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Coronavirus Defective-Interfering RNA as an Expression Vector: The Generation of a Pseudorecombinant Mouse Hepatitis Virus Expressing Hemagglutinin-Esterase

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We have developed an expression vector system using a defective-interfering (DI) RNA of mouse hepatitis virus (MHV), a prototype coronavirus, to deliver and express a foreign gene in MHV-infected cells. This vector contains an MHV intergenic sequence to promote the expression of foreign genes. In this study, we used this vector to introduce a hemagglutinin-esterase (HE) protein, an optional MHV structural protein, into the MHV-infected cells. The engineered HE protein could be efficiently incorporated into the virion which did not synthesize its own HE protein, thus generating a pseudorecombinant virus that expresses an exogenous HE protein. The engineered HE protein could be made distinguishable from the native protein by attaching an 8-amino-acid peptide tag at the carboxyl-terminus. Both the engineered and native HE proteins from the HE-producing virus train could be incorporated into the virion, thus generating phenotypically mixed virus particles. We also showed that the HE-expressing DI RNA could be incorporated into viruses, and the engineered HE protein expressed in the infected cells for at least three serial virus passages. Furthermore, we have made two mutants, in which parts of the external domain of the HE protein have been deleted, to study the sequence requirements for the stable expression of HE and its incorporation into MHV virions. Although both of the mutant HE proteins could be expressed in the MHV-infected cells, they failed to be incorporated into virions, suggesting the importance of the extracellular domain of HE protein for its incorporation into virus particles. This vector system enabled the first successful incorporation of a selected coronaviral protein into virions and demonstrates its utility as an expression vector for studying the molecular biology of coronaviruses.

INTRODUCTION

Mouse hepatitis virus (MHV), a prototype coronavirus, is an enveloped RNA virus, which contains a genomic RNA of 31 kilobases (kb) (Pachuk *et al.*, 1989; Lee *et al.*, 1991). The RNA genome associates with the nucleocapsid phosphoprotein (N) to form a helical nucleocapsid (Macnaughton *et al.*, 1978; Sturman *et al.*, 1980). The viral envelope contains two major glycoproteins, membrane protein (M) and spike protein (S) (Sturman and Holmes, 1985). The M protein is thought to bind to the nucleocapsid protein during virus budding (Sturman *et al.*, 1980). The S protein forms the spikes on the virion surface and interacts with the viral receptors on the surface of target cells (Collins *et al.*, 1982). An additional envelope glycoprotein, hemagglutinin–esterase (HE), approximately 65 kDa in size, is found in some, but not all, strains of MHV (Shieh *et al.*, 1989; Yokomori *et al.*, 1989, 1991); thus, HE protein is not required for virus infectivity. HE protein binds to 9-*O*-acetylated neuraminic acid on cell membranes (Herrler *et al.*, 1985, 1988; Roger *et al.*, 1986; Vlasak *et al.*, 1988b; Schultze *et al.*, 1991; Schultze and Herrler, 1992) and contains an esterase activity which cleaves the acetyl group from this neuraminic acid (Herrler *et al.*, 1985, 1988; Roger *et al.*, 1986; Vlasak *et al.*, 1988a; Yokomori *et al.*, 1989). The HE-coding sequence is present in the genomes of all MHV strains; however, in most of them, the HE gene cannot express a functional protein because of mutations either in the coding region or in the transcriptional initiation signal for the gene (Luytjes *et al.*, 1988; Shieh *et al.*, 1989; Yokomori *et al.*, 1991). Although the HE protein is not required for viral replication *in vitro*, its presence could conceivably alter the properties of MHV infections *in vivo*. Indeed, passive immunization of mice with HE-specific monoclonal antibodies has been shown to alter the neuropathogenic properties of MHV (Yokomori *et al.*, 1992). However, the precise role of HE protein in MHV infections remains to be elucidated. Since an infectious, full-length cDNA clone of coronavirus RNA is not yet available, the inability to manipulate MHV genomic RNA has been a major obstacle in unequivocally deciphering the functional roles of each viral protein, including HE protein, in viral infections.

