The Spike but Not the Hemagglutinin/Esterase Protein of Bovine Coronavirus Is Necessary and Sufficient for Viral Infection

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The spike (S) and hemagglutinin/esterase (HE) of bovine coronavirus (BCV) are the two envelope proteins that recognize the same receptor-determinant of 9-*O*-acetylneuraminic acid on host cells. However, the precise and relative roles of the two proteins in BCV infectivity remain elusive. To unequivocally determine their roles in viral cytopathogenicity, we developed a system in which phenotypically chimeric viruses were generated by infecting a closely related mouse hepatitis virus (MHV) in cells that stably express an individual BCV protein (S or HE). The chimeric viruses were then used to infect human rectal tumor (HRT)-18 cells that are permissive to BCV but are nonsusceptible to MHV. Using this approach, we found that the chimeric virus containing the BCV S protein on the virion surface entered and replicated in HRT-18 cells; this was specifically blocked by prior treatment of the virus with a neutralizing antibody specific to the BCV S protein, indicating that the BCV S protein is responsible for initiating chimeric virus infection. In contrast, chimeric viruses that contain biologically active and functional BCV HE protein on the surface failed to enter HRT-18 cells, indicating that the BCV HE protein alone is not sufficient for BCV infection. Taken together, these results demonstrate that the S protein but not the HE protein of BCV is necessary and sufficient for infection of the chimeric viruses in HRT-18 cells, suggesting that BCV likely uses the S protein as a primary vehicle to infect permissive cells. © 2002 Elsevier Science (USA)

INTRODUCTION

Coronaviruses are a group of enveloped RNA viruses that contain a positive-sense, single-strand RNA genome of 27–31 kb in length (Lai and Cavanagh, 1997). A typical coronaviral particle is round, ranging from 120 to 160 nm in diameter. The large peplomer that protrudes from the virion envelope forms the typical 20-nm spikes (Lai and Cavanagh, 1997). In some coronaviruses, a small peplomer composed of a hemagglutinin/esterase (HE) protein is also present on the virion surface (King *et al.,* 1985). Two additional proteins, the membrane glycoprotein (M) of 23–26 kDa (Armstrong *et al.,* 1984) and the small envelope protein (E) of ≈9–12 kDa (Yu *et al.,* 1994), are embedded in the envelope and are essential for virion envelopment, a required process during virion assembly (Rottier *et al.,* 1984; Vennema *et al.,* 1996). The nucleocapsid (N) protein of ≈50 kDa is associated with the RNA genome to form the nucleocapsid inside the viral envelope (Stohlman and Lai, 1979; Sturman *et al.,* 1980).

The spike (S) protein of coronavirus, as exemplified by bovine coronavirus (BCV), contains 1363 amino acids (aa) with an estimated molecular mass of 151 kDa (Abra-

ham *et al.,* 1990; Boireau *et al.,* 1990; Parker *et al.,* 1990; Zhang *et al.,* 1991b). With its 19 potential glycosylation sites, the S protein has a size of 190 kDa when fully glycosylated (Abraham *et al.,* 1990; Cavanagh, 1995). BCV S protein consists of two subunits: the N-terminal S1 (110 kDa) and the C-terminal S2 (100 kDa) (St. Cyr-Coats and Storz, 1988). The S1, which forms the bulb portion of the spike, contains domains responsible for viral attachment to the receptor of host cells, while the S2, which forms the stem anchoring the spike into the envelope, possesses regions essential for fusion between viral and cell membranes during infection (Storz *et al.,* 1992; Yoo *et al.,* 1991). Cleavage of the S protein into S1 and S2 subunits is mediated by cellular trypsin-like proteases and is believed an important process in viral infectivity (Storz *et al.,* 1981). In most coronaviruses, the S protein is the major and, probably, the only viral structural protein required for virus entry. For example, it has been shown that the S protein of transmissible gastroenteritis virus determines virus infectivity and tissue tropism (Sanchez *et al.,* 1999). In mouse hepatitis virus (MHV), some strains (such as JHM) contain the HE protein, while others (such as A59) do not (Shieh *et al.,* 1989; Yokomori *et al.,* 1989). Treatment of permissive cells with monoclonal antibody (mAb) specific to the MHV receptor molecules, which prevented the S protein from interacting with the receptor, also blocked JHM virus infection (Gagneten *et al.,* 1995); antibodies specific to the S protein neutralized MHV infectivity, whereas mAbs to the HE protein did not

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(Yokomori *et al.,* 1991). These findings indicate that the S but not the HE protein of MHV is responsible for viral infectivity.

In BCV, the HE protein contains 424 aa with an estimated molecular weight of 62 to 65 kDa. It usually exists in the virion as homodimers of 126 kDa linked by disulfate bonds (King *et al.,* 1985). It is evolutionarily closely related to the HEF protein of influenza C virus (Herrler *et al.,* 1985, 1988; Luytjes *et al.,* 1988). Like the HEF protein of influenza C virus (Herrler *et al.,* 1985, 1988; Herrler and Klenk, 1991), the BCV HE protein also contains receptor-binding and receptor-destroying activities, although it lacks fusion activity (Schultze *et al.,* 1990, 1991; Vlasak *et al.,* 1988). The receptor-destroying activity is associated with the acetylesterase that hydrolyzes an ester linkage to release the acetyl group from position C-9 of the *N*-acetyl-9-*O*-acetylneuraminic acid-containing receptors on host cells and erythrocytes (Vlasak *et al.,* 1988; Yokomori *et al.,* 1989). The 9-*O*-acetyl residue is crucial for influenza C virus recognition of the glycoprotein receptor, a major determinant for virus attachment and infection. By analogy to the HEF protein of influenza C virus, it was hypothesized that the HE protein of BCV is probably involved in virus attachment to the receptors of host cells, in addition to the S protein, and facilitates virus spread. This is in stark contrast to the MHV HE protein, which lacks the receptor-binding (hemagglutination) activity (Yokomori *et al.,* 1989). Several lines of evidence support an important role of the HE protein in BCV infectivity: (i) The HE protein of BCV was shown to induce mAbs that neutralized virus infectivity *in vitro* (in cell cultures) and *in vivo* (in animals); four neutralizing epitopes were identified on the HE protein of BCV (Deregt and Babiuk, 1987; Deregt *et al.,* 1989). (ii) Treatment of BCV with inhibitors that specifically inactivate acetylesterase reduced viral infectivity by 3 logs or greater, while the same treatment of influenza A virus did not affect virus infectivity, indicating that acetylesterase activity is required for BCV infectivity (Vlasak *et al.,* 1988). (iii) Both the S and HE proteins of BCV recognized the same receptor-determinant of the cultured cells and erythrocyte (Schultze *et al.,* 1991; Schultze and Herrler, 1992, 1994). Removal of the *N*-acetyl-9-*O*-acetylneuraminic acid from cultured cells by treatment with neuraminidase or acetylesterase rendered the cells resistant to BCV infection (Schultze and Herrler, 1992). Taken together, these findings strongly support the hypothesis that, as opposed to MHV, both the S and HE proteins of BCV are necessary for virus infection but that either protein alone is not sufficient for initiating infection.

To test this hypothesis, we developed a system that allowed us to make pseudotyped chimeric MHV containing either the S protein or the HE protein of BCV and to determine the infectivity of these chimeras in human rectal tumor (HRT-18) cells that are permissive to BCV infection but are nonsusceptible to MHV infection. Using this approach, we found that the chimeric MHV containing the BCV S protein entered and replicated in HRT-18 cells, whereas those containing the BCV HE protein did not. Our results clearly demonstrate that the S protein but not the HE protein of BCV is necessary and sufficient for virus infection in cultured cells.

