52, 61). MHV is highly transmissible from mouse to mouse but does not naturally spread from mice to other species. MHV strains have been experimentally transmitted to nonmurine species, although serial viral propagation in these animals did not occur. For example, intracerebral inoculation of neurovirulent MHV strain JHM can cause demyelinating encephalomyelitis in weanling rats and allow RNA replication but not virus production in owl monkeys (41, 61).

All strains of MHV utilize murine carcinoembryonic antigen cell adhesion protein 1a (mCEACAM1a) as their receptor in susceptible mice and murine cell lines (3, 11, 43, 66). MHV virions do not infect cell lines derived from nonmurine species. MHV strains differ in virulence, tissue tropism, and antigenicity in vivo and cytopathic effects in vitro. MHV-A59 and MHV-JHM infections kill more than 90% of murine cells, and in the surviving cells the surface expression of mCEACAM1a is markedly reduced (47, 50). Surviving cells establish long-term carrier cultures in which there is little or no cytopathic effect, and virus is produced from 10 to 20% of the cells (16, 20, 21, 26, 50).

Some viruses derived from persistently infected murine cells have an extended host range and can infect cell lines from hamsters, rats, cats, dogs, monkeys, and humans (1, 50, 51). Such in vitro selection of MHV variants with extended host range may model the molecular events that permit the adaptation of a coronavirus to a new host species in vivo. For example, the severe acute respiratory syndrome coronavirus apparently recently emerged in humans from a zoonotic reservoir (23, 31, 46).

The 180-kDa spike glycoprotein (S) of MHV is a type I viral fusion protein that mediates both virus attachment and membrane fusion (19). Trypsin and related serine proteases cleave S on virions to form the amino (N)-terminal S1 protein, which binds to the mCEACAM1a receptor, and the membrane-anchored S2 protein, which contains a coiled-coil domain and mediates membrane fusion and virus entry (4, 5). The S1 and S2 proteins remain noncovalently associated on virions, but MHV strains differ in the stability of S1 and S2 interactions (18, 34, 53). MHV variants that lack the trypsin cleavage site are infectious but cause little cytopathic effect or cell-cell fusion (21, 51).

Binding of the N-terminal 330 amino acids of S to residues in the CC′ loop and C′ β sheet in the N-terminal immunoglobulin-like domain of mCEACAM1a determines the limited host range of MHV strains (32, 48, 55, 58, 63). A monoclonal antibody (MAb) CC1, that binds the N-terminal domain of mCEACAM1a blocks infection of susceptible murine cells by all MHV strains (13). A dramatic conformational change in S2 is induced by the binding of recombinant, soluble mCEACAM1a proteins to S1 at 37°C (18, 54, 67, 68). Triggering of the conformation change in S2 by anchored mCEACAM1a probably leads to fusion of the viral envelope with cell membranes and virus infection as well as cell-to-cell fusion.

We previously showed that the host range variant MHV/

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TABLE 1. Primers used to amplify and sequence the S genes of MHV-A59, MHV/pi23, MHV/pi600, MHV/BHK, and recombinant viruses[a]

Primer no.	Position	Sequence
Sense primers		
1	−65 to −47	CGGTCATACTGCTAGGTG
3	344 to 365	AAGACAAGTACGCCATCAGGT G
5	1146 to 1167	AAGTGTATGGCAGGTGCTTTGG
7	1901 to 1920	AGGAGCTGTTATAGTGG
9	2657 to 2678	TCTGATGTTGGCTTTGTCGAGG
11	3459 to 3480	ATGGAGAATGGAAGTTCACAGG
13	1089 to 1110	GCCTGTTACGTTATGTTCAGGC
C4	Complement of 4	CCCCGAAGTAGGCAAGTTG
15	1492 to insert	CTCGGACTAAGAAGGTACC
17	3099 to 3118	TTCTAACTCGGCTTGAGGC
19	2334 to 2353	GTGCTGCATTTGTCTGTGG
21	Insert to nt 1510	AGAAGGTACCAA AGCCTAAGTCTGCTTTG
Antisense primers		
2	674 to 695	AAACAAAAACGTAGTAGCGG
4	1176 to 1199	TCAACTTGCCTACTTCGGGG
6	1956 to 1975	ATTACGATAGAGCAGAGCCG
8	2759 to 2780	TCTGACTCTCTGACAACACAGG
10	3496 to 3514	TCTGTAATGGGTTCAGGG
12	3990 to 4009	TCTTTCCAGGAGAGGCTGTG
C7	Complement of 7	CGGCCACTATAACAGCTCC
14	2107 to 2126	ATAAGCCAGCACCCATACGG
16	3175 to 3209	GCGTACTATCACTAAGTTGCTTGG
18	1473 to insert	TTTGGTACCTTCTTAGTCCGAGTAGTCTGAGTAGTGCAAGGGC
20	2390 to 2409	ACATTAACACAGAAAGAGCC
22	1629 to 1648	GCGATCATTAACAAGGCAGG

[a] S gene numbering is based on GenBank accession no. M18379 (35).

BHK, derived from passage 600 of a 17 Cl 1 line of murine fibroblasts persistently infected with MHV strain A59, infected a wide range of nonmurine cell lines, suggesting that this virus may use an as yet unidentified receptor on nonmurine cells (51). The anti-mCEACAM1a MAb CC1 only partially blocked MHV/BHK infection of murine cells, suggesting that this virus may use both mCEACAM1a and an alternative receptor on murine cells (51).

In this study, we compared the S genes of the parental strain MHV-A59 and the host range variants MHV/pi23, MHV/pi600, and MHV/BHK. Recombinant viruses between MHV-A59 and MHV/pi600 or MHV/BHK were selected on hamster cells. Analysis of the S genes of recombinant viruses with extended host range identified 21 amino acid substitutions and a 7-amino-acid insert in S1 that permit virus binding and entry into nonmurine cells.