Previous attempts at genetically manipulating coronavirus genomes or altering the patterns of viral protein expression have taken advantage of the potential recombination between wild-type MHV RNA and a transfected RNA or defective-interfering (DI) RNA to replace portions of the MHV genome (Koetzner *et al.*, 1992; Liao and Lai, 1992; Van der

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Most *et al.*, 1992; Masters *et al.*, 1994). However, the success of this approach so far has been limited to the 5′- and 3′-ends of the viral genome. It has not been possible to insert a foreign gene into the MHV genome or replace internal viral gene sequences, probably because these processes require multiple recombination events. Another common approach has been the expression of viral or foreign proteins by a heterologous viral or plasmid expression vector. However, this approach has not been very useful for coronaviruses, since the expressed proteins could not be incorporated into viral particles or passaged to subsequent viral populations, probably because of the low level of expression and different compartmentalization of the expressed proteins. Recently, infectious cDNA and RNA of MHV DI particles, which can replicate in the presence of a helper MHV, have been successfully developed (Makino *et al.*, 1988a, 1991; Van der Most *et al.*, 1991). These DI RNAs are small, the smallest being 2.2 kb (Makino *et al.*, 1988b), and retain both the 5′- and 3′-termini and scattered portions of the internal sequences of the wild-type viral genome (Makino *et al.*, 1985, 1988b, 1990; Van der Most *et al.*, 1991). Once transfected into the MHV-infected cells, they replicate efficiently. Insertion of an intergenic sequence (IG) into certain positions within the DI RNAs promoted subgenomic mRNA transcription from that site (Makino *et al.*, 1991; Makino and Joo, 1993). We have recently demonstrated that a modification of this DI system can be used to express the chloramphenicol acetyltransferase (CAT) protein in the MHV-infected cells (Liao and Lai, 1994). This DI expression vector can undergo both replication and transcription, and the CAT activity can be carried over through serial virus passages (Liao and Lai, 1994). The CAT activity can be expressed only in the cells in which MHV replication takes place, making the DI expression system an attractive tool for studying the molecular biology and pathogenesis of coronaviruses. We have also demonstrated that an internal ribosomal entry site (IRES) sequence (Jang *et al.*, 1989) can be used in lieu of the IG sequence in this vector RNA for the expression of the CAT activity (Lin and Lai, 1993).

In this study, we demonstrate the feasibility of using this DI expression system to express an engineered HE protein in MHV-infected cells. We show that this engineered HE protein can be incorporated into virions, thus generating pseudotyped virus particles. This is the first successful demonstration of the incorporation of an exogenously introduced viral protein into coronaviruses. This expression system provides a simple and efficient expression vector for studying the molecular biology and pathogenesis of viral gene products in MHV infection without the use of heterologous viral or plasmid vectors.

MATERIALS AND METHODS

Viruses and cells

Plaque-cloned MHV strains of A59 (Manaker *et al.*, 1961), JHM(2) (Makino and Lai, 1989), and At11f, which is a brain isolate from a JHM-infected Wistar–Furth rat (Morris *et al.*, 1989), were used throughout this study. Viruses were propagated in DBT cells (Hirano *et al.*, 1974), a mouse astrocytoma cell line, at a multiplicity of infection of 0.5. DBT cells were also used as the recipient cells for RNA transfection experiments.

Plasmid construction

To construct p25HE (Fig. 1), we used p25CAT (Liao and Lai, 1994), which contains a CAT gene behind an IG sequence in the DIssE RNA (Makino *et al.*, 1988b), as a starting plasmid. The cDNA fragment containing the HE gene was generated by RT–PCR amplification using the cytoplasmic RNAs from JHM(2)-infected cells as templates and two primers, 986 (5′-GGGACTAGTATTGTTG AGAATCTAATCTAAACTTTAAGGAATGGGCAGTAC-GTGC-3′) and 987 (5′-GGGCTGCAGTTATGCCTCATG-CAATCT-3′). In this cDNA fragment, the HE gene was placed behind the IG sequence of gene 7 (IG7) of MHV-A59 (Liao and Lai, 1994). After restriction digestion with *Spe*I and *Pst*I, a 1.4-kb cDNA fragment was purified from low-melting agarose gel and directionally cloned into the *Spe*I and *Pst*I sites of p25CAT (Liao and Lai, 1994), resulting in p25HE (Fig. 1). To construct p25HE/flag, the cDNA containing the HE gene plus the sequence encoding an 8-amino-acid (aa) peptide (Asp Tyr Lys Asp Asp Asp Asp Lys) was first generated by RT–PCR amplification using templates derived from the cytoplasmic RNAs of JHM(2)-infected cells and two primers, 986 (see above) and 86 (5′-CCCCTGCAGTTACTTGTCATCGTCGTCC-TTGTAGTCTGCCTCATGCAATCT-3′). The resulting cDNA fragment was digested with *Spe*I and *Pst*I and directionally cloned into the *Spe*I and *Pst*I sites of p25CAT, resulting in p25HE/flag. This clone encodes HE protein with the 8-aa tag at the carboxyl-terminus.

