The Spike Protein of Murine Coronavirus Regulates Viral Genome Transport from the Cell Surface to the Endoplasmic Reticulum during Infection[▽]

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We observed that the nonfusogenic mouse hepatitis virus (MHV) strain MHV-2 reached a titer of $\sim 2 \log_{10}$ higher than that of the fusogenic strain A59 in astrocytoma DBT cells. To determine whether the spike protein is responsible for the difference, a recombinant virus, Penn-98-1, that contains the A59 genome with a spike from MHV-2 was used to infect DBT cells. Results showed that Penn-98-1 behaved like MHV-2, thus establishing a role for the spike protein in viral growth. The inverse correlation between viral fusogenicity and growth was further established in four different cell types and with a fusogenic mutant, the S757R mutant, derived from isogenic Penn-98-1. While both A59 and Penn-98-1 entered cells at similar levels, viral RNA and protein syntheses were significantly delayed for A59. Interestingly, when the genomic RNAs were delivered directly into the cells via transfection, the levels of gene expression for these viruses were similar. Furthermore, cell fractionation experiments revealed that significantly more genomic RNAs for the nonfusogenic MHVs were detected in the endoplasmic reticulum (ER) within the first 2 h after infection than for the fusogenic MHVs. Pretreatment of Penn-98-1 with trypsin reversed its properties in syncytium formation, virus production, and genome transport to the ER. These findings identified a novel role for the spike protein in regulating the uncoating and delivery of the viral genome to the ER after internalization.

Murine coronavirus mouse hepatitis virus (MHV) is a member of the family *Coronaviridae*. It is an enveloped, positive-strand-RNA virus. The viral envelope contains three or four structural proteins, depending on the virus strain (21). The spike (S) protein is a glycoprotein with a molecular mass of approximately 180 kDa. For some MHV strains, such as JHM and A59, the S protein is cleaved by a furin-like proteinase into two subunits, the amino-terminal S1 and the carboxyl-terminal S2. The S1 subunit is thought to form the globular head of the spike and is responsible for the initial attachment of the virus to the receptor on the cell surface. The S2 subunit, which forms the stalk portion of the spike and which anchors the S protein to the viral envelope, facilitates the fusion between the viral envelope and the cell membrane and cell-cell fusion (4, 7, 20, 25, 39). In contrast, the S protein of some other MHV strains, such as MHV-2, does not undergo cleavage and usually does not cause cell-cell fusion (15, 34). It appears that the cleavability of the MHV S protein is associated usually, though not always, with its fusogenicity (10, 36). It has been suggested that the fusogenicity of the S protein may determine the route of virus entry, i.e., via direct fusion with plasma membranes or following endocytosis (11, 34), although the mechanism for virus-induced cell-cell fusion may differ from that for virus-cell fusion during entry (8). The S protein also elicits the induction of neutralizing antibodies and cell-mediated immunity in infected hosts (3). It is therefore an important determinant for

viral infectivity, pathogenicity, and virulence (2, 5, 31, 38). The hemagglutinin-esterase (HE) protein is present only in certain MHV strains (22, 42) and may play a role in viral pathogenesis (44, 45). The small envelope (E) protein and the membrane (M) protein play a key role in virus assembly (40). The nucleocapsid (N) protein is a phosphoprotein of approximately 50 kDa and is associated with the RNA genome to form the nucleocapsid inside the envelope (21, 37).

Infection of host cells by MHV is mediated through the interaction between the S protein and the cellular receptors that are members of the carcinoembryonic antigen (CEA) family of the immunoglobulin superfamily (9). This interaction then triggers fusion between the viral envelope and the plasma membrane or the endosomal membrane, the latter of which follows receptor-mediated endocytosis, thus allowing the nucleocapsid to deliver into the cytoplasm. Direct entry from the plasma membrane appears to be the predominant route for most MHV strains (19, 28), although entry by some mutant MHVs, such as OBLV60 and MHV-2, is low pH dependent, i.e., via endocytosis (11, 34). However, nothing is known about how the genomic RNA is transported to the rough endoplasmic reticulum (ER) for translation. Once on the ER, the viral genomic RNA is translated into a polymerase polyprotein from the 5'-end two open reading frames (two-thirds of the genome) via ribosomal frameshifting. The polymerase polyproteins in turn synthesize genomic and multiple species of subgenomic mRNAs. These mRNAs are then translated into nonstructural and structural proteins, the latter of which are essential for generation of progeny viruses.

MHV can infect rodents, causing hepatitis, enteritis, nephritis, and central nervous system diseases. In the mouse central nervous system, some MHV strains, such as JHM and A59, are neurovirulent, causing acute encephalitis and chronic demyeli-

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nation (1, 13), while others, such as MHV-2, exhibit extremely low neurovirulence, causing only meningitis without apparent encephalitis and demyelination (6, 16, 41). Extensive mutagenesis studies in combination with targeted RNA recombination have identified that the S protein is the major determinant of MHV pathogenicity in animals, although other viral genes also appear to modulate viral pathogenicity (17, 32). For example, the recombinant MHV Penn-98-1, which contains the S protein of MHV-2 in an A59 genome background, causes acute meningoencephalitis similar to that caused by A59 but does not cause demyelination similar to that observed for MHV-2 (6). It has also been shown that the amounts of antigen staining and necrosis in the liver correlate with the viral titer, which is determined largely by the S protein (29). However, how the S protein affects viral titer in cell culture and in animals is not known.

In the present study, we initially observed that the levels of production of infectious viruses in an astrocytoma DBT cell line were markedly different among three MHV strains. Using the recombinant MHV Penn-98-1 and its isogenic S757R mutant, we further established that the S protein is responsible for the observed difference. The difference in virus production between A59 and Penn-98-1 was detected as early as 4 to 6 h postinfection (p.i.) and likely occurred during the early stages of the virus life cycle but after virus internalization. Interestingly, when the genomic RNAs were delivered directly into the cells via transfection, the levels of gene expression for these viruses were similar. Furthermore, cell fractionation experiments revealed that significantly more genomic RNAs for non-fusogenic MHVs were delivered to the ER within the first 2 h after infection than for fusogenic MHVs. These results demonstrate that the spike protein of MHV can regulate the intracellular transport of the viral genome to the ER following internalization. To our knowledge, this is the first study identifying a role for a coronavirus S protein in genome delivery in addition to its well-established role in receptor binding and virus-cell and cell-cell fusions during infection.