MATERIALS AND METHODS

Antibodies. A mouse MAb to the MHV nucleocapsid protein (N) was generously provided by Julian Leibowitz (Texas A&M University, College Station, Tex.). The anti-mCEACAM1a MAb CC1 was used for flow cytometry (13). A mouse MAb (5B19) directed against the S2 protein of MHV, kindly provided by Michael Buchmeier (Scripps Research Institute, La Jolla, Calif.), was used to detect binding of virus by flow cytometry. An isotype-matched control mouse MAb directed against an irrelevant antigen was used in all immunofluorescence and fluorescence-activated cell sorting (FACS) experiments. Fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) was used for immunofluorescence analysis. Phycoerythrin-conjugated goat anti-mouse immunoglobulin antibody (Dako Corporation, Carpinteria, Calif.) was used for FACS analysis.

Cell lines. The 17 Cl 1 line of BALB/c fibroblasts, swine testis (ST) cells, the BHK-21 line of baby hamster kidney fibroblasts, and 17 Cl 1 cells persistently infected with MHV-A59 were obtained and propagated as previously described (16, 51).

Viruses. MHV-A59 and the host range variants MHV/pi600 and MHV/BHK, derived from 17 Cl 1 cells persistently infected with MHV-A59, were propagated as previously described (51). The host range variant MHV/pi23, from passage 23 of 17 Cl 1 cells persistently infected with MHV-A59, was plaque purified twice in 17 Cl 1 cells and propagated as described above. The titers of infectious viruses were determined by plaque assay as previously described (17) in 17 Cl 1 cells or BHK cells.

Restriction enzyme digestion and sequencing analysis. RNA was extracted from viruses in tissue culture supernatant medium and amplified with a one-step reverse transcription (RT)-PCR adapted from Noble and coworkers (44). Amplification products were treated with restriction enzymes and/or purified by centrifugation in 30,000 nominal molecular weight limit filter units (Millipore Corporation, Bedford, Mass.). RT-PCR products of the S genes of the parental MHV-A59, the host range variants MHV/BHK (passage 2), MHV/pi600, and MHV/pi23, and the recombinant viruses were sequenced by the University of Colorado Cancer Center DNA Sequencing and Analysis Core Facility with ABI Prism kits as previously described (62). The oligonucleotides used for amplification and sequencing of the S genes are shown in Table 1. Each base position was sequenced from at least two independently derived RT-PCRs. S gene sequences in this paper were numbered according to GenBank accession number M18379 (35).

Generation and characterization of recombinant viruses. Eleven independent cultures of 17 Cl 1 cells (named a through k) were inoculated with the parental MHV-A59 and MHV/BHK viruses at an multiplicity of infection of 10 PFU/cell for each virus. The supernatant medium was diluted 1:50 at 15 h postinoculation and used to inoculate fresh 17 Cl 1 cells. Viruses harvested at 15 h postinoculation (samples a to f) and at 36 h postinoculation (samples g to k) were inoculated onto BHK cells and grown at 39.5°C. Viruses that formed both large and small plaques, picked from BHK cells at 39.5°C, were amplified in 17 Cl 1 cells at 37°C, plaque purified twice in BHK cells at 39.5°C, and propagated in 17 Cl 1 cells at 37°C to titers between 10^6 and 10^7 PFU/ml. The growth of the plaque-purified viruses in BHK cells at 39.5°C was confirmed by indirect immunofluorescence. The recombinant virus Rec2A was derived from a 12th independent culture of 17 Cl 1 cells inoculated with the parental MHV-A59 and MHV/pi600 viruses.

Flow cytometric analysis of murine CEACAM1a expression. Cells were trypsin treated, washed once, and incubated with anti-mCEACAM1a MAb CC1 or isotype control MAb for 1 h. After two washes, cells were incubated with phy-

coerythrin-conjugated goat anti-mouse immunoglobulin antibody for 1 h. After one wash, cells were resuspended in fix buffer, consisting of phosphate-buffered saline (PBS, pH 7.4) supplemented with 0.1% bovine serum albumin and 2% paraformaldehyde. All incubations and washes were performed at 4°C in FACS buffer (PBS [pH 7.4] supplemented with 0.1% bovine serum albumin). FACS analysis was performed on a Beckman-Coulter XL flow cytometer at the University of Colorado Cancer Center FACS Core Facility.

Flow cytometric analysis of virus binding. The 17 Cl 1, BHK, ST, and BHK and ST cells expressing murine CEACAM1a were trypsin treated and washed as described above. Cells (10^6) were incubated for 2 h with 10^7 PFU of either MHV-A59, MHV/BHK, or the recombinant virus k6c in 500 μl of medium. Bound virus was detected with anti-S2 MAb 5B19 or isotype control MAb followed by phycoerythrin-conjugated goat anti-mouse immunoglobulin antibody. All incubations were performed for 1 h at 4°C in FACS buffer except as indicated above.

Immunofluorescence detection of virus infection. Cells grown on coverslips were inoculated with MHV-A59, MHV/BHK, or one of the recombinant viruses as previously described (51). At 10, 24, or 48 h postinoculation, cells were washed in PBS and fixed in acetone at −20°C. Fixed cells were blocked for 30 min with 2% normal goat serum, and expression of nucleocapsid protein in infected cells was detected with mouse anti-N MAb followed by fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin antibody as previously described (12). Controls that showed no immunofluorescence included virus-inoculated cells incubated with isotype control MAb and mock-infected cells incubated with anti-N MAb. Each cell line and virus was tested in three independent experiments.

Transfection of swine testis cells with murine CEACAM1a cDNA. The murine CEACAM1a[1-4] cDNA (12) was digested with the restriction enzymes HindIII and NotI and ligated into plasmid pRc/CMV (Invitrogen, Carlsbad, Calif.) under a cytomegalovirus promoter. ST cells in six-well plates were transfected at 50% confluency with the mCEACAM1a[1-4] plasmid with PFX-1 lipid (Invitrogen, Carlsbad, Calif.) according to the manufacturer's instructions. Three days after transfection, the medium was aspirated, and the cells were fed with medium supplemented with 700 μg of G418 per ml. The selection medium was replaced every 3 days. BHK cells stably transfected with mCEACAM1a[1-4] cDNA were described previously (12). BHK and ST cells expressing mCEACAM1a[1-4] were sorted by flow cytometry with anti-mCEACAM1a MAb CC1 prior to use in the experiments in this study.