RESULTS

Establishment of cell lines that stably express the BCV S protein

In a preliminary study, we attempted to express the BCV S protein using the MHV defective-interfering (DI) RNA system that has been successfully used for expressing several viral and cellular proteins, including the MHV HE protein, interferon-γ, and chloramphenicol acetyltransferase (Liao and Lai, 1994; Liao *et al.,* 1995; Zhang *et al.,* 1994, 1997, 1998). However, the expression of the BCV S protein was extremely low using this system, as determined by both the fluorescence intensity and the number of fluorescence cells with flow cytometry (data not shown). Chimeric MHV containing the BCV S protein generated by this system failed to infect BCV-susceptible cells. We assumed that such failure was likely attributed by two major factors: limitation of the capacity of the DI RNA system in efficiently expressing large genes (longer than 4 kb) and the low efficiency of RNA transfection. To overcome such limitations we explored the possibility of stably expressing the BCV S protein in cells via eukaryotic expression vector pcDNA3. Therefore, we cloned the complete S gene of BCV virulent strain LY-138 into the pcDNA3 vector under the control of a cytomegalovirus (CMV) immediate early gene promoter (Fig. 1A). Because this vector also contains a neomycin resistance gene, stably transfected cells can be selected with the drug geneticin (G418). Initially, we obtained 50 DBT cell clones that were resistant to G418 treatment for 3–5 weeks. To identify cells that were expressing the BCV S protein from these drug-resistant clones, mAb 43C2 specific to the BCV S protein was used in an immunofluorescence (IF) assay. The IF assay was the method of choice because mAb 43C2 only reacts with conformational epitope and does not react with the S protein in immunoprecipitation or Western blot assay when the S protein is denatured. We grouped these clones based on the intensity of fluorescence as directly compared with that in DBT/V cells (Fig. 1B, a) that were stably transfected with the vector alone as a negative control. Out of the 50 cell clones, 7 exhibited a low level of fluorescence, 27 a medium level, 11 a high level, and 5 the highest level. Figure 1B shows 2 representative cell clones that exhibited the highest level of expression of the BCV S protein (c and e). Within the same clone, all cells expressed the BCV S protein, as evidenced by comparison of the fluorescence images with bright field
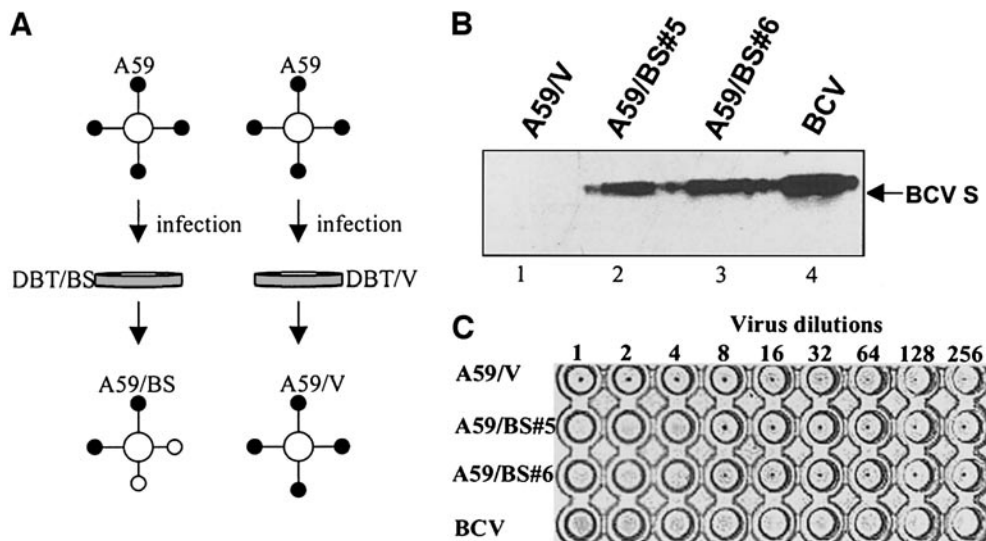
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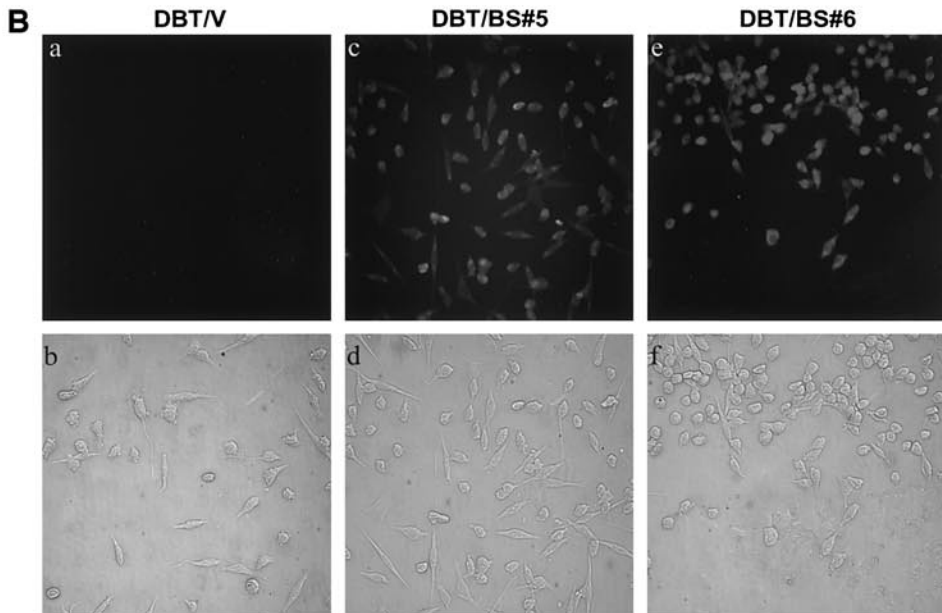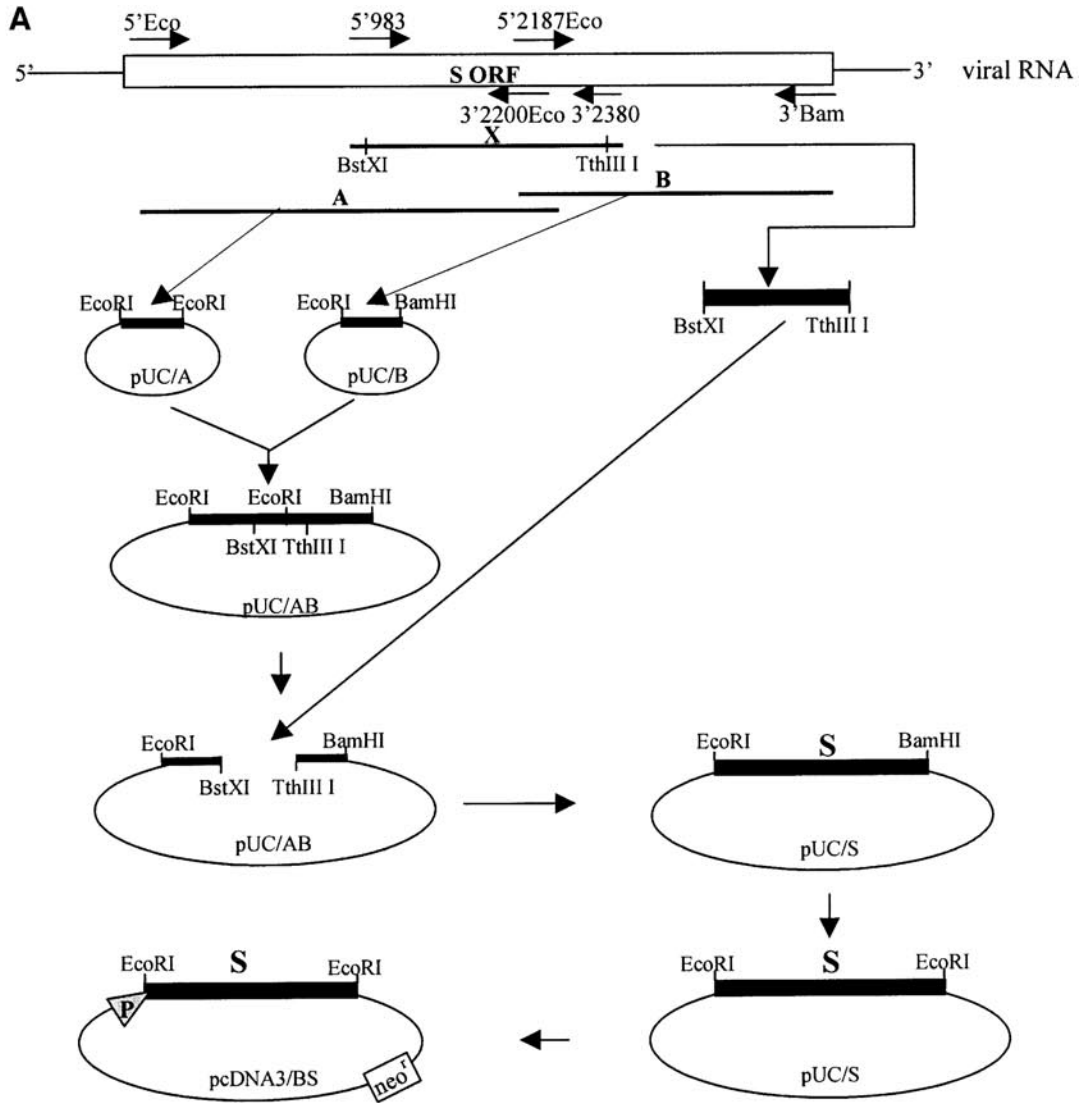
FIG. 2. Production of chimeric viruses. (A) Scheme of chimeric virus production. DBT cells stably expressing the BCV S protein (DBT/BS) or the vector alone (DBT/V) were infected with MHV strain A59 at a m.o.i. of 10. Viruses were harvested when the cytopathic effect reached ~90%. Chimeric viruses that were produced from DBT/BS cells are termed A59/BS while those from DBT/V cells are designated A59/V. Unfilled circles represent the BCV S protein; filled circles denote the MHV S protein. (B) Western blot analysis. All four viruses (indicated at the top of the gel) were purified through sucrose gradients. Viral proteins were then separated by PAGE (10%) under nondenaturing condition (see details under Materials and Methods), transferred to nitrocellulose membranes, and detected with mAb 43C2 specific to the BCV S protein and the ECL system. (C) Hemagglutination assay showing the hemagglutination activity of the chimeric viruses. Viruses were purified through ultracentrifugation on a 30% sucrose cushion and then on a 20 to 60% sucrose gradient. A twofold serial dilution of the virus preparation (indicated at top) was made in PBS in a V-shaped 96-well microplate. An equal volume (50 μl) of 0.5% mouse erythrocytes was added and incubated at 4°C for 2 h or overnight. HA titers were determined and images were taken using a UVP Transluminator with visible light. A59/BS#5 and A59/BS#6 indicate two chimeric viruses generated from two separated cell clones; BCV was a positive control, with a HA titer of >256 HA units.