MATERIALS AND METHODS

Viruses, cells, and reagents. MHV strains A59 and MHV-2 were a gift of Michael Lai (University of Southern California, Keck School of Medicine, Los Angeles). Penn-98-1 is a chimeric virus on the background of the A59 genome with a replacement of the S gene from MHV-2. Penn-98-1 was kindly provided by Ehud Lavi (University of Pennsylvania School of Medicine). The recombinant S757R mutant has a single-amino-acid serine-to-arginine mutation at amino acid position 757 of the S protein of Penn-98-1 (kindly provided by Susan Weiss, University of Pennsylvania School of Medicine). All viruses were propagated in the astrocytoma cell line DBT (16), and virus titers were determined by plaque assay as described previously (15). DBT cells were grown in minimum essential medium (MEM) with 7.5% newborn calf serum and 10% tryptose-phosphate broth, while the other murine cell lines, SAC, 17Cl-1, and NIH 3T3, were maintained in Dulbecco's minimal essential medium with 10% fetal bovine serum. SAC and 17Cl-1 cells were kindly provided by Kathryn Holmes (University of Colorado Health Science Center, Denver) and Susan Baker (Loyola University of Chicago), respectively.

Radiolabeling and purification of virions. DBT cells were grown to confluence and were infected with various MHV strains at a multiplicity of infection (MOI) of 5. At 1 h p.i., following removal of the virus inoculum, cells were washed with phosphate-buffered saline (PBS) and fed with fresh MEM. Actinomycin D was added to the cells at a concentration of 10 μ g/ml. Viral genomic RNAs were labeled with [³H]uridine (50 μ Ci/ml) from 2 to 16 h p.i. Radiolabeled viruses were harvested from culture medium, clarified from cell debris by low-speed centrifugation at 4,000 rpm for 30 min in a benchtop centrifuge (Marathon 2500; Fisher Scientific), and purified through a 30% (wt/vol) sucrose cushion by ultra-

centrifugation at 27,000 rpm for 3 h with an SW27 rotor (8000L; Beckman). The virus pellets were resuspended in PBS and were used for infection.

Virus internalization assay. DBT cells were grown on 60-mm dishes to sub-confluency. [³H]-labeled and sucrose cushion-purified viruses were then used to infect DBT cells. The virus internalization assay was performed as described previously (24). Briefly, cells were infected with A59 or Penn-98-1 (containing 5,000 cpm) at 4°C for 1 h. Unbound virus was then removed by five washes with PBS containing 0.5% bovine serum albumin (BSA) and 0.05% Tween 20. Infected cells were then moved to 37°C. At 45 min following the temperature shift, infected cells were washed with cold PBS and incubated with proteinase K (0.5 mg/ml) at 4°C for 45 min to remove the bound but uninternalized virus particles. Cells were collected with a rubber scraper, mixed with equal volumes of a buffer containing 2 mM phenylmethylsulfonyl fluoride and 6% BSA to inactivate proteinase K, and centrifuged for 30 s at 14,000 rpm in a microcentrifuge (Micro-max; Fisher Scientific). Cell pellets were washed with MEM containing 2% BSA. Cells were then resuspended in MEM, and the radioactivity was determined with a liquid scintillation counter (model T2500; Beckman). Three replicates per sample were analyzed.

Infectious-center assay. Approximately 3×10^5 DBT cells were infected with MHV A59 or Penn-98-1 at an MOI of 5 at 4°C for 1 h. Unbound virus was removed by five washes with PBS. Infected cells were then moved to 37°C for 1 h to allow virus entry into cells. Cells were then trypsinized, counted, serially diluted with MEM, mixed with uninfected DBT cells, and seeded onto a six-well plate. After cells were attached for 2 to 3 h, soft agar was overlaid as in the plaque assay, and the plates were incubated for 2 days. The number of infectious centers (plaque) was counted following neutral red staining.

Detection of viral RNA synthesis. DBT cells were grown in six-well plates and infected with MHV at an MOI of 5, washed with serum-free medium twice, and incubated in medium containing 1% fetal bovine serum. Actinomycin D (5 μ g/ml) was added 1 h prior to infection. At 1 h p.i., [³H]uridine (50 μ Ci per ml) (NEN, Boston, MA) was added to the medium. Cells were labeled for 6 h, collected, and washed twice with cold PBS. Intracellular RNAs were extracted by use of Trizol reagent (Gibco) and precipitated by 10% trichloroacetic acid (TCA). Following three washes with 10% TCA on fiberglass filter paper, the radioactivity of the TCA precipitates was determined in a scintillation counter (model T2500; Beckman) and expressed as counts per minute.

Western blot analysis. For detection of viral proteins, DBT cells were infected with MHV strains at an MOI of 5. At various time points p.i., cells were washed five times with PBS and lysed by use of a radioimmunoprecipitation assay buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% sodium dodecyl sulfate) containing proteinase inhibitors. Intracellular proteins in the lysates were separated by electrophoresis on 10% polyacrylamide gels and were then transferred to nitrocellulose or polyvinylidene difluoride membranes (MSI). After a blocking step with 5% nonfat milk in TBST buffer (10 mM Tris-Cl [pH 7.5], 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature, the membranes were incubated overnight at 4°C with a monoclonal antibody specific to the viral N protein (1:2,000 dilution) or anti- β -actin antibody (1:2,000 dilution; Sigma) in TBST-milk. The anti-N monoclonal antibody was kindly provided by Stephen Stohman (The Cleveland Clinic) and John Fleming (University of Wisconsin Medical Center at Madison). Following extensive washing of the membranes with TBST buffer for 10 min, horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (1:2,000 dilution; Sigma) was added, and the reaction mixture was incubated for 1 h at room temperature. Proteins were detected using Renaissance Western blot chemiluminescence reagent (NEN, Boston, MA) and exposed to X-ray film (Kodak). For detection of cellular proteins, primary antibodies specific to β -actin (Sigma), the rough ER marker calnexin (1:2,000 dilution; GeneTex), and the endosome marker Rab5 (1:2,000 dilution; Abcam) were used.