Nucleotide sequence accession numbers. The S gene sequences for parental MHV-A59 (AY497328), MHV/pi23 (AY497329), MHV/pi600 (AY497330), and MHV/BHK (AY497331) determined in this study were submitted to the GenBank database.

RESULTS

Replication in hamster cells of viruses derived from persistently infected murine cells. We previously reported that the MHV/pi600 and MHV/BHK viruses derived from persistently infected murine 17 Cl 1 cells can be propagated in hamster BHK cells and cell lines from many other nonmurine species (51). To explore the development of the extended host range phenotype, we also examined a virus, MHV/pi23, derived from passage 23 of persistently infected 17 Cl 1 cells. In 17 Cl 1 cells, plaques of MHV/pi23 were markedly smaller and more turbid than those of the parental MHV-A59 virus but larger than the plaques of MHV/BHK or MHV/pi600 (data not shown). MHV-A59 can only be propagated in murine cell lines, as shown by immunofluorescence with a monoclonal antibody (anti-N MAb) that binds the viral nucleocapsid protein (51). In marked contrast, MHV/pi23 was also able to infect BHK cells, as determined by immunofluorescence with the anti-N MAb (Fig. 1), although less than 1% of the cells in a confluent monolayer of BHK cells were infected.

Since BHK cells transfected with murine CEACAM1a are susceptible to infection with MHV-A59 and produce infectious virus (12), it is clear that this virus can replicate in BHK cells once it has entered. Therefore, the low efficiency of MHV/pi23 infection of BHK cells suggests that entry of this virus into BHK cells was inefficient. No plaques were observed in BHK cells inoculated with MHV/pi23, and, unlike MHV/BHK, the MHV/pi23 virus could not be serially propagated in BHK cells. The inefficient infection of BHK cells by MHV/pi23 suggests that this extended host range variant lacks determinants that permit MHV/pi600 and MHV/BHK to efficiently bind and/or enter nonmurine cells.

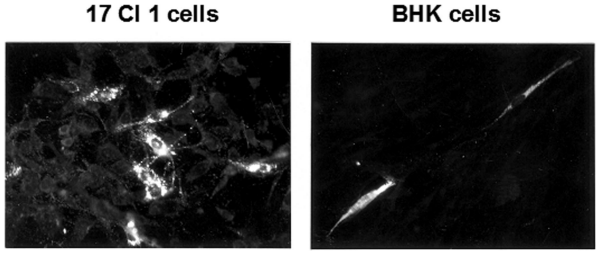

FIG. 1. MHV/pi23 inefficiently infects hamster BHK cells. 17 Cl 1 cells and BHK cells were inoculated with MHV/pi23 at a multiplicity of infection of 3 to 5 (as determined by plaque assay in 17 Cl 1 cells) and fixed at 24 h postinoculation Viral nucleocapsid protein was detected by immunolabeling with anti-N MAb followed by fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin antibody. Magnification, 300×.

Comparison of the sequences of the spike genes of MHV-A59 and viruses from persistently infected murine cells. The spike genes of the parental MHV-A59 and the host range variants were sequenced to identify mutations in the S gene that are responsible for the ability to infect hamster cells (Table 2). Three nucleotide differences were found between the published S gene sequence of MHV-A59 (35) and the parental MHV-A59 used for our experiments and all viruses derived from it. Two of the nucleotide changes resulted in single-amino-acid substitutions R82T and N98S, while the third nucleotide change was noncoding (Table 2). Relative to the parental MHV-A59 virus, the S gene of MHV/pi23 had seven point mutations and a 12-bp insert (Table 2). The 12-bp insert at nucleotide 1492 resulted in a 4-amino-acid insert, KKVL, in the S1 domain (Fig. 2). One of the point mutations in MHV/pi23 resulted in a Q493R substitution just upstream of the insert. The S gene of MHV/pi23 also had a point mutation just downstream of the cleavage site that changed the amino acid sequence RRAHR/S of MHV-A59 to RRAHR/L.

In comparison to the parental MHV-A59 virus, the S gene of MHV/pi600 had 62 point mutations (30 in S1 and 32 in S2) and a 21-bp insert at nucleotide 1492, where the 12-bp insert was located in MHV/pi23. The 21-bp insert in MHV/pi600 shares all but one of the 12 nucleotides in the insert of MHV/pi23 and results in a 7-amino-acid insert, TRTKKVP, in the S1 domain (Table 2). The S gene of MHV/BHK had 63 point mutations and a 21-bp insert compared to the S gene of MHV-A59 (Fig. 2). The 21-bp insert and all but three of the point mutations in the S gene of MHV/BHK were identical to those in MHV/pi600 (Table 2). Fifty-seven of the 63 point mutations in the S gene of MHV/BHK resulted in amino acid substitutions. The S genes of MHV/pi600 and MHV/BHK had a coding point mutation just downstream of the cleavage site like that in the S gene of MHV/pi23.

TABLE 2. Mutations in the S genes of MHV-A59, MHV/pi23, MHV/pi600, and MHV/BHK compared to MHV-A59 (Genbank accession no. M18379)[a]