RNA transcription and transfection

All plasmid constructs were linearized with *Xba*I, and RNA was obtained by *in vitro* transcription using T7 RNA polymerase according to the manufacturer's recommended procedure (Promega). RNA transfection was carried out using the DOTAP method (Boehringer-Mannheim). MHV-infected DBT cells were used as the recipient cells for RNA transfection. Briefly, monolayers of DBT cells at approximately 80% confluence in 6-cm petri dishes were infected with A59 at a multiplicity of infection of 5.0. At 1 hr postinfection, virus-infected cells were washed once with serum-free Eagle's minimal essential medium (MEM) and then covered with 2 ml of prewarmed MEM containing 1% newborn calf serum. Ten micrograms of *in vitro*-transcribed RNAs was dissolved in a final volume of 200 μl of 10% (v/v) DOTAP (Boehringer-Mannheim) mixture. The RNA–DOTAP mixtures were incubated at 25° for 15 min and then added slowly to the cell cultures.

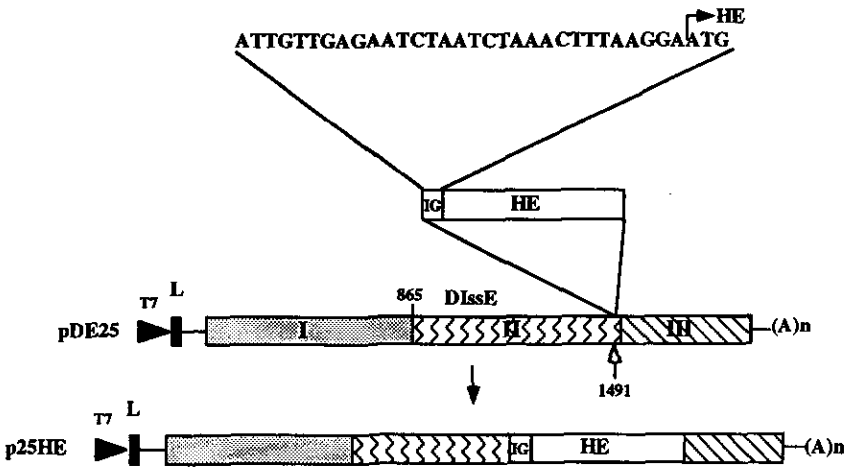

FIG. 1. Structure of p25HE. The IG segment is derived from the region between genes 6 and 7 of MHV-JHM genome. The original ATG in IG is used as the initiation codon for HE gene (marked with a bent arrow). The cDNA fragment containing the HE ORF was inserted into pDE25 at nt 1491 (marked with an open arrow) near the end of domain II (Makino *et al.*, 1988b), resulting in p25HE. L denotes the MHV leader, and the solid arrowheads represent T7 promoter. The thin horizontal lines in front of and behind the rectangular boxes denote untranslated regions.

[^{35}S]Methionine labeling of viral proteins and preparation of cell lysates and virus particles