In vitro RNA transcription and transfection. Plasmid p25CAT DNA, which contains the MHV defective-interfering-chloramphenicol acetyltransferase (DI-CAT) reporter RNA (44), was linearized by XbaI digestion, purified with a gel purification kit (Qiagen), and used as a template for in vitro transcription. The in vitro transcription reaction was carried out with T7 RNA polymerase according to the manufacturer's protocol (mMessage mMachine kit; Ambion). Briefly, the reaction mixture was assembled in an RNase-free microfuge tube containing 1 μ g of linearized template DNAs, 2 μ l of enzyme mixtures, 10 μ l of 2 $\times$ nucleoside triphosphate cap analog, and 2 μ l of 10 $\times$ reaction buffer. The reaction mixture was incubated at 37°C for 4 h. For real-time quantitative reverse transcription-PCR (qRT-PCR), plasmid pMHV-A59-A, which contains the T7 promoter and the 5'-end 4.8-kb sequence of the A59 genome, was used as the template for in vitro transcription. The in vitro-transcribed RNA was then quantified and used as a control and as a standard for real-time RT-PCR with the

same pair of primers. RNA transfection was carried out using Lipofectamine reagent (Invitrogen) according to the manufacturer's protocol.

CAT assay. The CAT assay was carried out as described previously (46). Briefly, cells were cotransfected with viral genomic and DI-CAT RNAs. At various time points posttransfection, cells were washed twice with PBS, followed by the addition of 125 μ l of Tris-HCl (0.25 M, pH 8.0), and were lysed by being frozen and thawed three times. The lysates were incubated at 60°C for 10 min and then clarified from debris by centrifugation at 12,000 $\times$ g for 5 min. The CAT assay reaction mixture, which contained 5 μ l of *N*-butyl coenzyme A (5 mM), 50 μ l cell lysate, 66 μ l Tris-HCl (0.25 M, pH 8.0), and 4 μ l [14 C]chloramphenicol (50 μ Ci/ml), was incubated at 37°C for 16 h. The reaction was terminated by the addition of 300 μ l of mixed xylenes (Sigma). The mixture was vortexed for 30 s and spun at 12,000 $\times$ g for 3 min. The upper phase (xylenes) was transferred to a new tube and was extracted twice with 100 μ l of fresh Tris-HCl (0.25 M, pH 8.0). Finally, 200 μ l of the upper phase was transferred to a scintillation vial containing scintillation fluid. The radioactivity of the butyrylated chloramphenicol products, which represents the CAT activity, was measured in a liquid scintillation counter. The CAT activity is indicated as the increase over the background level, which is set at one-fold and which is obtained by measuring the radioactivity in the lysates from mock-transfected cells. At least three independent experiments were performed for each sample.

Isolation of the ER fraction. The ER fraction was isolated with an ER isolation kit (Sigma) according to the manufacturer's instructions (12). Briefly, DBT cells were infected with MHVs at an MOI of 5 at 4°C for 1 h and then washed thoroughly with cold PBS. Infected cells were moved to 37°C for various times to allow virus entry. Note that 0 h p.i. indicates that the infected cells were not incubated at 37°C. Mock-infected cells were used as a negative control. Cells were collected and homogenized in isotonic extraction buffer (10 mM HEPES, pH 7.8, 250 mM sucrose, 25 mM potassium chloride, and 1 mM EGTA) by Dounce homogenization. The homogenates were centrifuged at 1,000 $\times$ g for 10 min at 4°C, and the supernatants were further centrifuged at 12,000 $\times$ g for 15 min at 4°C to remove the nuclei and mitochondria. The microsomal fraction containing the ER was precipitated with a calcium chloride (8 mM) solution (7.5 times the volume of the supernatants) for 15 min, followed by centrifugation at 8,000 $\times$ g for 10 min at 4°C. The purity of the ER fractions was determined by Western blot analysis with antibodies specific to the ER marker calnexin and the endosome marker Rab5. The protein content of the ER fraction was further quantified using a Bio-Rad protein assay kit.

RNA isolation and real-time qRT-PCR. Following ER isolation as described above, the amount of the ER fraction for each sample was normalized based on the protein content. RNAs were then extracted from the ER fraction using Trizol reagent and were treated with DNase I (Invitrogen, Carlsbad, CA). RNAs were then used for qRT-PCR analyses. The primer pairs specific to MHV genomic RNA (5'IAA59-leader [5'-AAG AGT GAT TGG CGT CCG TAC-3'] and 3'IAA59-177 [5'-ATG GAC ACG TCA CTG GCA GAG-3']) and subgenomic mRNA7 (for the N gene) (5'IAA59-leader and 3'IAA59-N29766 [5'-TGG TCA GCC CAA GTG GTC TTC-3']) were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The specificity and efficiency of detection have been validated in other studies (unpublished data). The RT reaction was carried out using an iScript Select cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer's instructions. Real-time PCRs were performed with a total reaction volume of 25 μ l using an IQ SYBR Green Supermix kit (Bio-Rad Laboratories), containing a final concentration of 200 nM of forward and reverse primers and 6 μ l of cDNA from the RT step, and results were analyzed using an iCycler IQ multicolor real-time PCR detection system (Bio-Rad Laboratories). The detection levels following the various experimental treatments were calculated after normalizing cycle thresholds against the in vitro-transcribed MHV RNA template as a standard with a known amount and are presented in nanograms.

Statistical analysis. Where possible, data were analyzed for statistical significance and are expressed as means $\pm$ standard deviations. The mean values were compared using Student's *t* test. *P* values of <0.01 or <0.05 were considered statistically significant.