Nucleotide change	Amino acid change	Change present			
		MHV-A59	MHV/pi23	MHV/pi600	MHV/BHK
A139C	E43D			X	X
G255C	R82T	X	X	X	X
A303G	N98S	X	X	X	X
C354T	T115M			X	X
G408A	G133D			X	X
C477A	T156N			X	X
G639A	G210D			X	X
C645T	T212I			X	X
T712G	D234E			X	X
A810G	Q267R			X	X
T820G	F270L			X	X
A821T	N271Y			X	X
T909C	L300S			X	X
T932G	L308V			X	X
G1023A	R338K			X	X
G1049A	E347K			X	X
C1097T	R363S			X	X
G1098C	R363S			X	X
T1122C	F371S			X	
T1151C	Y381H			X	X
T1358C	Y450H			X	X
A1359G	Y450C		X		
C1402T	464, silent			X	X
T1431C	V474A			X	X
T1436G	S476A			X	X
A1464G	D485G		X	X	X
T1467A	I486K		X	X	X
T1486A	492, silent			X	X
A1488G	Q493R		X		
1492AAGAAGGTACTA1493	KKVL		X		
1492ACTCGGACTAAGAAGGTACCA1493	TRTKKVP			X	X
G1563T	G518V			X	X
C1878T	T623I			X	X
C1961T	651, silent			X	X
C2075T	R689C			X	X
T2101C	697, silent			X	X
C2163T	S718L		X	X	X
T2166G	V719G			X	X
A2423G	N805D			X	X
C2525T	P839S			X	X
A2535G	D842G			X	X
C2574G	T855R			X	X
T2602G	S864R			X	X
G2612A	G868R			X	X
C2702A	Q898K			X	X
A2706G	E899G		X	X	X
G2773T	E921D			X	X
A2816T	M936L			X	X
G2829T	W940L			X	X
C2835T	A942V				X
G2844C	G945A			X	X
A2855C	S949R		X	X	X
T2885G	L959V				X
G2965T	Q985H			X	X
A2967G	D986G			X	X
A2976G	D989G			X	X
C2979T	A990V			X	X
G3055A	1015, silent	X	X	X	X
T3369C	I1120T			X	X
A3442G	1144, silent			X	X
C3597A	P1196Q			X	X
G3627T	W1206L			X	X
T3644G	S1212A			X	X
C3654T	P1215L			X	X
G3679T	K1223N			X	X
C3732T	A1241V			X	X
T3893A	C1295S			X	X
A3906C	K1299T			X	X
G3943T	Q1311H			X	X
A3963C	N1318T			X	X
T3966G	I1319S			X	X

[a] Nucleotides 11, 12, and 13 constitute the start codon of the S gene (35).

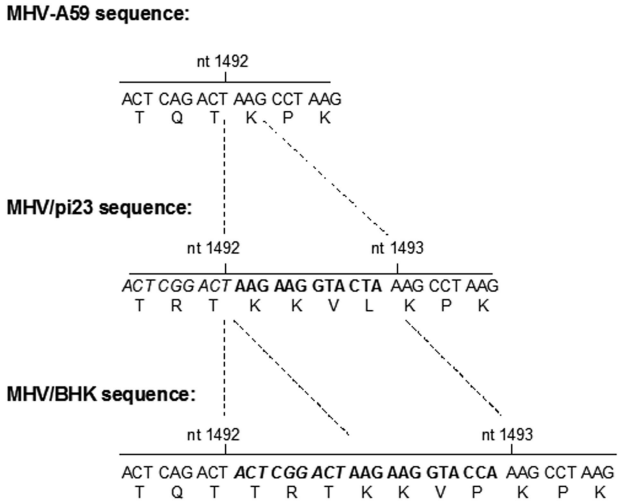

FIG. 2. Comparison of the 12-bp insert of MHV/pi23 with the 21-bp insert of MHV/BHK. Compared to MHV-A59, MHV/pi23 has a 12-bp insert (bold). Eight of the nine nucleotides immediately upstream of the 12-bp inset of MHV/pi23 (italicized) match the 5′-most nine nucleotides (italicized) of the 21-bp insert (bold) of MHV/BHK.

Isolation of viruses that are recombinants between MHV-A59 and MHV/BHK or MHV/pi600. To determine which mutations or insertion in the S gene could permit the MHV/BHK and MHV/pi600 viruses to bind to and/or enter BHK cells, 12 cultures of murine 17 Cl 1 cells (a to k) were coinfected with MHV-A59 and MHV/BHK or MHV-A59 and MHV/pi600, and 74 recombinant viruses were selected on BHK cells at 39.5°C for further study. MHV-A59 can grow in 17 Cl 1 cells at 37 or 39.5°C but cannot infect BHK cells at either 37 or 39.5°C (data not shown). In contrast, the host range variants MHV/BHK and MHV/pi600 can infect 17 Cl 1 and BHK cells at 37°C but cannot grow in either 17 Cl 1 or BHK cells at 39.5°C (data not shown).

Characterization of plaque morphologies and host ranges of recombinant viruses. The plaques formed by all 74 of the recombinant viruses were compared. In murine 17 Cl 1 cells at 37°C, most of the recombinants, such as k6c, k8c, and h4b, formed small plaques approximately 50% of the diameter of MHV-A59 plaques, while a few of the recombinants, such as g5a, formed plaques the same size as MHV-A59 plaques (Fig. 3). None of the recombinant viruses formed plaques as small as those of MHV/BHK in 17 Cl 1 cells. In BHK cells at 37°C, some recombinants, such as k6c and k8c, formed very small, diffuse plaques. Although most of the recombinant viruses formed smaller plaques in BHK cells at 39.5°C than at 37°C, the recombinant h4b formed larger plaques in BHK cells at 39.5°C than at 37°C (Fig. 3).

Recombinant viruses with a variety of plaque morphologies were plaque purified twice in BHK cells at 39.5°C. Seventy-four plaque-purified recombinants were then grown in 17 Cl 1 cells at 37°C for 48 h. To confirm that recombinants could still infect BHK cells after amplification in 17 Cl 1 cells, fresh BHK cells were inoculated at 37°C with one of the plaque-purified viruses at a multiplicity of infection of 3 to 5 PFU/cell. Immunolabeling of the inoculated cells with antinucleocapsid (N) antibody revealed differences between infection of BHK cells and 17 Cl 1 cells. Most of the recombinants caused moderate fusion on 17 Cl 1 cells, while some of the recombinants, such as k6c, Rec2A, and k16e, caused extensive fusion of 17 Cl 1 cells, and a few of the recombinants, such as g5a and h4b, did not cause cell fusion (Fig. 4).

Although most of the recombinants induced the formation of N protein in the cytoplasm of 17 Cl 1 cells, cells infected with Rec2a consistently expressed N antigen in the nuclei of syncytia (Fig. 4). In BHK cells, most of the recombinants had an even distribution of viral N expression, but BHK cells infected by a few recombinants, such as Rec2a and g5a, had viral N expression in bright foci in 20 to 30% of cells (Fig. 4). The differences in plaque morphology, cell fusion, and viral N protein expression seen for the recombinant viruses may reflect alterations in binding and/or fusion due to mutations in S or differences in replication due to mutations in other viral genes.