images (compare c with d and e with f in Fig. 1B). However, the expression level varied slightly from cell to cell depending on the shape and size of the cells at the time of fixation (Fig. 1B, c and e). Furthermore, the BCV S protein was expressed predominantly, if not exclusively, in the cytoplasm. Cytoplasmic localization became more pronounced when a higher magnification of the microscope lens was used (data not shown). Interestingly, while in some cells the S protein distributed relatively evenly throughout the cytoplasm, it concentrated more in certain compartments (such as Golgi) in other cells (Fig. 1B). The exact reasons for these variations are unknown, but it is likely that these cells were in different phases of the cell cycle, since the cell cultures were not synchronized for these experiments. Because the expression levels of clones #5 and #6 were similar to those in

BCV-infected cells (see Fig. 3A, c), we used these 2 cell clones for all subsequent experiments.

Generation and characterization of pseudotyped chimeric viruses

To explore the possibility that phenotypically chimeric viruses can be generated by infecting a virus into cells that stably express a heterologous viral envelope protein, we used MHV A59 to infect DBT cells that stably express the BCV S protein (DBT/BS). We expected that such infections would result in the generation of chimeric viruses the surface proteins of which would be a mixture of the MHV S protein and the BCV S protein, while its genome and other structural proteins would be the same as MHV A59 (Fig. 2A). As a control, DBT cells that stably express the vector alone (DBT/V) were in-

FIG. 1. Cloning, construction, and stable expression of the BCV S protein in DBT cells. (A) Schematic diagram for cloning and construction of the BCV S gene. The names and relative locations of the three pairs of primers used for RT–PCR are indicated at the top of the figure. Only the S gene ORF is shown. The resulting three RT–PCR fragments A, B, and X are also indicated. Critical restriction enzymes used for cloning and construction are shown. S, complete BCV S gene. The CMV immediate early gene promoter (P) and neomycin resistance gene (neo^r) in pcDNA3 are indicated. (B) Stable expression of the BCV S protein in DBT cells. DBT cells were transfected with pcDNA3/BS or pcDNA3 vector alone, and stable expressing cells were selected with G418 (see Materials and Methods). Two cell clones that express the BCV S protein [(DBT/BS#5 (c, d), DBT/BS#6 (e, f)] and a cell clone that expresses vector alone [(DBT/V (a, b)] were grown on chamber slides, fixed, and labeled with mAb (43C2) specific to BCV S and FITC conjugates. Images of both fluorescence (a, c, e) and bright field (b, d, f) were taken using an inverted fluorescence microscope (Olympus IX70) with an attached digital camera (Magnifier) at a magnification of 20×. Images were saved as TIFF files and labeled in PowerPoint (version 5).

fected with MHV A59. The resulting progeny virus (A59/V) would be genotypically and phenotypically the same as MHV A59 (Fig. 2A). To establish that BCV S proteins are indeed incorporated into the chimeric virions, virus preparations were purified through sucrose gradients, separated by SDS–PAGE under the nondenaturing conditions, and analyzed by Western blotting using mAb 43C2 specific to the BCV S protein. As shown in Fig. 2B, the BCV S protein was present in both chimeric virions (lanes 2 and 3), but not in A59/V (lane 1). The same S protein was also detected in wild-type BCV (lane 4). We thus conclude that the BCV S protein was incorporated into the chimeric virions.

To verify whether the incorporated BCV S protein retained its biological function, we determined the hemagglutination activity of the chimeric viruses. It was reported previously that the S protein purified from the BCV virion possessed hemagglutinin activity when erythrocytes from several animal species were used in hemagglutination (HA) assay (Schultze *et al.,* 1991). Moreover, the BCV S protein is a stronger hemagglutinin that its HE protein (Schultze *et al.,* 1991). Indeed, both chimeric viruses, A59/BS#5 and A59/BS#6, which were generated by infecting MHV A59 into DBT/BS#5 and DBT/BS#6 cells, respectively, exhibited hemagglutination activity (4 HA units, Fig. 2C). Because MHV A59 lacks the HE protein and its S protein does not recognize the sialic acid receptor on erythrocytes, MHV A59 would not agglutinate erythrocytes. This was verified in A59/V (Fig. 2C). Therefore, the HA activity of the two chimeric viruses must have been conferred from BCV S proteins that were incorporated into the chimeric viruses during virus assembly in DBT cells stably expressing the BCV S protein. It is worth noting that the HA activity observed in the chimeric virus was less likely conferred by free BCV S in the virus preparations because the BCV S protein is known to be a type I membrane protein and the virus preparations were purified through a sucrose gradient prior to HA assay. Samples prepared from mock-infected DBT/BS#5 cells through the same sucrose gradient centrifugation method did not contain the BCV S protein in Western blot and did not exhibit HA activity (data not shown, see Fig. 6 for an example). These results support the conclusion that the chimeric viruses retained their biological (receptor-binding) activity. However, the HA titer of the chimeric viruses was lower than that of wild-type BCV (256 HA units, Fig. 2C) even though an equivalent number of virus particles [based on the radioactivity of the N proteins in a labeling experiment (data not shown)] for all four viruses was used for the HA assay, suggesting that the number or the binding activity of the S protein or both in chimeric viruses are lower than those of the S proteins in wild-type BCV virions. It should be noted that the HA activity of the wild-type BCV is partially contributed by the HE protein (see Fig. 6C).
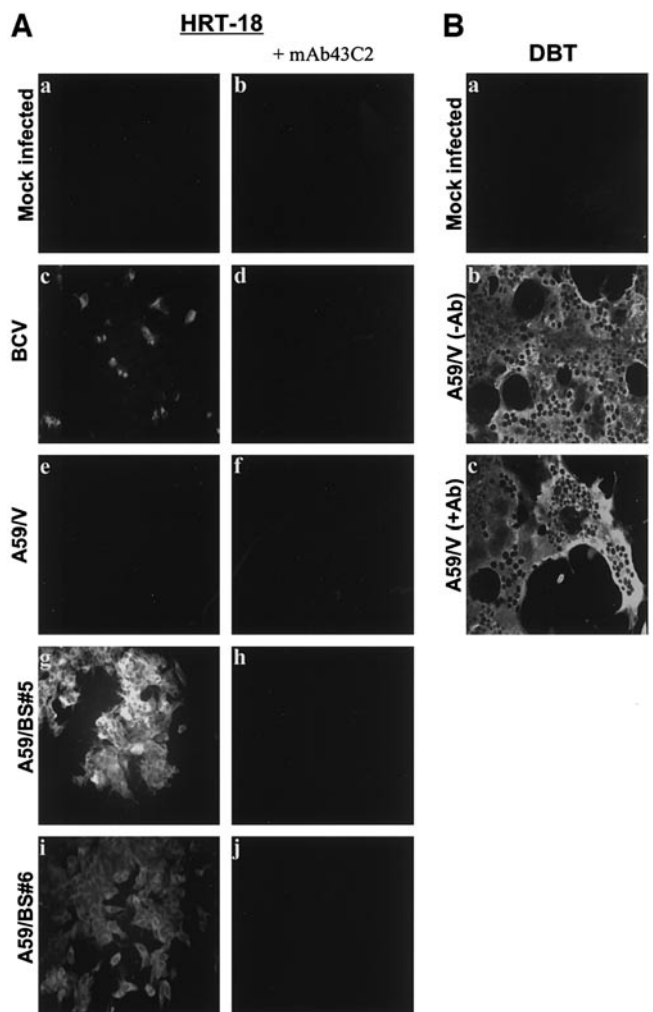

FIG. 3. Immunofluorescence detection of viral gene expression in HRT-18 cells (A) and DBT (B). (A) Left column: HRT-18 cells were grown on chamber slides and infected with BCV (c), A59/V (e), A59/BS#5 (g), and A59/BS#6 (i) or were mock-infected (a). Cells were fixed and labeled with mAb J3.3.1 specific for the MHV N protein (a, e, g, i) or with mAb 43C2 specific for the BCV S protein (c) and FITC conjugates. Right column (b, d, f, h, j): Respective viruses (indicated at left) were incubated with a neutralizing mAb (43C2) prior to inoculation. (B) DBT cells were grown on chamber slides and infected with A59/V with (c) or without (b) prior treatment with mAb 43C2 or were mock-infected (a). Cells were fixed and labeled with the MHV N-specific mAb J3.3.1 and FITC conjugates. All images were taken using an inverted fluorescence microscope (Olympus IX70) with an attached digital camera (Magnifier) at a magnification of 20×.