RESULTS

Inverse correlation between viral growth and syncytium formation in DBT cells. During our initial study, we observed that MHV-2 had a titer of approximately 2 log₁₀ higher than that of A59 in DBT cells at 16 h p.i. (*P* < 0.01) (Fig. 1A) and that the viral titers inversely correlated with the ability to induce cell-

FIG. 1. Differential virus production inversely correlates with MHV fusogenicity in DBT cells. DBT cells were infected with three MHV strains (A59, MHV-2, and Penn-98-1) at an MOI of 5. (A) Culture media were collected at 16 h p.i., and virus titers were determined by a plaque assay with DBT cells. The virus titer is expressed as the mean log₁₀ PFU/ml from three independent experiments. Error bars indicate the standard deviations of the means. (B) Cell fusion was observed under a microscope (Olympus IX70), and representative photographs were taken at 10 h p.i. with an attached digital camera (Optronics, Inc.).

cell fusion (syncytia) in DBT cells (Fig. 1B). Because it is well documented that the S protein of coronaviruses is responsible for cell-cell fusion, this observation led us to hypothesize that the S protein may be responsible for the difference in virus propagation among the two MHV strains. To test this hypothesis, we used a recombinant MHV, Penn-98-1, which contains an MHV-2 S gene in the background of the A59 genome. Thus, the major difference between Penn-98-1 and A59 is the S gene. Following infection of DBT cells with Penn-98-1 for 16 h, the recovered virus had a titer similar to that of MHV-2 (approximately 8 log₁₀ PFU/ml) (*P* > 0.05) but approximately 2 log₁₀ higher than that of A59 (*P* < 0.01) (Fig. 1A), thus establishing that the S protein is the determinant for the observed difference in virus titer. Penn-98-1 also lacked the ability to induce cell-cell fusion (syncytia) in DBT cells (Fig. 1B), which is consistent with the property of an MHV-2 S protein. These results demonstrate that the S protein of MHV plays a role in virus production.

Fusogenicity of the viral S protein is associated with differential virus production. To extend the above-described observation, we used a recombinant S757R mutant virus, which contains a single-amino-acid mutation at the cleavage site of the S protein of Penn-98-1 (34). This mutation renders the recombinant S757R virus fusogenic in DBT cells, as opposed to the nonfusogenic S protein of the parental, isogenic strain Penn-98-1 (Fig. 2A). Interestingly, the titers for Penn-98-1

FIG. 2. The efficiency of virus production is associated with the fusogenicity of the viral spike protein. (A) DBT cells were infected with A59, Penn-98-1, and the S757R mutant at an MOI of 5, and cell fusion was observed at 12 h p.i. (B) One-step virus growth curve. DBT cells were infected with A59, Penn-98-1, or the S757R mutant at an MOI of 5. At various time points p.i., culture media were collected and virus titers determined by plaque assay. Results are expressed as the mean log₁₀ PFU/ml from an experiment done in triplicate. Error bars indicate standard deviations of the means.

were approximately 2 log₁₀ higher than those for the S757R mutant and the wild-type A59 beginning at 6 h p.i., and this difference maintained throughout the 24-h experimental period (Fig. 2B). This result suggests that the fusogenicity of the S protein is associated with the virus growth property.

To further determine whether the fusogenicity of the S protein is directly associated with virus production, we used various cell types for MHV infection, since Frana and coworkers have shown previously that the fusion activity of A59 is dependent on cell type (10). Consistent with their observations, our results showed that A59 induced extensive cell-cell fusion in DBT and Sac cells but minimal fusion in 17Cl-1 and NIH 3T3 cells (Fig. 3A). In contrast, Penn-98-1 did not induce cell-cell fusion in any of the four types of cells tested (Fig. 3A). Interestingly, the difference in virus titer between A59 and Penn-98-1 was much greater with DBT and Sac cells (>2.5 log₁₀) ($P < 0.01$) than with 17Cl-1 and NIH 3T3 cells (<1 log₁₀) ($P > 0.05$) (Fig. 3B). Thus, there appears to be a negative (inverse) correlation between the fusogenicity of the viral S protein and the efficiency of virus propagation.

MHV A59 and Penn-98-1 enter DBT cells at similar levels.

It is well known that the S protein of coronavirus is responsible for virus attachment and penetration to the host cell. It is thus conceivable that if the S proteins of the two viruses have

FIG. 3. Inverse correlation of virus production with virus-induced cell-cell fusion in different types of mouse cells. Various types of cells (DBT, Sac, 17Cl-1, and NIH 3T3 cells) were infected with A59 or Penn-98-1 at an MOI of 5. (A) Cell fusion was observed under a microscope, and photographs were taken with an attached digital camera. (B) Virus titers were determined by plaque assay at 24 h p.i. The results are expressed as the mean log₁₀ PFU/ml from three independent experiments. Error bars indicate standard deviations of the means.

different properties related to virus attachment and penetration, subsequent levels of virus production would likely also be different. To test this possibility, we performed a virus internalization assay. [³H]uridine-labeled MHV A59 and Penn-98-1 viruses (with equivalent amounts of radioactivity) were used to infect DBT cells at 4°C for 1 h. After removal of unbound virus, cells were placed at 37°C for 45 min to allow virus internalization. Uninternalized viruses were removed by treatment with proteinase K, and cell-associated radioactivity was

FIG. 4. Parental A59 and recombinant Penn-98-1 viruses enter cells at similar levels. (A) Virus internalization assay. DBT cells were infected with radiolabeled MHV strains A59 and Penn-98-1, with equivalent levels of radioactivity. Viral genomic RNAs were prelabeled with [³H]uridine, and the radiolabeled viruses were purified as described in Materials and Methods. Virus attachment was carried out at 4°C for 1 h. Following extensive washing of unbound viruses, cell cultures were either kept at 4°C as a negative control or moved to 37°C for an additional 45 min, the latter of which allows the virus to internalize. Bound but uninternalized viruses on the cell surface were then removed by treatment with protease K. Cells were then lysed, and intracellular radioactivity was determined with a liquid scintillation counter. The results are expressed as the mean counts per minute from three independent experiments. Error bars indicate the standard deviations of the means. (B) Infectious-center assay. Equivalent numbers of DBT cells were infected with MHV A59 and Penn-98-1 at an MOI of 5 at 4°C for 1 h to synchronize the infections. Cells were then moved to 37°C for 1 h to allow virus to enter the cells. Infected cells that remained at 4°C were used as a negative control. An infectious-center assay was then performed to determine the number of infected cells (infectious centers) for the original virus inoculum, as described in Materials and Methods. Error bars indicate the standard deviations of the means.

then determined. Figure 4A shows that there was no significant difference in radioactivity between the cells infected with the two viruses ($P > 0.05$). This result indicates that both viruses, with equivalent numbers of physical particles, bind and enter cells at similar levels of efficiency, which further indicates that both viruses have similar infectivities.