Detection of crossover events in the spike genes of recombinant viruses. Restriction enzyme sites that differ between the

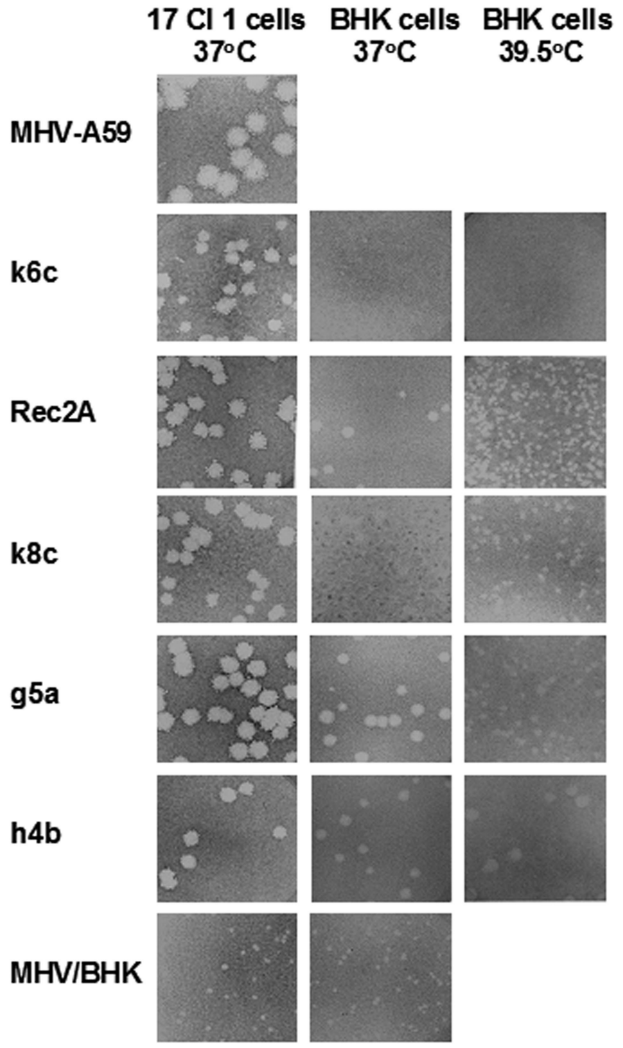

FIG. 3. Plaque morphology differs between recombinant viruses. Twice-plaque-purified recombinant viruses were plaqued in 17 Cl 1 cells at 37°C and BHK cells at 37 or 39.5°C. Plaques were visualized by neutral red staining and photographed 4 days postinoculation.

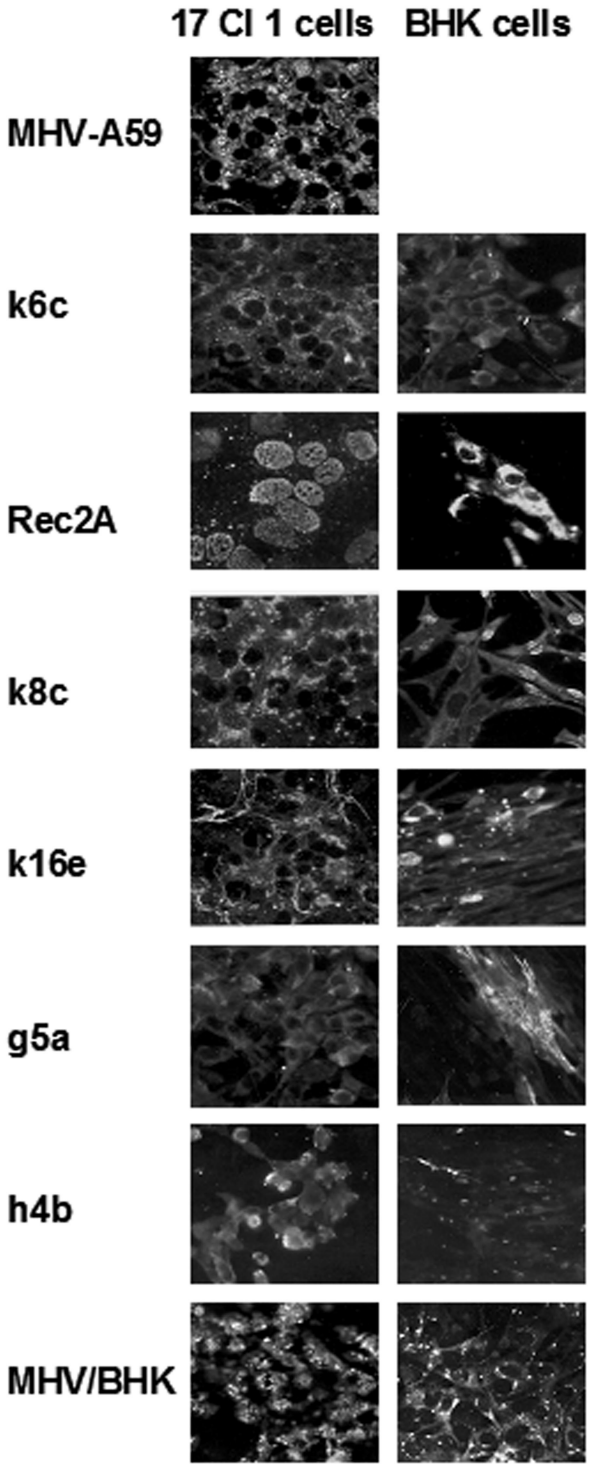

FIG. 4. Expression of viral nucleocapsid protein differs between recombinant viruses. Following plaque purification, recombinant viruses were inoculated onto 17 Cl 1 or BHK cells at a multiplicity of infection of 3 to 5 PFU/cell, and the cells were fixed 24 h postinoculation. Viral nucleocapsid protein was visualized by labeling with anti-N MAb followed by fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin antibody. Magnification, 300×.