The BCV S protein conferred the infectivity upon chimeric MHV/BS virus

To determine whether the BCV S protein alone is sufficient for initiation of viral infection, we used the pseudotyped chimeric virus MHV/BS that contains the BCV S protein on the virion surface (Fig. 2) to infect HRT-18 cells. HRT-18 cells are permissive to BCV infection (Fig. 3A, c) and are widely used for BCV propagation, but are not susceptible to MHV A59 infection (Fig. 3A, e). Therefore, replication of the chimeric viruses in HRT-18

cells would indicate a successful entry of the viruses, which must be mediated solely by the BCV S protein. To monitor the replication and gene expression of the chimeric viruses in cells, we used mAb J3.3.1 specific to MHV N protein in IF assay. As expected, both chimeric viruses replicated in HRT-18 cells at 24 h p.i., as demonstrated by the expression of the MHV N protein (Fig. 3A, g and i). These fluorescence stainings were specific because neither the mock-infected nor the MHV A59-infected HRT-18 cells were stained with the N protein mAb and fluorescein isothiocyanate (FITC) conjugates (Fig. 3A, panels a and e).

To ensure that entry of chimeric viruses was indeed mediated by the BCV S protein, we used neutralizing mAb 43C2 specific to the BCV S protein (Hussain et al., 1991) in a virus-neutralizing assay. mAb 43C2 was mixed with different virus preparations and incubated for 1 h at 37°C prior to inoculation into HRT-18 cells. At 24 h p.i. the same IF assay was used for detecting viral gene expression using mAb J3.3.1 for MHV A59 and the chimeric viruses and mAb 43C2 for BCV. As shown in Fig. 3A (d, h, and j), prior incubation with neutralizing mAb 43C2 completely blocked the infection of HRT-18 cells by BCV and the two chimeric viruses. However, this result did not exclude the possibility that mAb 43C2 may also have been able to neutralize MHV infectivity by cross-reacting with the MHV S protein. To rule out this possibility, MHV A59 was incubated with mAb 43C2 for 1 h at 37°C prior to infection of DBT cells. Regardless of the incubation with mAb 43C2, MHV A59 replicated equally well in DBT cells, as evidenced by the cytopathic effect, cell fusion, syncytium formation, and strong fluorescence staining with the N mAb (Fig. 3B), indicating that mAb 43C2 did not neutralize MHV infectivity. Taken together, these results clearly demonstrated that the BCV S protein but not the other structural proteins of MHV origin conferred the infectivity of the chimeric viruses in HRT-18 cells.

We assumed that the chimeric viruses so generated should contain a mixture of two S proteins (MHV S and BCV S), while the remaining structural proteins and the genome should be identical to those of the wild-type MHV A59. If the BCV S protein is used for chimeric virus infection into HRT-18 cells, then BCV S protein should be left outside the infecting cells during virus uncoating and entry, and the progeny virus recovered from HRT-18 cells should be identical to wild-type MHV A59 (Fig. 4A). To test this hypothesis, medium was collected from cultures of HRT-18 cells infected with the chimeric viruses, and the recovered virus, designated passage I virus, was used for infection of both DBT and HRT-18 cells. The IF assay using the MHV N-specific mAb was performed to detect viral gene expression in these cells. As shown in Fig. 4B, both passage I viruses replicated in DBT cells (e and f), but not in HRT-18 cells (b and c), supporting our hypothesis. Furthermore, these results support our con-
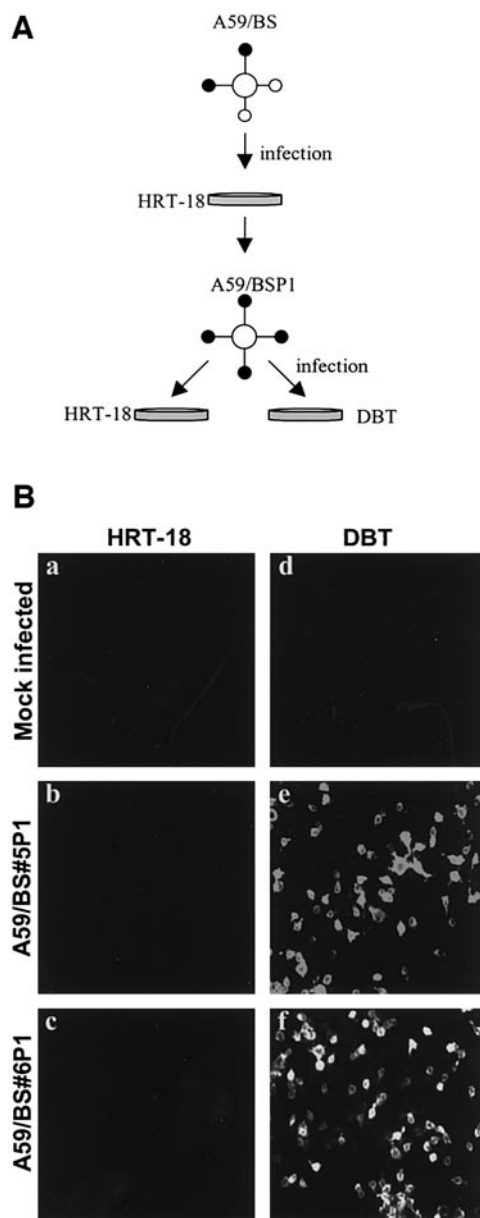

FIG. 4. Inability in continuous passage of the chimeric viruses in HRT-18 cells. (A) Schematic diagram for generating passage one (P1) viruses. HRT-18 cells were infected with chimeric viruses (A59/BS). The resulting virus, designated A59/BSP1, was used to infect HRT-18 and DBT cells. (B) Fluorescence detection of P1 viral gene expression in HRT-18 (a–c) and DBT (d–f) cells. Cells were grown on chamber slides and infected with both P1 viruses or were mock-infected. At 24 h p.i. (for HRT-18) or 9 h p.i. (DBT), cells were fixed and labeled with MHV N-specific mAb J3.3.1 and FITC conjugates. Images were taken using an inverted fluorescence microscope (Olympus IX70) with an attached digital camera (Magnifier) at a magnification of 20×.

clusion that entry of the chimeric viruses into HRT-18 cells is mediated solely by the BCV S protein.

Establishment of cell lines that stably express the BCV HE protein

Having established the system for stably expressing viral spike protein and for generating chimeric viruses,
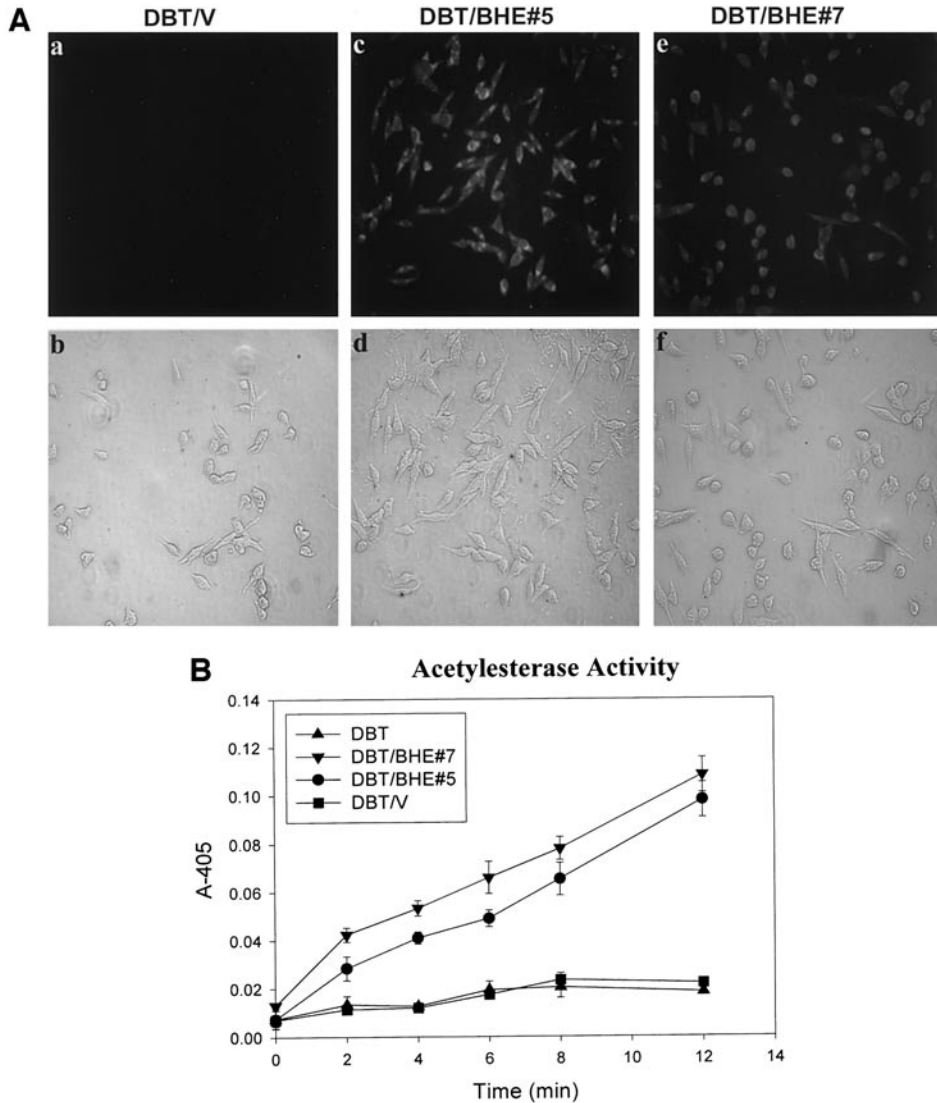
FIG. 5. Stable expression of the BCV HE protein in DBT cells. (A) DBT cells were transfected with pcDNA3/BHE or pcDNA3 vector alone and stable expressing cells were selected with G418 (see Materials and Methods). Two cell clones that express the BCV HE protein [DBT/BHE#5 (c, d), DBT/BHE#7 (e, f)] and a cell clone that expresses vector alone [DBT/V (a, b)] were grown on chamber slides, fixed, and labeled with polyclonal antibody specific to the Flag and FITC conjugates. Images of both fluorescence (a, c, e) and bright field (b, d, f) were taken using an inverted fluorescence microscope (Olympus IX70) with an attached digital camera (Magnifier) at a magnification of 20×. (B) Acetylesterase activity of DBT/BHE#5 and DBT/BHE#7 cell lines. The activity was determined by hydrolysis of p-nitrophylacetate (PNPA) using cellular protein from total cell lysates. Five micrograms protein of each sample was incubated with 1 mM PNPA for various times at room temperature. Absorbency at 405 nm was read every 2 min and values were recorded after the subtraction of PNPA values. Each sample was assayed in triplicate. The SigmaPlot graphics program (IBM) was used to generate the graph.