To further rule out the possibility that a fusogenic virus, such as MHV A59, is less infectious than the nonfusogenic strain Penn-98-1, we carried out a complementary infectious-center assay in which DBT cells were infected with A59 or Penn-98-1 at an MOI of 5. As shown in Fig. 4B, the numbers of infectious centers formed at 1 h following the temperature shift to 37°C were similar for A59 and Penn-98-1 ($\sim 5 \log_{10}$), indicating similar efficiencies of internalization by the two viruses. The lower number of infectious centers ($\sim 3 \log_{10}$) for cells kept at 4°C following infection likely resulted from residual virions

FIG. 5. Comparative analysis of viral RNA and protein syntheses. (A) Detection of viral RNA synthesis. DBT cells were infected with A59 or Penn-98-1 at an MOI of 5 in the presence of actinomycin D. Intracellular viral RNAs were labeled with [³H]uridine, extracted and precipitated by use of TCA at 5 h p.i., and quantified with a liquid scintillation counter. The results are expressed as mean counts per minute from three independent experiments. Error bars indicate the standard deviations of the means. (B) Western blot detection of viral protein synthesis. DBT cells were infected with A59 or Penn-98-1 at an MOI of 5. At various times p.i., as indicated, cell lysates were extracted. Proteins were separated by polyacrylamide gel electrophoresis (10%), transferred to nitrocellulose membranes, and detected with a monoclonal antibody specific to the MHV N protein by Western blotting. β-Actin was used as an internal control for normalization. The arrows indicate the specific protein bands.

remaining on the cell surface due to incomplete removal and subsequent entry during the assay at 37°C. Nevertheless, the levels for both viruses were also similar. Thus, these results suggest that the particle-to-PFU ratios are similar for A59 and Penn-98-1 and that the observed difference in virus titer between the two viruses does not result from a lower infectivity of A59.

Differential viral gene expression by MHV A59 and Penn-98-1. Next, we determined whether MHV A59 and Penn-98-1 have different levels of gene expression. We compared viral RNA and protein syntheses between A59 and Penn-98-1. DBT cells were infected with A59 and Penn-98-1 at an MOI of 5. Viral RNA synthesis was monitored by the incorporation of [³H]uridine in the presence of actinomycin D. As shown in Fig. 5A, [³H]uridine incorporation was significantly higher in Penn-98-1-infected cells than in A59-infected cells ($P < 0.01$). We then compared viral protein synthesis between the two viruses. Because the N protein is one of the most abundant viral proteins synthesized in infected cells, we used a monoclonal antibody specific to the N protein to monitor intracellular viral

protein synthesis. As shown in Fig. 5B, N protein was detected in Penn-98-1-infected DBT cells as early as 4 h p.i. and was increased drastically from 6 to 12 h p.i. In contrast, the N protein in A59-infected DBT cells was hardly detectable at 6 h p.i., and the levels of N-protein synthesis from 8 to 24 h p.i. were significantly lower than those for Penn-98-1. These results indicate that the two viruses exhibit a distinct difference in level of viral gene expression.

Difference in uncoating and/or intracellular transport between MHV A59 and Penn-98-1. The combined results shown in Fig. 2B and 5B suggest that the difference in virus production between the two viruses likely occurs either at the step of viral gene expression or at steps prior to gene expression but following internalization. To distinguish these two possibilities, we isolated viral genomic RNAs from sucrose gradient-purified viral particles and then cotransfected them with the DI-CAT reporter RNA in DBT cells. The DI-CAT reporter system has been well established to monitor the gene expression level for MHV (Fig. 6A) (23, 46). The DI RNA encodes a CAT gene, which is under the control of a functional MHV transcriptional regulatory sequence. At 5 h posttransfection, cells were harvested and the CAT activities determined. The results showed that the CAT activities from cells cotransfected with A59, MHV-2, Penn-98-1, or S757R mutant genomic RNAs were significantly higher than those from cells transfected with the DI-CAT reporter RNA alone ($P < 0.01$) (Fig. 6B), indicating that the expression of the CAT activity specifically requires helper virus replication. However, there was no significant difference in CAT activity from cells cotransfected with each of the four viral genomes ($P > 0.05$) (Fig. 6B). In contrast, when the DI-CAT RNA was transfected into DBT cells that had been infected with the respective live virus for 1 h, the CAT activity was significantly (~ 5 -fold) higher in MHV-2- and Penn-98-1-infected cells than in A59- and S757R mutant-infected cells at 6 h p.i. ($P < 0.01$) (Fig. 6C). These data combined demonstrate that there is no difference in viral gene expression among the four viruses once their genomic RNAs bypass the uncoating and delivery steps during infection, thus suggesting that there must be a difference in uncoating and delivery of genomic RNAs between the fusogenic MHVs (A59 and the S757R mutant) and the nonfusogenic MHVs (Penn-98 and MHV-2) following penetration.