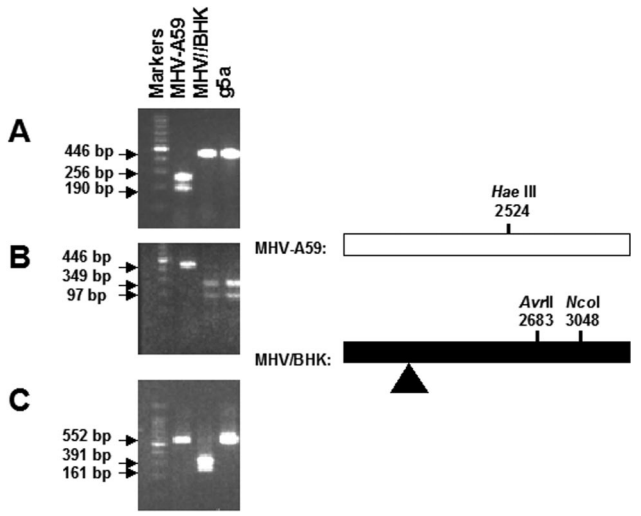

FIG. 5. Restriction enzyme digestion analysis of recombinant viruses with HaeIII, AvrII, and NcoI. RT-PCR of the S genes of recombinant viruses was performed with primers 8 and 19 (Table 1). The RT-PCR products were digested with either HaeIII (A) or AvrII (B). In addition, RT-PCR of the S genes was performed with primers 9 and 16 (Table 1), and the RT-PCR products were digested by NcoI (C). MHV-A59 has one HaeIII site that yielded two fragments of 256 and 190 bp. MHV/BHK has one AvrII site that yielded two fragments of 349 and 97 bp and one NcoI site that yielded two fragments of 391 and 161 bp.

S genes of the parental MHV-A59 and MHV/BHK viruses were used to evaluate whether a crossover event had occurred within the S gene of each of the 74 recombinant viruses. Viral RNA from selected regions of the S gene was amplified by RT-PCR. The resulting cDNA products were examined for the presence of the 21-bp insert with a KpnI site at nucleotide 1510 and for sequences downstream of the insert with an MboI site at nucleotide 2160, an HaeIII site at nucleotide 2524, an AvrII site at nucleotide 2683, an MspI site at nucleotide 2842, an NcoI site at nucleotide 3048, or an AatII site at nucleotide 3644. Examples of HaeIII, AvrII, and NcoI restriction enzyme digestion patterns are shown in Fig. 5.

A summary of the restriction enzyme digestion analysis (Fig. 6) shows that a single recombination event occurred within the S gene of each of the 74 recombinant viruses selected for growth in BHK cells at 39.5°C. Sequencing analysis of the crossover regions of the S genes of representatives of the 74 recombinant viruses showed that each crossover event occurred within one of three regions on the S gene. One of the 74 recombinants had an entire S gene like that of MHV/BHK. Sixty of the 74 recombinants had a single crossover event in the S gene between the AvrII site at nucleotide 2683 and the MspI site at nucleotide 2842. Eight of the 60 recombinants had a crossover event between nucleotide 2706 and nucleotide 2773. Eight of the 74 recombinants had a single crossover between nucleotide 1563 and nucleotide 1878. The remaining five recombinants had crossovers between nucleotide 1493 and nucleotide 1563. Thus, every recombinant virus selected for growth in BHK cells at 39.5°C contained the 21-bp insert and 24 point mutations upstream of the insert found in the 5′ region of the S gene of MHV/BHK.

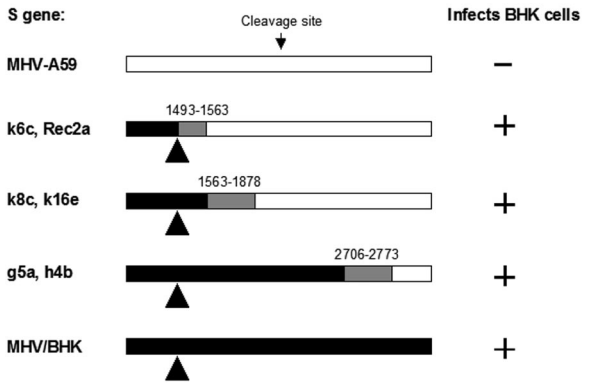

FIG. 6. Recombinant viruses that infect BHK cells have the 5′ region of the S gene from MHV/BHK. Recombinant viruses had a single crossover event in one of three regions of the S gene, as determined by restriction enzyme digestion analysis and sequencing of RT-PCR products. Each of the representative recombinants shown retained the 21-bp insert (triangles) and 24 point mutations upstream of the insert found in the S gene of MHV/BHK.

Binding of MHV-A59, MHV/BHK, and the recombinant virus k6c to cells of different species. To explore the role of the 5′ region of the S gene of MHV/BHK in binding to and entering cells of nonmurine species, we compared the virus-binding activities of three viruses to several cell lines. MHV-A59, MHV/BHK, and the recombinant virus k6c (which had a single crossover in the S gene between nucleotide 1493 and nucleotide 1563) were allowed to bind (10 PFU/cell) to five cell lines: murine 17 Cl 1, hamster BHK, porcine ST, and BHK or ST cells expressing recombinant mCEACAM1a[1-4]. Binding of virions to cells was determined by flow cytometry with anti-S2 MAb 5B19. MHV-A59 bound to 37% of 17 Cl 1 cells (Fig. 7K) but did not significantly bind to BHK or ST cells, which are resistant to infection with this virus (Fig. 7L and N). However, MHV-A59 bound to 12% of BHK and 42% of ST cells that expressed recombinant mCEACAM1a[1-4] (Fig. 7M and O). The increased level of virion binding to ST cells expressing mCEACAM1a[1-4] compared to BHK cells expressing mCEACAM1a[1-4] correlated with a markedly increased level of mCEACAM1a expression (Fig. 7C and E). Thus, MHV-A59 bound to cells that express murine CEACAM1a and did