we sought to extend our investigation and to define the role of the HE protein in BCV pathogenicity. For this purpose, the HE protein of BCV virulent strain LY-138 was expressed in DBT cells via the eukaryotic expression vector pcDNA3, as described for the BCV S protein (Fig. 1A). A total of 50 individual cell clones that were resistant to G418 treatment for approximately 4 weeks was obtained. To identify the expression of the HE protein, we used a rabbit polyclonal antibody specific to the 8 aa Flag epitope that was fused to the carboxyl terminus of the HE protein (see Materials and Methods) in an IF assay. Of the 50 cell clones, 7 exhibited a low level of

expression, 19 a medium level, 19 a high level, and 5 the highest level of expression. Figure 5A (c and e) shows 2 representative clones (DBT/BHE#5 and DBT/BHE#7) that had the highest expression of the BCV HE protein as judged by fluorescence intensity. The intracellular staining appeared to be specific, since it was not observed in DBT/V cells that were stably transfected with the vector alone (Fig. 5A, a). All cells expressed the HE protein, as determined by comparing the fluorescence images with their bright field images (compare c with d and e with f). The intracellular localization and the abundance of the HE protein varied from cell to cell, and the expressed
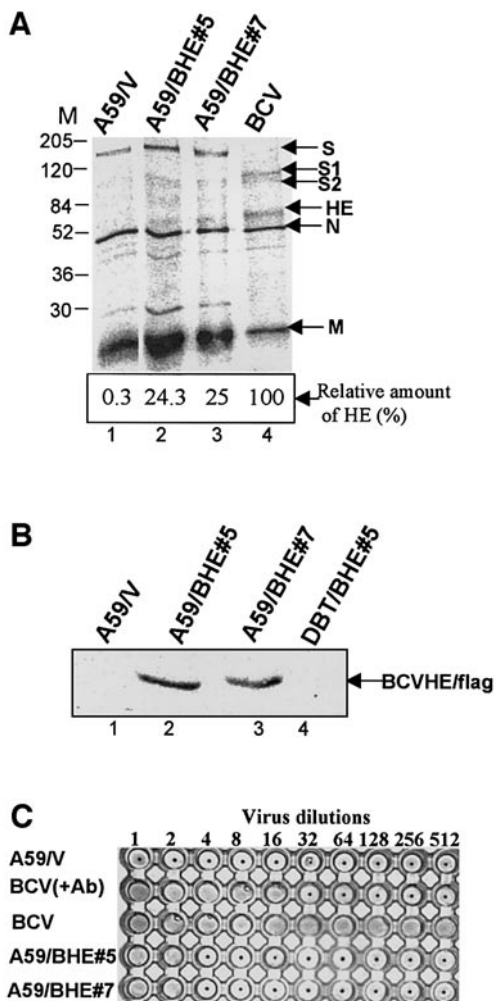
FIG. 6. Characterization of MHV/BCV HE chimeric viruses. (A) Detection of BCV HE/Flag in chimeric viruses. DBT cells stably expressing the BCV HE protein or the vector alone were infected with MHV A59, and HRT-18 cells were infected with BCV LY-138. Cells were then labeled with [^{35}S]methionine (100 μCi/ml). Viruses were collected from the medium and purified through a sucrose gradient. Viral proteins were separated by SDS–PAGE on a 10% gel and visualized by PhosphorImager (Molecular Dynamics). Lanes 1–3, as in the figure; lane 4, BCV LY-138. The viral structural proteins are indicated by arrows at right. The radioactivity of the N and HE bands was determined with the PhosphorImager software (ImageQuant, version 4.2a, build 13, Molecular Dynamics). The amount of HE protein in each virus was normalized by its N protein based on radioactivity and is presented at the bottom of the gel as a percentage relative to the HE protein in wild-type BCV. M, molecular weight standards in kDa. (B) Detection of BCV HE/Flag in chimeric viruses by Western blot analysis. Purified virions were separated by SDS–PAGE on a 10% gel and were transferred to nitrocellulose membrane. The viral proteins were labeled with antibody to Flag followed by peroxidase (see Material and Methods). Lanes 1–3, as in the figure. Lane 4, "virus" preparation from mock-infected DBT cells expressing the BCV HE protein (DBT/BHE#5). The band representing BCV HE/Flag is indicated by an arrow at right. (C) Hemagglutination assay showing the hemagglutination activity of the A59/BHE chimeric viruses. Viruses were purified through ultracentrifugation on a 30% sucrose cushion and then on a 20 to 60% sucrose gradient. Twofold serial dilutions (indicated at top) of the virus preparations (as indicated at left) were made in a V-shaped 96-well microplate. An equivalent number of virions for all viruses (based on the radioactivity of the N protein shown in A) was used for the assay. An equal volume (50 μl) of

characteristics were similar to those of the S protein (compare Fig. 5A with Fig. 1B).

Although the IF assay detected the expression of the BCV HE protein, it could not determine whether the expressed HE protein retains its biological activity. One of the biological activities of the BCV HE protein is the receptor-destroying function by its acetylesterase. Therefore, DBT cells expressing the BCV HE protein were lysed by repeated freezing and thawing. The parental DBT cells and DBT cells expressing vector alone were used as negative controls. Cell lysates containing equivalent amounts of protein (5 μg) for each sample were subjected to acetylesterase assay. As shown in Fig. 5B, both of the cell clones (DBT/BHE#5 and DBT/BHE#7) that express the BCV HE protein had a significantly higher esterase activity ($\sim$fivefold higher OD values at 12 min) than those of the parental cells (DBT) or cells expressing vector alone (DBT/V), indicating that the expressed HE protein retained its biological function with respect to the esterase activity.

Generation and characterization of MHV/BCV HE chimeric viruses.

Since the biologically functional HE protein was stably expressed, we proceeded to make chimeric viruses by infecting these cells with MHV A59. MHV A59 was chosen for this experiment because it does not contain the HE protein. To ensure that the HE protein was indeed incorporated into the chimeric virus particles, several approaches were employed. First, we used radiolabeling of chimeric viral proteins. Cells were infected with MHV A59 and proteins radiolabeled with [^{35}S]methoinine. Chimeric viruses were then purified through sucrose gradients, and viral structural proteins were separated by SDS–PAGE. As shown in Fig. 6A, both representative chimeric viruses contained the BCV HE protein (lanes 2 and 3). The amount of HE protein incorporated into the chimeric virions was approximately 25% of the wild-type BCV HE, when it was normalized by the N proteins (Fig. 6A, bottom, compare lanes 2 and 3 with lane 4). This protein did not appear in A59/V (Fig. 6A, lane 1). These results indicated that the BCV HE protein is likely incorporated into the chimeric virions. Second, we carried out Western blot analysis on chimeric virion proteins. The engineered BCV HE protein was detected in relatively similar amounts in both sucrose-gradient purified chimeric virions by the anti-Flag antibody in Western blot

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0.5% mouse erythrocytes was added to each well and incubated at 4°C for 2 h or overnight. HA titers were then determined and images were taken using a UVP transluminator with visible light. A59/BHE#5 and A59/BHE#7 indicate two chimeric viruses generated from two separated cell clones; BCV, a positive control, with an HA titer of 512 units. BCV (+Ab), BCV was treated with mAb 43C2 for 1 h at 37°C prior to incubation with erythrocytes.