The viral S protein regulates the transport of the viral genome to the ER following penetration. To further determine whether there is indeed a difference in uncoating and genome transport to the ER (the initial site for viral genome translation) between the fusogenic and nonfusogenic MHV strains, DBT cells were infected with the four viruses at 4°C for 1 h. Unbound viruses were washed with PBS, and the bound viruses were allowed to penetrate into cells by a shift in temperature to 37°C. At various time points, cells were lysed and fractionated, and the ER fraction was isolated with a specific ER isolation kit. The purity of the ER fraction was determined by Western blot analysis using antibodies specific to the ER marker calnexin and the endosome marker Rab5 (as a negative control). The ER marker, calnexin, was clearly detected in the ER fractions at all time points and in the unfractionated lysates, while the endosome marker, Rab5, was detected only in the unfractionated lysates (Fig. 7A, lane C). These data confirmed that the preparation was a highly enriched ER fraction with

FIG. 6. Comparative analysis of viral gene expression by use of a reporter system. (A) Diagram of the coronavirus DI-CAT reporter system. The structure of the bicistronic DI-CAT RNA, with a 5' leader (L) and a 3' poly(A) tail [(A)n], is shown. Expression of the CAT activity depends on the transcription of a subgenomic CAT mRNA from the coronavirus intergenic (IG) sequence, which is in turn dependent on helper virus to provide polymerase function. UTR, untranslated region; ORF, open reading frame. (B and C) CAT activities. DBT cells were transfected with the genome of one of the MHV strains (A59, MHV-2, Penn-98-1, or the S757R mutant) (B) or infected with one of the four viruses (C) and cotransfected with in vitro-transcribed DI-CAT RNA or cotransfected with DI-CAT RNA alone as a background control (mock). Cell lysates were collected at 5 h posttransfection, and CAT activity was determined. Values are means from three independent experiments. Error bars represent standard deviations.

minimal, if any, contamination with other cellular organelles, such as the endosome. Real-time qRT-PCR was then performed to quantify the viral genomic RNAs in the ER by use of a primer pair corresponding to the most-5'-end 177 nucleotides of the viral genome (Fig. 7B). Results showed that viral genomic RNAs were undetectable at 0 h p.i. (Fig. 7C), demonstrating the high specificity of the real-time RT-PCR method and the high purity of the ER preparation, because the

FIG. 7. Detection of viral genomic RNAs in the ER by real-time qRT-PCR. DBT cells were infected with four MHV strains (A59, the S757R mutant, MHV-2, and Penn-98-1) at an MOI of 5. At various time points p.i., as indicated, cells were lysed and fractionated. The ER fractions were then isolated with an ER isolation kit. (A) The purity of the ER fractions was determined by Western blot analysis with an antibody specific to the ER marker calnexin or an antibody to the endosome marker Rab5 as a negative control. Unfractionated cell lysates were loaded as a control (lane C). (B) Schematic diagram showing the relative locations of the primers used in RT-PCR to the templates (viral genomic RNA and subgenomic mRNA) and the lengths of the RT-PCR products. (A)n, 3' poly(A) tail. (C and D) Detection of viral genomic RNA and subgenomic mRNA in the ER. Following isolation of the ER at the indicated time points p.i., RNAs were extracted from equivalent amounts of ER for each sample with Trizol reagent and were used for detection of viral genomic RNA (C) or subgenomic mRNA (D) by real-time qRT-PCR. The level of viral RNA detected was calculated after normalizing cycle thresholds against the in vitro-transcribed MHV genomic RNA from a plasmid with a known amount as the standard. Values are means from two to three independent experiments. Error bars represent standard deviations. Statistical significance between the groups is indicated with one asterisk ($P < 0.05$) or two asterisks ($P < 0.01$).

presence of virions on the cell surface at this time point apparently did not result in false-positive detection in the ER following fractionation and purification. Viral genomic RNAs began to be detected in the ER at 1 h p.i. (after the temperature shift to 37°C) and increased only slightly at 2 h p.i., indicating that most of the transport of the virion genome from the cell surface to the ER takes place in 1 h. The amount of genomic RNAs in the ER drastically increased at 3 and 4 h p.i. Importantly, significantly more genomic RNAs for MHV-2 and Penn-98-1 were detected in the ER through 4 h p.i. than for A59 and the S757R mutant ($P < 0.05$) (Fig. 7C). When the same real-time RT-PCR method was employed for detecting subgenomic mRNA (Fig. 7B), no subgenomic mRNA was detected in the ER within the first 2 h p.i., but it was detected at 3 h p.i. and drastically increased at 4 h p.i. (Fig. 7D). The combined data shown in Fig. 7C and D indicate that the amounts of genomic RNAs detected in the ER within the first 2 h p.i. most likely reflected the virion genomes transported to the ER during the initial infection, while the majority of those detected at 3 and 4 h p.i. were the products of secondary replication/transcription. Thus, we conclude that the genomes of MHV-2 and Penn-98-1 arrived at the ER for translation of

the polymerase gene earlier or more efficiently than those of A59 and the S757R mutant, suggesting that the MHV S protein has the ability to regulate the uncoating and genome transport/delivery processes.

Pretreatment of virions with trypsin reversed the phenotype of Penn-98-1 regarding syncytium formation, virus production, and genome transport to the ER. To further test the hypothesis that the S protein and more specifically its cleavage and fusogenic properties are associated with the genome transport from the cell surface to the ER during infection, Penn-98-1 was subjected to pretreatment with trypsin. DBT cells were infected with trypsin-treated or mock-treated Penn-98-1 at 4°C for 1 h. Infected cells were then washed and fed with fresh medium in the presence or absence of trypsin and placed at 37°C to allow virus entry. Cells were observed for syncytium formation at 30 h p.i., and culture medium was collected at 16 h p.i. for the determination of virus production. In parallel experiments, the ER was isolated at 1 and 2 h p.i. for detection of viral genomic RNAs. As shown in Fig. 8A, pretreatment of Penn-98-1 with trypsin converted its nonfusogenic phenotype to fusogenic, as revealed by the appearance of extensive syncytia at 30 h p.i., while little to no syncytia were observed when