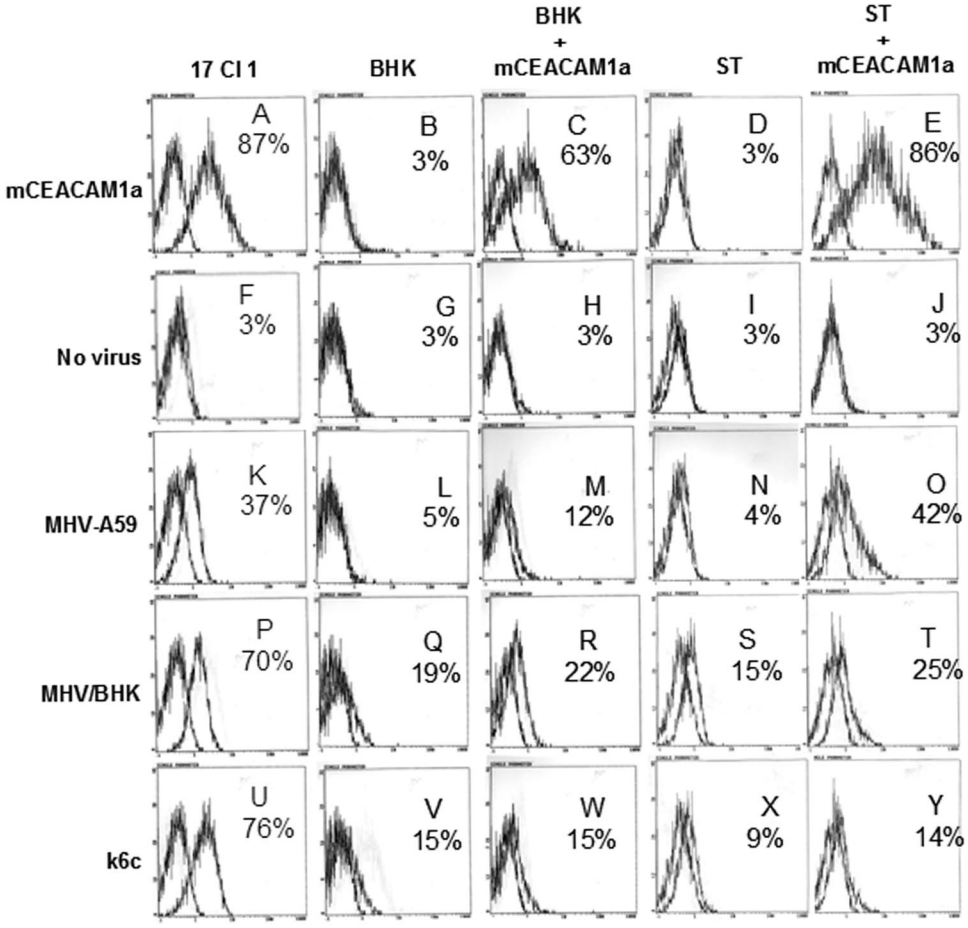

FIG. 7. Binding of viruses to cells of different species. Expression of mCEACAM1a on cells treated with the blocking anti-mCEACAM1a MAb CC1 or an isotype control MAb followed by phycoerythrin-conjugated goat anti-mouse immunoglobulin antibody is illustrated in panels A, B, C, D, and E. The percentage of cells positive for mCEACAM1a expression compared to the isotype control is also indicated. Binding of medium alone (no virus), MHV-A59, MHV/BHK, or the recombinant virus k6c to cells was detected with anti-S2 MAb 5B19 or an isotype control MAb followed by phycoerythrin-conjugated goat anti-mouse immunoglobulin antibody (F through Y). The percentage of cells positive for bound virus compared to the isotype control is also indicated. Flow cytometry data shown are representative of two or more independent experiments.

not bind to nonmurine ST and BHK cells, which do not express murine CEACAM1a.

MHV/BHK bound to 70% of 17 Cl 1 cells, 19% of BHK cells, and 15% of ST cells (Fig. 7P, Q and S). The expression of recombinant mCEACAM1a[1-4] in BHK and ST cells slightly increased the levels of MHV/BHK virus binding (Fig. 7R and T). The recombinant virus k6c, containing the 21-bp insert and 24 point mutations found in the 5′ region of the S gene of MHV/BHK, bound to 76% of 17 Cl 1 cells, 15% of BHK cells, and 9% of ST cells (Fig. 7U, V, and X). Thus, the ability to bind to nonmurine cell lines that do not express mCEACAM1a lies within the 5′ region of the S gene of MHV/BHK. The reduced binding of the recombinant virus k6c compared to MHV/BHK to BHK and ST cells suggests that some of mutations downstream of the insert in the S gene may enhance the binding of MHV/BHK to nonmurine cells.

DISCUSSION

During the course of virus evolution, coronaviruses have adapted to grow in a variety of host species. The interaction of coronaviruses with receptors on host cell membranes is a major determinant of the species specificity of coronavirus infection (8, 12, 24, 33, 57, 65). Some coronaviruses, such as bovine coronavirus, can apparently infect not only their natural bovine host but also other ruminants, sheep, turkeys, and even, rarely, humans (29, 37, 56, 59, 69). Although the virus can cause epizootics in turkeys, it is not transmitted from one infected human to another. In contrast, murine coronavirus MHV-A59 naturally infects only susceptible mice and murine cell lines. Interaction between the S glycoprotein of MHV-A59 and murine CEACAM glycoproteins is required for virus binding and entry (40, 58, 67).

This paper describes mutations in the S gene of MHV-A59 that were selected during persistent infection of murine cells in vitro and were associated with acquisition of extended host range. Similarly, Baric and coworkers isolated and characterized extended host range mutants of MHV-A59 and/or MHV-JHM from persistently infected murine cells and cocultures of murine and hamster cells (1, 2). In this paper, we report the nucleotide sequences of the S genes and the deduced amino acid sequences of the S glycoproteins of three viruses generated during persistent MHV-A59 infection. The MHV/pi23 and MHV/pi600 viruses were isolated from passages 23 and 600, respectively, of murine cells persistently infected with MHV-A59 (50) and twice plaque purified in murine cells. MHV/BHK was derived from MHV/pi600 that was passaged 12 times in hamster cells and twice plaque purified in hamster cells (51).