When the cytopathic effect of MHV-infected DBT cells in 60-mm plates reached approximately 85%, cells were washed once with serum-free MEM, and media were replaced with 2 ml of methionine-free MEM containing 1% dialyzed fetal calf serum. After 30 min of incubation, ^{35}S-Translabel (100 μCi/ml, 1193 Ci/mmol; ICN Biochemical) was added, and the cells were incubated for another 60 min. Cell lysates were made as described previously (Yokomori *et al.*, 1989), using RIPA buffer (50 m*M* Tris–HCl, pH 7.4, 0.3 *M* NaCl, 4 m*M* EDTA, 0.5% Triton X-100, 0.1% SDS) containing 1 m*M* phenylmethylsulfonyl fluoride. For harvesting of virus particles, the cells were labeled with ^{35}S-Translabel in methionine-free MEM for 2 hr, and then incubated for an additional 12 hr following addition of 2 ml of MEM containing 1% newborn calf serum. Culture media were collected and clarified of cell debris by centrifugation at 5000 rpm for 30 min. Supernatants were then further clarified by centrifugation through a discontinuous sucrose gradient (60 and 20%, w/v) at 27,000 rpm in a Beckman SW28 rotor for 3 hr. The band from the interface was extracted, diluted in TNE buffer, and pelleted by centrifugation at 35,000 rpm in an SW41.Ti rotor (Beckman) for 1 hr. Pellets were then resuspended in 1 ml of TNE buffer, loaded on a sucrose step gradient [60, 50, 40, 30, and 20% (w/v) sucrose in TNE], and centrifuged at 35,000 rpm in an SW41.Ti rotor for 16 hr. Following centrifugation, 1 ml of each fraction was collected from the top of the tube, diluted with TNE buffer, and pelleted at 35,000 rpm in an SW41.Ti rotor for 1 hr. The pellets of each fraction were resuspended in 50 μl of electrophoresis sample buffer and analyzed by electrophoresis in 10% polyacrylamide gel (Laemmli, 1970). For most of the experiments, a simplified virus purification procedure (Shieh *et al.*, 1989) was used. Briefly, the culture media were collected and clarified of cell debris by centrifugation in an SW-55 Ti rotor (Beckman) at 8000 rpm for 30 min. Virus particles in supernatants were then pelleted through a 30% sucrose cushion at 27,000 rpm in a Beckman SW-28 rotor for 3.5 hr. The pellets were dissolved in RIPA buffer for immunoprecipitation.

Immunoprecipitation

Immunoprecipitation was carried out as described previously (Baker *et al.*, 1989). Briefly, preparations of cell lysates or virions in RIPA buffer were incubated with 5 μl of polyclonal antibody against JHM(2) (Yokomori *et al.*, 1989), HE-specific monoclonal antibody (3B4) (Yokomori *et al.*, 1992), or M2 monoclonal antibody specific for the 8-aa (flag) tag (IBI Kodak) for 2 hr at 4°, and then 100 μl of 30 mg/ml suspension of protein A–Sepharose 4 Fast Flow (Pharmacia) in RIPA buffer was added and the samples were incubated for an additional 1 hr at 4°. The antigen–antibody complex was precipitated by brief centrifugation and then washed three times with 1 ml of RIPA buffer each time. The immunoprecipitated proteins were then eluted from protein A–Sepharose by boiling for 3 min in 20 μl of electrophoresis sample buffer (0.1 *M* β-mercaptoethanol, 1% SDS, 0.08 *M* Tris–HCl, pH 6.8, 10% glycerol). Protein A–Sepharose was removed by centrifugation, and the supernatant was analyzed by electrophoresis on 7.5 to 15% gradient polyacrylamide gels containing 0.1% SDS (Laemmli, 1970).

RESULTS

Establishment of an MHV DI vector which expresses an HE protein

Previously, we used a DI vector (25CAT) to express CAT activity in MHV-infected cells (Liao and Lai, 1994). In the current study, we attempted to examine whether this vector could be used to express viral proteins. We