FIG. 8. Effect of trypsin treatment on virus-induced cell-cell fusion, virus production, and transport of the viral genome to the ER. Penn-98-1 was treated with trypsin at a concentration of 5 μ g/ml for 20 min at 37°C or was mock treated as a control. DBT cells were infected with trypsin- or mock-treated Penn98-1 at an MOI of 5 in the presence or absence of trypsin, respectively. (+) indicates that the virus was treated with trypsin; (-) indicates that the virus was untreated and that the infected cells were cultured in the absence of trypsin. (A) Infected cells were observed under a microscope for fusion formation. (B) Culture medium was used for determining virus production (titer) by plaque assay. The virus titer is expressed as the mean $\log_{10}$ PFU/ml from independent experiments. Error bars represent standard deviations of the means. (C) ER isolation and real-time qRT-PCR were performed to detect viral genomic RNAs in the ER, as described in the legend for Fig. 7 and in Materials and Methods. Error bars represent standard deviations of the means. Statistical significance between trypsin-treated and mock-treated samples is indicated with an asterisk ($P < 0.05$).

cells were infected with mock-treated Penn-98-1 for the same period of time. Conversely, when DBT cells were infected with untreated Penn-98-1 in the presence of trypsin, syncytia formed, while no syncytia were detected in cells infected with

trypsin-treated Penn-98-1 when trypsin was absent from the culture medium (data not shown). These results support the notion that cleavage of the S protein of nonfusogenic Penn-98-1 by trypsin redirects the virus to enter cells through fusion with the plasma membrane, as in the case of A59. Similarly, the virus titer decreased by approximately 2 $\log_{10}$ following trypsin treatment (Fig. 8B). Consistent with the reverse in virus production, fewer genomic RNAs for Penn-98-1 were detected in the ER at 1 and 2 h p.i. after trypsin treatment, while trypsin treatment did not significantly affect the transport of viral genomic RNAs to the ER for A59, since the S protein of A59 is already cleaved (Fig. 8C). These results demonstrate that the S protein is responsible at least in part for the observed difference in virus production between the fusogenic and nonfusogenic MHV strains by regulating the transport of the viral genome from the cell surface to the ER during virus infection.

DISCUSSION

In the present study, we have shown that the levels of growth of three MHV strains (A59, MHV-2, and Penn-98-1) in DBT cells varied significantly (Fig. 1). This difference is due to the viral S protein, because the recombinant MHV Penn-98-1, which contains the S gene from MHV-2 and the rest of the genome sequence from A59, exhibited a growth property similar to that of MHV-2 (Fig. 1). This conclusion is further supported by the finding that a single-amino-acid mutation in the S protein of Penn 98-1 reverses the growth property (Fig. 2). Thus, these results raise an interesting question as to how the viral envelope S protein influences viral titer in addition to its known functions for virus attachment and virus-cell fusion during entry.

Previous studies have shown that the levels of fusogenicity of MHV A59 vary among different cell types in which the virus is grown and that the fusogenicity of different MHV strains correlates with the degree of proteolytic cleavage of the respective S proteins (10, 16, 18). A careful reexamination of these data reveals that there also appears to be a general correlation between the fusogenicity of the S proteins and the growth of the given virus strains (10). Our direct comparison of fusogenicity and growth property between A59 and Penn-98-1 in different cells reaffirmed and further extended these observations (Fig. 3). Moreover, by using a recombinant S757R mutant MHV, which has a single-amino-acid mutation from serine to arginine at the cleavage site of the S protein and which becomes fusogenic in DBT cells, as opposed to the nonfusogenic parental strain Penn-98-1, we were able to pinpoint that the fusogenicity of the S protein is inversely correlated with the production of infectious viruses (Fig. 2). However, exactly how the fusogenicity of the S protein influences viral growth remains a mystery. Since the S protein is the virion surface protein that induces virus-cell and cell-cell fusion during infection, we initially hypothesized that the cell fusion (syncytia) induced by infection with fusogenic MHVs may damage cell membrane integrity and cause cell death, resulting in reduced production of infectious viruses. However, results from analyses of membrane integrity and cell viability following virus infection indicate that there is no correlation between cell death and viral growth, at least before 8 h p.i. (data not shown). Thus, the fusogenicity of the S protein is not the cause

of reduced virus production, even though these events appear to be tightly coupled.

We therefore searched for other factors that may be associated with the S protein and that may contribute to the observed difference in viral growth and gene expression at early stages of the virus life cycle. Contrary to our expectation, results from virus internalization and infectious-center assays showed that both A59 and Penn-98-1 penetrated cells at similar levels (Fig. 4), which appear to depend on CEA engagement since pretreatment of DBT cells with the CC1 antireceptor antibody blocked the infection with both viruses (data not shown). However, viral RNA and protein syntheses were significantly higher for Penn-98-1 than for A59 (Fig. 5). Thus, these results suggest that the difference in viral gene expression between the two viruses must result from events that occur during or after virus internalization but before or during viral RNA synthesis. Interestingly, when the viral genomic RNAs and the DI-CAT reporter RNA were cotransfected into DBT cells, the levels of viral gene expression as measured by CAT activity were similar among all four viruses (Fig. 6), suggesting that the difference in viral gene expression must be due to events that take place prior to viral genome translation. Real-time qRT-PCR detection of viral genomic RNAs in the ER further indicates that the delivery of the viral genome to the ER was significantly less efficient in cells infected with fusogenic viruses (A59 and the S757R mutant) than in cells infected with nonfusogenic viruses (Penn-98-1 and MHV-2). Taken together, our studies identified a previously unrecognized role for the MHV S protein in viral infection, i.e., regulating the transport or delivery of viral genomic RNAs to the ER following receptor binding and penetration (internalization). Although Yokomori et al. (43) have previously suggested that an S-protein-associated cellular factor is likely involved in virus penetration, a role for the coronavirus S protein in genome delivery has not been described before.