(Fig. 6B, lanes 2 and 3), but not in A59/V (lane 1), confirming that the BCV HE protein was present in the chimeric virions. Since no HE protein was detected in the sucrose-gradient preparation purified from mock-infected, HE-expressing DBT/HE#5 cells (Fig. 6B, lane 4), this further established that the HE protein detected in the chimeric virus preparations (lanes 2 and 3) was truly incorporated into the virion rather than a contaminant of HE-containing cellular vesicles. Third, we used the hemagglutination assay to assess the receptor-binding activity of the BCV HE protein in the chimeric viruses. Indeed, both chimeric viruses A59/BHE#5 and A59/BHE#7 possessed hemagglutination activity (2 HA units), whereas control virus A59/V did not (Fig. 6C), demonstrating that the HE protein of the chimeric virus retained its receptor-binding activity. Finally, to provide a relatively quantitative assessment, we performed a hemagglutination inhibition assay. Because both the S and HE proteins of BCV agglutinate erythrocytes, the relative contribution of each individual protein needs to be determined. We used mAb 43C2 to block the hemagglutination activity mediated by the S protein. As a result, the HA titer of BCV was reduced from 512 to 16 units [Fig. 6C, compare row BCV with BCV(+Ab)], indicating that the remaining 16 HA units are likely contributed by the HE protein. This estimate is reasonable because the concentration of the mAb was in greater excess to completely block S protein binding (data not shown). If this estimate is correct, then the HA titers of the chimeric viruses would be eightfold less than that contributed by the HE in wild-type BCV (Fig. 6C). An equivalent number of virus particles for each virus preparation as estimated from radiolabeled N protein (Fig. 6A) was used for the HA and hemagglutination inhibition (HI) assays. Thus, these data indicate that the BCV HE protein was incorporated into the chimeric virions and retained its receptor-binding activity but that its amount in the chimeric viruses was lower than that in wild-type BCV. It is important to note, however, that the relative receptor-binding activity of the HE protein in the A59/BHE chimeras may be similar to that of the S protein in the A59/BS chimeras compared with those in BCV, since A59/BS had a twofold higher HA titer (4 HA units) than A59/BHE (2 HA units) and the S protein had a twofold higher HA titer (32 HA units) than the HE protein (16 HA units) in BCV (compare Fig. 6C with Fig. 2C).

The BCV HE protein alone is not sufficient for chimeric virus infection in HRT-18 cells

To define the role of the HE protein in BCV pathogenicity, two chimeric viruses (A59/BHE#5 and A59/BHE#7) were used to infect HRT-18 cells, which are permissive to BCV infection but not to MHV infection. The mAb specific to the MHV N protein was used in an IF assay to monitor the gene expression of the chimeric viruses. As shown in Fig. 7, none of the HRT-18 cells infected with the chimeric
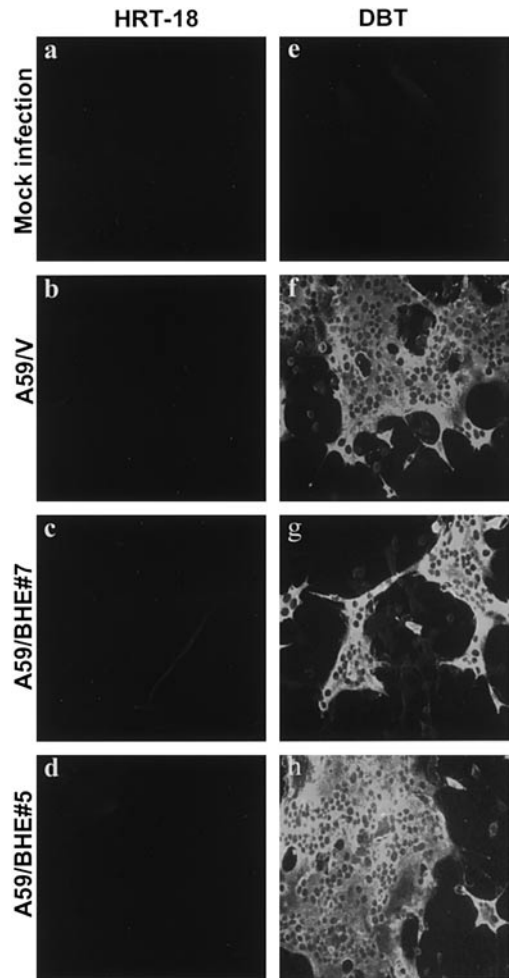

FIG. 7. Chimeric virus that contained MHV S and BCV HE on the viral surface failed to infect HRT-18 cells. Cells were grown on chamber slides and were infected with two chimeric viruses, A59/BHE#5 (d, h) and A59/BHE#7 (c, g), or the control virus A59/V (b, f) or were mock-infected (a, e). At 24 h p.i. (HRT-18, a–d) or 9 h p.i. (DBT, e–h), cells were fixed and labeled with MHV N-specific mAb J3.3.1 and FITC conjugates. Images were taken using an inverted fluorescence microscope (Olympus IX70) with an attached digital camera (Magnifier) at a magnification of 20×.

viruses exhibited any fluorescence, nor did those infected with A59/V or the mock-infected cells (Fig. 7, a–d), indicating that the chimeric viruses could not infect HRT-18 cells. To rule out the possibility that the chimeric viruses lost infectivity during virus preparation, DBT cells were also infected with these viruses. The result showed that both chimeric viruses were viable at the time of infection (Fig. 7, g and h). These results indicate that the BCV HE protein on chimeric virions was unable to facilitate chimeric virus infection in HRT-18 cells, suggesting that the HE protein alone was not sufficient for BCV infection.

DISCUSSION

In this study, we developed a system in which a viral surface protein (spike or hemagglutinin/esterase) of a

closely related BCV was stably expressed in MHV-permissive cells and chimeric viruses were generated by infecting MHV in these stably expressing cells. The chimeric virus contains the genome of wild-type MHV, but the proteins present on the virion surface are a mixture of two types of proteins derived from MHV and BCV. Such a virus is pseudotyped, i.e., only phenotypically chimeric. Using this system, we generated two types of chimeric viruses: one containing a mixture of the MHV S and BCV S proteins (A59/BS) and the other containing a mixture of the MHV S and BCV HE proteins (A59/BHE) on the virion surface. Because the chimeric virus contains a single BCV envelope protein, the role of the individual protein in BCV cytopathogenicity can be unequivocally determined by infecting the chimeric virus (A59/BS or A59/BHE) into cells (i.e., HRT-18) that are nonpermissive to MHV infection but that are susceptible to BCV infection. If the chimeric virus were to enter the HRT-18 cells and replicate, this would indicate that the BCV envelope protein confers infectivity upon the chimeric virus. Our rationale for developing such a system is based on the fact that the surface glycoproteins (S and HE) of MHV and BCV recognize distinct classes of receptors on the cell surface and thus have different cell-type specificity. We also took advantage of the availability of two different cell lines that are permissive to infection by only one type of virus (MHV or BCV). The phenotypically chimeric viruses thus allow us to address questions of BCV–cell interactions in the current study.

To date, the question of whether the S protein alone is sufficient for BCV infection has remained unanswered. Our present results clearly demonstrated that the chimeric MHV/BCV S entered and replicated in HRT-18 cells, which are nonpermissive to infection by the parental MHV (Fig. 3). Infection of HRT-18 cells by the chimeric virus was completely blocked by prior treatment of the virus with mAb 43C2, which is specific to the BCV S protein, but the infection of DBT cells by the chimeric virus was not inhibited by the same mAb treatment (Fig. 3). This supports the conclusion that entry of the chimeric virus to HRT-18 cells is mediated specifically and solely by the BCV S protein present on the surface of the chimeric virus. Our data provide an unequivocal answer that the S protein is necessary and sufficient for BCV infection. These findings thus suggest that, regardless of the presence of the HE protein, all coronaviruses likely use the S protein as the primary vehicle for infection in permissive cells.

The precise role of the HE protein in BCV infection has also remained unclear. We addressed this issue by infecting HRT-18 cells with the chimeric MHV containing the BCV HE protein on the virion surface. We found that the chimeric virus (A59/BHE) could not enter into HRT-18 cells (Fig. 7) despite the fact that the HE protein of the chimeric virus retains its receptor-binding (hemagglutination) and receptor-destroying (acetylesterase) activi-

ties (Fig. 6C and data not shown). Our results thus suggest that the HE protein alone is not sufficient for BCV infection. Alternatively, it is possible that the low incorporation of the HE protein into chimeric MHV/BHE (Fig. 6) may contribute to the inability of the chimera to enter HRT-18 cells. However, the finding that chimeric MHV/BS is sufficient for viral infection (Fig. 3) further suggests that the HE protein is not necessary for BCV infection. This is similar to MHV, in which the HE protein is found to be dispensable (Gagneten et al., 1995), thus negating the hypothesis that the HE protein is required for BCV infectivity. However, our results do not exclude the possibility that the HE protein may play other roles in BCV entry. The demonstration of the receptor-binding (hemagglutination) and esterase activities of the BCV HE protein clearly supports the notion that the HE protein plays a role in BCV pathogenicity. This may be achieved by its ability to bind to sialic acid-containing receptors and to facilitate the release of bound virus from the receptors on the cell surface. That the hemagglutination of BCV was partially contributed by the HE protein (Fig. 6 in this study; Schultze et al., 1991) further supports such an assumption. Whether and to what extent the HE protein contributes to BCV infection in cell culture and in animals remains to be investigated further.