Compared to the parental MHV-A59, the S gene of MHV/pi23 has seven point mutations and a 12-bp insert encoding seven amino acid substitutions and a 4-amino-acid insertion; the S gene of MHV/pi600 has 62 point mutations and a 21-bp insert encoding 55 amino acid substitutions and a 7-amino-acid insert; and the S gene of MHV/BHK has 63 point mutations and a 21-bp insert encoding 56 amino acid substitutions and a 7-amino-acid insert (Table 2). Mutations in the S gene appear to be strongly selected for during persistent infection in vitro, since the ratio of nonsilent to silent mutations is approximately 12. In the absence of selection, the ratio of nonsilent to silent mutations would be approximately 3 (36). All three viruses isolated from the persistently infected murine cell cultures caused minimal cytopathic effects in and produced small plaques on murine cells that differed from the large plaques produced by the parental MHV-A59 virus. MHV/pi23 caused nonproductive infection of less than 1% of BHK cells (Fig. 1), while MHV/pi600 and MHV/BHK caused productive infection of all BHK cells.

To identify the region of the genome that is required for the extended host range of the MHV variants, recombinant viruses between MHV-A59 and MHV/pi600 or MHV/BHK were selected in hamster cells at 39.5°C. All of the recombinant viruses retained at least the 21-bp insert and 25 or 24 point mutations upstream of the insert in MHV/BHK and MHV/pi600, respectively, as well as genes upstream of the S gene. MHV/BHK and the recombinant virus k6c bound hamster cells in addition to cells expressing mCEACAM1a (Fig. 7). Surprisingly, MHV/BHK and k6c also bound a swine testis cell line that is resistant to infection by MHV/BHK (51), suggesting that these cells may have a postentry defect. The HE genes of MHV-A59 and MHV/BHK are not functional (51), and mutations in gene 1 are not likely to cause differences in virus binding and entry. Thus, mutations in the N-terminal region of S1 are most likely responsible for the extended host range of MHV/BHK and MHV/pi600. Although the N-terminal 330 amino acids of the S of MHV are sufficient for binding to the murine CEACAM1 receptor (32, 58), a region of S responsible for extended host range had not previously been identified. Interestingly, 12 amino acid substitutions in the minimal mCEACAM1a receptor binding domain were found in MHV/pi600 and MHV/BHK. In addition, 10 and 11 amino acid substitutions lay downstream of the minimal receptor binding domain but before the 7-amino-acid insert in the S of MHV/pi600 and MHV/BHK, respectively.

The differences in plaque morphology, cell fusion, and viral N protein expression seen for the recombinant viruses strongly suggest that these viruses had a variety of mutations in the S gene or in the rest of the viral genome in addition to the 21-bp insert and 25 or 24 point mutations. The recovery of 60 recombinant viruses with a single crossover event between nucleotide 2683 and nucleotide 2842 in the S gene supports the idea that some of the mutations in the S gene downstream of the 21-bp insert may enhance the binding and/or entry of these viruses into nonmurine cells. Due to the variety of phenotypes observed for the recombinant viruses, viruses with the 21-bp insert and the 25 point mutations in the N-terminal region of the S gene have been generated by targeted RNA recombination and are currently being characterized to confirm the role of these mutations in the extended host range of MHV/BHK.

The S gene of MHV-A59 may contain a secondary structure at or near nucleotide 1492 that favors insertion of additional nucleotides. Recombination between a cellular gene and S gene sequences may have generated the 12-bp insert found in MHV/pi23 and the 21-bp insert found in MHV/pi600 and MHV/BHK (Fig. 2). The ACTAAGAAGGTAC motif found in the insert of all three viruses is identical to sequences found in the p55 and p85 β regulatory subunits of murine phosphatidylinositol 3-kinase. In MHV/pi23, the 12-bp insert encodes a KKVL amino acid sequence. In MHV/pi600 and MHV/BHK, the insert acquired nine additional nucleotides and encodes a

TRTKKVP amino acid sequence (Fig. 2). The TRTKKV motif is found not only in MHV/pi600 and MHV/BHK but also in MHV/pi23 due to a Q493R substitution directly upstream of the insert. This positively charged amino acid sequence may bind to negatively charged moieties, such as heparin sulfate, on host cell membranes. This motif may also provide an additional protease cleavage site in S1 that could facilitate membrane fusion and virus entry.

The introduction of a virus into a new host species is a complex and poorly understood process. The emergence of viruses such as the human immunodeficiency virus in the 1960s, canine parvovirus in 1978, a seal morbillivirus in 1988, and strains of influenza virus that have caused human pandemics (6, 9, 22, 27, 42, 45, 70) are thought to be due to the introduction of a virus to a new host species. We propose that persistent infection of murine cells which reduces mCEACAM1a expression (7, 47, 50) selects for virus variants with mutations in the S gene that optimize binding and entry into murine cells that express little mCEACAM1a. These mutations may incidentally also permit binding and entry into nonmurine cells. Similarly, selection for host range variants might arise in vivo during persistent infection of tissues that express low levels of CEACAM1a. Persistent coronavirus infection in vivo has been documented for weeks and months after initial infection of cats, pigs, and chickens (25, 28, 64). The RNA quasispecies in persistently infected animals may include virus variants with mutations in the S gene that can utilize alternative receptors on cells of a new host species. During adaptation in a new host species, a coronavirus may lose the ability to infect its original host.

Coronaviruses have emerged in both swine and human hosts. The devastating epizootics of porcine epidemic diarrhea virus in Europe in the 1980s appeared to be due to the adaptation of a coronavirus from an unknown host species to swine (30, 60). The recent severe acute respiratory syndrome (SARS) epidemic was caused by a novel coronavirus (SARS-CoV) that can cause lower respiratory tract infection in humans and adapted to serial human transmission (10, 38, 49). Antibodies to SARS-CoV were not present in human sera banked prior to the epidemic (31, 46). Viruses closely related to SARS-CoV were recovered from Himalayan palm civets and raccoon dogs in exotic meat markets in southern China and contained mutations in the S gene and other viral genes (23). The roles of ecological changes and/or genetic changes in SARS-CoV must be evaluated in order to explain the emergence of SARS-CoV as a human virus. The host range of SARS-CoV appears to be much broader than that of MHV in that SARS-CoV isolated from humans can infect humans, monkeys, cats, ferrets, and mice (14, 15, 39). Functional studies are needed to elucidate the importance of amino acid substitutions in the S glycoprotein of SARS-CoV in determining the viral host range.

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