How is the fusogenicity of the S protein associated with genome delivery? One potential mechanism is that the route of virus entry regulates the efficiency of genome delivery. It has recently been suggested that the fusogenicity of the MHV S protein may determine the route of virus entry (34). For example, the nonfusogenic strain MHV-2 requires endosomal cathepsins A/B to cleave the S protein for entry, suggesting that MHV-2 enters cells via endocytosis. In contrast, the fusogenic A59 strain and the S757R mutant strain are insensitive to cathepsin A/B inhibitors, suggesting that A59 and the S757R mutant enter cells via direct fusion between the viral envelope and the plasma membrane (34). Interestingly, pretreatment of MHV-2 with trypsin results in cell-cell fusion and abrogation of cathepsin dependence (34). Indeed, upon trypsin treatment, Penn 98-1 induced cell-cell fusion and exhibited reduced titers and less efficient RNA transport to the ER in DBT cells, which is consistent with a fusogenic phenotype of A59 (Fig. 8). This suggests that the route of virus entry may determine the efficiency of genome delivery and hence virus production. In further support of this interpretation is the evidence from the S757R mutant, which becomes fusogenic but exhibits less efficient growth and RNA transport to the ER than the nonfusogenic parental strain Penn-98-1 (Fig. 2 and 7). Thus, the reversion of the viral growth property correlates well with the change in fusogenicity of the S protein and the route of entry

(32; this study). It is thus conceivable that the two entry pathways differentially utilized by fusogenic and nonfusogenic MHVs may have very different effects on RNA transport to the ER.

Our data show that the genomes of nonfusogenic viruses (MHV-2 and Penn-98-1), which utilize endocytosis to enter cells (33), can reach the ER more rapidly or efficiently than those of fusogenic viruses (A59 and the S757R mutant), which enter cells via direct fusion with the plasma membrane (33). Why is the endocytic entry pathway more efficient in transport of the viral genome from the cell surface to the ER? One possible explanation is that intracellular transport by endocytic vesicles may allow the virus-loaded cargo to easily bypass many of the barriers in the cytoplasm such that it can move deep into the cytoplasm and rapidly deliver the genome to the destination, which, for positive-strand-RNA viruses, is the ER. Alternatively, the processes involving penetration, uncoating, and genome transport may require specific cellular factors residing at specific locations that interact with the viral "cargo." The endocytic pathways may favor such processes. For example, infection of Chinese hamster ovary (CHO) cells with Semliki Forest virus is usually established via the endocytic pathway. Even when the virions were experimentally induced to fuse with the plasma membrane of CHO cells, viral RNA and protein synthesis failed to initiate (26). Similar results were observed when CHO cells were fused with vesicular stomatitis viruses (26). It was found that the low-pH environment at the endosomes allows Semliki Forest virus to use the decreasing pH as a cue to activate the penetration process (14). However, since fusogenic MHVs can still penetrate and replicate to significant levels, albeit less efficiently than the nonfusogenic MHVs, and since pretreatment of a nonfusogenic MHV with trypsin alters its entry pathway, it is less likely that a defect in penetration for MHV, which enters from the plasma membrane, is the determinant for the reduced efficiency of genome transport, as demonstrated by the internalization study (Fig. 4). A third possibility is that the intracellular-signaling events induced during virus-cell engagement (virus-cell fusion) at the plasma membrane by fusogenic viruses and at the endosomal membrane by nonfusogenic viruses are very different, which may influence viral genome uncoating and intracellular transport. Indeed, viruses that use different entry pathways induce or inhibit both common and unique subsets of kinases and phosphatases during entry, as exemplified by simian virus 40 and vesicular stomatitis virus (30). These molecules may engage in viral uncoating/transport processes directly or indirectly through activation or inhibition of the signal transduction pathways that can modulate cell metabolism, cytoskeleton rearrangement, cycles, differentiation, and death. In this regard, it is tempting to postulate that the regulatory steps reside at the site of uncoating during the transport of the viral genome to the ER. To date, we do not know which form of the viral genomic RNA (naked or associated with the N protein in a capsid) is transported from the cell surface to the ER or which precise path (along the microtubule or through other transport molecules) the viral genome travels before reaching the ER. Recent data from our lab indicated, however, that although A59 enters from the plasma membrane, microtubules are not required for transport of A59 since the disruption of microtubule filaments by specific drugs does not affect A59

infectivity (33). It is also possible that the A59 preparation used for infection may have at least two different populations. While the minor population may preferentially utilize endocytosis for entry, the major population enters from the plasma membrane, since treatment of cells with lysosomotropic agents only partially inhibited A59 infectivity ($<1 \log_{10}$ reduction in titer) (33). Although it has been speculated that the endocytic pathway may be a more efficient route for delivery of the viral genome (27), direct evidence is still lacking. Most of the previous studies used different viruses for studying the two pathways, rendering a direct comparison meaningless. Our current studies use the same MHVs differing in only a single gene or a single amino acid, thus underscoring the significance of our comparative analysis. Our findings offer the first direct experimental evidence in support of this hypothesis. The pairs of MHVs and mutants that exhibit contrasting features in fusogenicity, route of entry, and genome delivery provide an ideal tool for further probing the precise entry pathways and dissecting the molecular interactions along the pathways, which may govern the efficiency of genome delivery. These investigations are under way.

It is worth noting, however, that our results cannot distinguish whether the ER is the site for virus translation and/or replication, although the cell fractionation experiment was primarily based on the assumption that the ER is the first destination for the viral genome to be delivered for translation of the polymerase gene upon entry into cells. The detection of genomic RNA and at a later time the subgenomic mRNA in the ER supports this assumption. However, it is possible that not all viral genomes are destined for the ER and that many of them may move to other organelles, such as the lysosome, for degradation. If so, the detected genomic RNA in the ER may represent only a fraction of the infecting genomic RNAs. The isolated ER can also be the site for virus replication. Recent evidence shows that MHV replication complexes are located in double-membranous vesicles that are rich in ribosomes (35), suggesting that these vesicles are either the ER or derived from the ER, which is modified by viral nonstructural proteins following translation. Currently it is not known whether the coronavirus genome moves back and forth between the sites for translation and replication or whether the ribosome-containing double-membranous vesicles formed upon virus infection can serve for both virus translation and virus replication. Thus, the real-time qRT-PCR developed in this study may offer a sensitive means for tracing the movement of the viral genome inside the cells.

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