We have also tested indirectly the hypothesis that both the S and HE proteins of BCV bind to the same receptor on permissive cells. We found that when HRT-18 cells were incubated with the HE protein from lysates of HE-expressing DBT cells prior to infection, the infectivity of both MHV/BCV S chimeras (A59/BS#5 and A59/BS#6) was inhibited, which correlated with increased amounts of the HE protein (data not shown). This suggested that either the HE protein competed with the S protein for the same receptor on HRT-18 cells or the receptors were destroyed by the acetylesterase of the HE protein. Similar results were obtained with the HA assay (data not shown). In all cases, the data are consistent with previous findings that both S and HE of BCV recognized the same receptor on cultured cells and erythrocytes (Schultze et al., 1991; Schultze and Herrler, 1992, 1994).

Our conclusion that the S protein but not the HE protein of BCV is necessary and sufficient for BCV infection in permissive cells is consistent with the biology of viral infection in general and coronavirus structural proteins in particular. For almost all enveloped viruses, entry into cells requires two major steps: attachment of viral envelope proteins to receptors on host cells and fusion between viral envelope and cytoplasma or endosomal membranes. The S protein of all coronaviruses possesses both such functions, while the HE protein of BCV has the receptor-attachment function but lacks the fusion activity. This is in contrast to the HEF protein of influenza C virus, which possesses the receptor-binding, esterase, and fusion activities (Herrler et al., 1985, 1988). The lack of the fusion activity in the BCV HE protein may indicate

that the HE protein alone is insufficient for BCV infection. Our data contradict the conclusions by Deregt and Babiuk (1987) drawn from experiments on monoclonal antibody to the HE protein. One possible interpretation of this discrepancy is that the HE-specific mAbs they used may have interfered with the binding of the S protein to the receptor by steric hindrance or by other undefined mechanisms. Experiments are currently underway to test whether a chimeric virus containing both the BCV S and HE proteins has increased infectivity in HRT-18 cells compared to a virus expressing BCV S alone. These experiments will likely identify auxiliary roles of the HE protein, if any, in BCV infection.

The issue of how the BCV S and HE proteins were incorporated into the chimeric viruses was not addressed in this study. Godeke *et al.* (2000) showed that interaction between the C-terminal cytoplasmic domains of the S protein and the M protein is essential for the S protein to be incorporated into the virion. Sequence comparison of the cytoplasmic tail of the S proteins showed a high degree of amino acid identity between MHV and BCV (data not shown). This suggests that the cytoplasmic tail of the BCV S protein might have interacted with the MHV M protein during virus assembly. Such chimeras are common, especially between related viruses, such as those between feline coronavirus and MHV (Kuo *et al.,* 2000). However, nothing is known about HE sequences in virion assembly. Apparently, the expression of the 8-aa Flag epitope at the carboxyl terminus did not block its incorporation, since the Flag-containing HE protein was clearly detected in the chimera (Fig. 6), although its effect on the efficiency of incorporation has not been determined.

MATERIALS AND METHODS

Virus, cells, and antibodies

MHV strain A59 was used throughout this study. For some experiments, MHV strain JHM and BCV virulent strain LY-138 (Zhang *et al.,* 1991a,b) were also used. The murine astrocytoma cell line (DBT) (Hirano *et al.,* 1974) was used for propagation of MHV, expression of recombinant proteins, production of chimeric virus, and other experiments involving MHV infection. DBT cells were grown in 1× Minimum Essential Medium (MEM) containing 7.5% newborn calf serum (GIBCO BRL). The G clone of human rectal tumor (HRT-18) cells [kindly provided by J. Storz, Louisiana State University (LSU), Baton Rouge, LA] was used for experiments involving BCV infection. HRT-18 cells were grown in Dulbecco's modification of Eagle's medium (DMEM) containing 10% fetal bovine serum.

Monoclonal antibody J3.3.1 specific to the MHV N protein and mAb 43C2 specific to the BCV S protein were kindly provided by Drs. S. A. Stohlman (University of Southern California, Los Angeles, CA) and J. Storz, LSU,

respectively. mAb 43C2 contains neutralizing activities to BCV and it only recognizes the conformational epitope (Hussain *et al.,* 1991). The rabbit polyclonal antibody specific to the 8-aa Flag epitope was purchased from Neomarkers (San Diego, CA).

Plasmid construction

For expressing the BCV S protein in mammalian cells, the full-length BCV S gene was cloned into eukaryotic expression vector pcDNA3 (Promega) under the control of an immediate early gene promoter of CMV. Briefly, HRT-18 cells were grown to monolayers and were infected with BCV virulent strain LY-138 at a multiplicity of infection (m.o.i.) of 10 in the presence of actinomycin D (10 μg/ml). At 24 h postinfection, cells were lysed and total intracellular RNAs were isolated with the Trizol RNA isolation reagent (GIBCO BRL). RNAs were subjected to reverse transcription (RT) reaction with antisense primers 3′BCVS2200Eco [5′-TTT <u>GAA TTC</u> AGG TTG CAG CTG TCG TGA AAG A-3′, complementary to the sequence of the BCV S gene open reading frame (ORF) at nucleotide (nt) positions 2178–2200] and 3′BCVSBam (5′-AAC <u>GGA TCC</u> AAT ATA TCG TCA GGA GCC AAT A-3′, complementary to the sequence of the BCV S gene at the last 22 nt). The restriction enzyme sites incorporated into the primer sequences are underlined throughout this section. cDNAs were then amplified by PCR with sense primers 5′BCVS2187Eco (5′-GCT <u>GAA TTC</u> TCT TTC ACG ACA GCT GCA ACC T-3′, corresponding to nt 2187–2209 of the BCV S gene) and 5′BCVSEco (5′-GCT <u>GAA TTC</u> GAT AAT GGT ACT AGG CTG CAT GAT-3′, corresponding to the sequence at the 5′-end of the BCV S gene) (see Fig. 1A). RT–PCR was performed as described previously (Zhang *et al.,* 1991b). PCR fragment A, which contains the 5′-end 2.2 kb of the S gene, was digested with *Eco*RI and cloned into the *Eco*RI site of pUC19 (GIBCO BRL), resulting in pUC/A; fragment B, which contains the 3′-end 2.0 kb of the S gene, was digested with *Eco*RI and *Bam*HI and cloned into the *Eco*RI-*Bam*HI sites of pUC19, resulting in pUC/B. Fragment A was then subcloned into the *Eco*RI site of pUC/B, resulting in pUC/AB. The orientation of fragment A in pUC/AB was verified by internal restriction enzyme digestions. pUC/AB was then digested with *Bst*XI and *Tth*III I, and the smaller *Bst*XI-*Tth*III I fragment was replaced by an additional fragment X, which was made in RT–PCR with primers 5′BCVS983 (5′-TTG CAG ATG TTT ACC GAC GTA TAC-3′, corresponding to nt 983–1007 of the BCV S gene) and 3′BCVS2380 (5′-CAG TAA ATG GCT CAA AAT TAG T-3′, complementary to nt 2358–2380 of the BCV S gene), generating pUC/BCVS (the full-length 4.2 kb S gene in pUC19). Sequences of fragments A, B, and X were determined and published previously (Zhang *et al.,* 1991b). For subcloning into the pcDNA3 expression vector, the 3′-end *Bam*HI site was converted into an

*Eco*RI site by digestion of pUC/BCVS with *Bam*HI, blunt-ending with T4 DNA polymerase, dephosphorylation with calf intestinal phosphatase (CIP), and ligation with T4 DNA ligase with an *Eco*RI linker. The resulting plasmid pUC/BCVSee contains an *Eco*RI site at both ends. pUC/BCVSee was digested with *Eco*RI and cloned into the *Eco*RI site of pcDNA3 vector, resulting in pcDNA3/BS. The orientation of the insert was confirmed by restriction enzyme digestions.

For constructing plasmid containing the BCV HE gene, all procedures were similar to those used for the BCV S gene except for the following steps. cDNA fragments containing the entire BCV HE gene were synthesized from viral RNA by RT–PCR with primers 3′BCVHE/Flag [5′-TTT GAA TTC TTA CTT GTC ATC GTC GTC CTT GTA GTC AGC ATC ATG CAG CCT-3′, containing a sequence complementary to the last 15 nt of the BCV HE gene, the coding sequence for the 8-aa Flag epitope (Asp Tyr Lys Asp Asp Asp Asp Lys), the stop codon, and an *Eco*RI site] and 5′SpeIG/BCV HE (5′-TAA CTA GTG AAT CTA ATC TAA ACT TTA AGG AAT GTT TTT GCT TCC-3′, containing an *Spe*I site, an MHV intergenic consensus sequence, and the first 15 nt of the BCV HE gene). RT–PCR products were gel purified, digested with *Spe*I and *Eco*RI, and cloned into the *Spe*I-*Eco*RI site of previously constructed MHV defective-interfering RNA vector pDE25CAT (Liao and Lai, 1994), resulting in pDE/BCV-HE. For stably expressing the BCV HE protein, pDE/BCV-HE was digested with *Spe*I, blunt-ended with T4 DNA polymerase, and then digested with *Eco*RI. The *Spe*I (blunt-ended) *Eco*RI fragment of the BCV HE gene was cloned into the *Hin*dIII (blunt-ended) *Eco*RI sites of the pcDNA3 vector, resulting in pcDNA3/BHE.

DNA transfection and selection of stable transfectants

DBT cells were grown in MEM in 60-mm culture plates (Sarsted) to 60–70% confluence and were transfected with plasmid DNAs containing the pcDNA3 vector alone or vectors with the BCV S gene (pcDNA3/BS) or the HE gene (pcDNA3/BHE) by use of the cationic liposome transfection reagent DOTAP according to the manufacturer's instructions (Boehringer Mannheim). Briefly, 7 μg of each construct was mixed with 14 μg of DOTAP in 65 μl of 20 mM HEPES (pH 7.4). The mixture was then added to DBT cells. Following incubation for 4 h, geneticin (G418) (GIBCO BRL) was added to the medium at the final concentration of 800 μg/ml, and cells were grown for 2 to 3 days. Cells that died off during this period were removed, while surviving cells were allowed to grow further in the presence of G418. Individual cells were cloned into 96-well culture plates and were selected by G418. Individual clones that were resistant to G418 treatment for 3–5 weeks were screened for expression of the S or HE protein of BCV.

Immunofluorescence assay

Indirect immunofluorescence assay was performed for detection of recombinant gene expression or viral replication according to protocol described previously (Wang and Zhang, 1999). Briefly, cells were grown to subconfluence on 8-well chamber slides (Lab Tak, Nunc, and Nalgene), fixed with 2% formaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature, and permeabilized with acetone for 15 min at −20°C. Cells were washed three times with PBS containing 5% horse serum, air dried, and incubated with a primary antibody (mAb 43C2 for the BCV S protein or polyclonal Ab anti-Flag for BCV HE). For detection of viral replication, mAb J3.3.1 and mAb 43C2 were used for MHV and BCV, respectively. Cells were then washed three times with PBS containing 5% horse serum and stained with an appropriate secondary antibody conjugated with FITC at a 1:1000 dilution for 2 h at 37°C. After a washing with PBS containing 5% horse serum, slides were mounted and observed under an inverted fluorescence microscope (Olympus IX70, filter BA515IF nm). All images were taken using a digital camera (Magnifier) with a magnification of 20×.

Generation of pseudotyped chimeric viruses

Individual cell clones that stably express the BCV S or HE protein were grown to confluence. Cells that were stably transfected with the vector alone were also grown to confluence and used as a control. These cells were then infected with MHV A59 at a m.o.i. of 10. When complete fusion of the monolayer appeared, culture medium was taken and clarified from cell debris by centrifugation at 8000 rpm for 30 min in a benchtop centrifuge (Marathon 3200R, Fisher Scientific). Clear supernatants were used as chimeric virus preparations.

Radiolabeling and analysis of virion proteins

Cell lines were infected with MHV A59 or BCV LY-138 at a m.o.i. of 10. At 2 h p.i. medium was replaced with methionine-free MEM (for DBT cells) or DMEM (for HRT-18 cells) and cells were incubated for an additional 2 h. Medium was then removed and replaced with fresh methionine-free MEM or DMEM containing [^{35}S]methionine at 100 μCi/ml (800 Ci/mmol, Amersham). Virus was harvested from the medium when the cytopathic effect reached approximately 80–90% of the cells. Medium was purified from cell debris by centrifugation at 8000 rpm (Marathon 3200R) for 30 min. For virus purification, viruses were pelleted over a 30% (w/v) sucrose cushion by centrifugation at 27,000 rpm in an SW40.Ti rotor (Beckman) for 3.5 h at 4°C. The pellets were resuspended in 1 ml of PBS and subjected to a sucrose step gradient [(20, 30, 40, 50, and 60% (w/v)] centrifugation at 35,000 rpm in an SW40.Ti rotor for 16 h at 4°C. After centrifugation, the

gradients were fractionated at 1 ml per fraction. The radioactivity of each fraction was determined in a liquid scintillation counter (Beckman LS6000TA). Fractions that contained high radioactivity counts (usually between 40 and 50% sucrose) were combined and pelleted by centrifugation at 35,000 rpm for 1 h in an SW40.Ti rotor. Nonradiolabeled viruses were purified using the same procedure as for radiolabeled viruses. Pelleted virus was resuspended in 50 μl of Lammeli's protein electrophoresis sample buffer. Equivalent counts for each sample were loaded and proteins were separated by sodium dodecyl sulfate–polyacrylamide gel (10%) electrophoresis (SDS–PAGE). To quantify viral proteins, the gel was exposed to a PhosphorImager screen and analyzed by a PhosphorImager (445SI Molecular Dynamics, Sunnyvale, CA). The amount of radioactivity in each band was determined with the software program ImageQuaNT (version 4.2a, build 13).

Western blot analysis

For Western blot analysis, purified virus was resuspended in PBS and the concentration of viral proteins was determined with the Bio-Rad protein assay kit. Under denaturing conditions, 10 μg of protein for each sample was resuspended in 20 μl of Lammeli's electrophoresis sample buffer, boiled for 3 min, and resolved by SDS–PAGE on a 10% gel. Under nondenaturing conditions, proteins were resuspended in a buffer containing 0.1% SDS without dithiothreitol; the samples were not boiled prior to loading, and proteins were separated by SDS (0.1%)–PAGE as described previously (Zhang *et al.,* 1994). Proteins were then transferred to nitrocellulose membrane (MSI, Westborough, MA) for 2 h at 100 V in a transfer buffer (25 mM Tris, 200 mM glycine, 20% methanol, 0.02% SDS). After being blocked with 5% skim milk in PBS for 2 h at room temperature, a nitrocellulose membrane was washed three times in Tris-buffered saline containing 0.5% Tween 20 and immunoblotted with Ab specific to Flag (5 μg/ml) for 2 h at room temperature, followed by a secondary Ab coupled to horseradish peroxidase (1:1000 dilution) (Sigma) for 1 h at room temperature. The presence of the BCV HE protein was detected by enhanced chemiluminescence (ECL) using peracid as a substrate (Amersham) followed by autoradiography with exposure times ranging from 30 s to 1 min.

Hemagglutination assay

The hemagglutination assay was performed as described previously (Herrler *et al.,* 1988). Briefly, mouse erythrocytes were collected from BALB/c mice. A 0.5% (v/v) erythrocyte suspension prepared in PBS was used for the HA assay. A twofold serial dilution of virus samples in PBS was made in a V-shaped 96-well microtiter plate (Falcon). An equal volume of the erythrocyte suspension was added to each well, mixed, and incubated

at room temperature for 1–2 h. The HA titer was then determined based on the reciprocal value of the maximum dilution that caused complete agglutination of erythrocytes.

Hemagglutination inhibition assay

A modified hemagglutination inhibition assay was performed. Briefly, 98 μl of BCV preparations was mixed with 2 μl of mAb 43C2. Following incubation at 37°C for 1 h, the virus–antibody mixture was serially diluted twofold with PBS, and an equal volume (50 μl) of erythrocyte suspension was added to determine the HA titer.

Virus neutralization assay

Several dilutions (1:40, 1:80, 1:160, and 1:320) of mAb 43C2, which is specific to BCV S, were made in PBS and incubated with 200 plaque-forming units of BCV LY-138, MHV A59, or the chimeric viruses for 1 h at 37°C. The antibody–virus mixtures were inoculated onto appropriate cells as indicated. Replication of the virus was monitored by immunofluorescence staining with mAb J3.3.1 for MHV A59 and mAb 43C2 for BCV. Alternatively, replication of the viruses was determined by viral plaque assay (Spaan *et al.,* 1981).

Acetylesterase assay

The acetylesterase assay was carried out as described previously (Storz *et al.,* 1992; Vlasak *et al.,* 1988). Briefly, cells were grown to confluence, scraped from the plate using cell scrapers in 500 μl of PBS, and washed three times with PBS. For extraction of cellular proteins, cells were frozen and thawed three times and centrifuged at 14,000 rpm in a microcentrifuge (IEC) for 5 min at 4°C. The supernatants were transferred to new microcentrifuge tubes. The protein concentration of cell lysates was determined with the Bio-Rad protein assay kit. Five micrograms protein of each sample was incubated with 1 ml of PBS containing 1 mM *p*-nitrophenol (Sigma) as a substrate. The absorbency of each sample was determined at a 2-min interval in a spectrophotometer (U-2001) at 405 nm. Each sample was determined in triplicate for each time point and the absorbency values were plotted using SigmaPlot software (version 5.0, IBM).

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