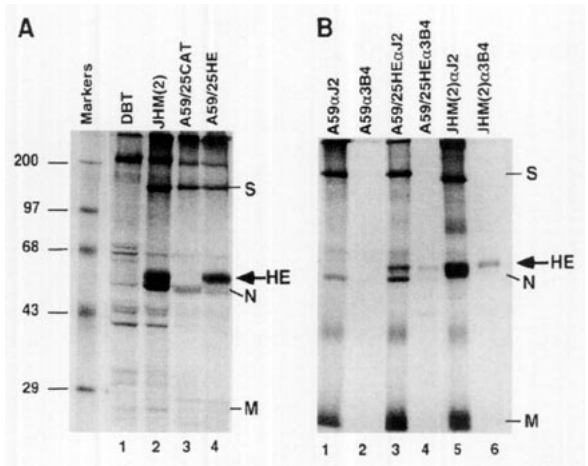

FIG. 2. The expression of HE in the cells. Cell lysates were prepared from [^{35}S]methionine-labeled cells and immunoprecipitated with various antibodies. The precipitated proteins were analyzed by SDS–PAGE on 7.5 to 15% gradient polyacrylamide gel. (A) [^{35}S]methionine-labeled cell lysates were precipitated with anti-JHM(2) antibodies. The numbers on the left of the gel are molecular sizes in kilodaltons. DBT, DBT cells without infection or transfection; JHM(2), DBT cells infected with JHM(2) only; A59/25CAT, A59-infected and 25CAT-transfected cells; A59/HE, A59-infected and 25HE-RNA-transfected cells. (B) Cellular lysates as in (A) were immunoprecipitated with anti-JHM(2) antibody (αJ2) (lanes 1, 3, and 5) or anti-HE monoclonal antibody (α3B4) (lanes 2, 4, and 6). The MHV structural proteins S, N, and M are indicated; the positions of HE proteins are indicated by the arrows on the right of the gels.

first studied HE protein, since it is an optional viral protein. We reasoned that an optional structural protein will have fewer genetic or structural constraints for its expression and incorporation into virus particles than other viral structural proteins. To establish an HE expression system, we placed the HE gene behind the IG7 sequence inserted in the DIssE RNA (see Materials and Methods, Fig. 1). The resulting 25HE RNA contains two major open reading frames (ORFs): the 5′ ORF is derived from the original ORF of DIssE RNA (Makino *et al.*, 1988b), and the 3′ ORF from the HE gene. Between the two ORFs is an IG, which has been shown to be a necessary *cis*-element for mRNA transcription (Makino *et al.*, 1991; Makino and Joo, 1993).

To examine whether 25HE RNA can express HE protein in MHV-infected cells, we transfected the *in vitro*-transcribed 25HE RNA into DBT cells which had been infected with MHV-A59, an HE-deficient strain (Luytjes *et al.*, 1988; Shieh *et al.*, 1989). These transfected and infected cells were labeled with [^{35}S]methionine, and cell lysates were used for immunoprecipitation using anti-JHM(2) polyclonal antibodies (Yokomori *et al.*, 1989), which recognize MHV structural proteins, including S, N, M, and HE. As shown in Fig. 2A, HE protein was detected in the A59-infected and 25HE-RNA-transfected cells (lane 4), but not in the A59-infected and 25CAT-RNA-transfected cells (lane 3) or uninfected DBT cells (lane 1). The protein has an electrophoretic mobility similar to that of the HE protein synthesized by JHM(2) virus (lane 2). The nature of the engineered HE protein was further studied by immunoprecipitation using an anti-HE monoclonal antibody, 3B4 (Yokomori *et al.*, 1992). Figure 2B shows that the engineered HE protein could be recognized by both anti-JHM(2) and 3B4 antibodies (lanes 3 and 4), similar to the native HE protein synthesized by JHM(2) virus (lanes 5 and 6). The amount of HE protein expressed in the A59-infected and 25HE-RNA-transfected cells (Fig. 2A, lane 4) was comparable to that in the JHM(2)-infected cells (Fig. 2A, lane 2), indicating that 25HE DI RNA was capable of expressing a large amount of HE protein in A59-infected cells, which normally do not synthesize HE proteins (Fig. 2B, lanes 1 and 2). Thus, this vector system is a very efficient means of expressing viral HE proteins.

The expression of an engineered HE/flag protein which can be distinguished from native HE proteins

It is of interest to compare the properties of native and engineered HE proteins. Thus, we examined whether engineered HE protein could be expressed and function properly in cells infected with an HE-producing virus strain, such as JHM(2) (Shieh *et al.*, 1989; Yokomori *et al.*, 1989). For this purpose, we constructed p25HE/flag, which is identical to p25HE, except that it has a tag of 8 amino acids (flag epitope) attached to the C-terminus of its expressed HE protein (see Materials and Methods). Thus, the engineered HE/flag protein can be immunologically distinguished from the native HE protein produced by the helper virus by using a flag-specific monoclonal antibody, M2.

To examine the expression of HE/flag protein, we used three different MHV strains as helper viruses: JHM(2), which is an HE-producing strain, and A59 and At11f (Morris *et al.*, 1989), neither of which synthesize HE protein (La Monica *et al.*, 1991). The [^{35}S]methionine-labeled cellular lysates were immunoprecipitated with either anti-JHM(2) polyclonal antibodies or anti-flag monoclonal antibody M2. As shown in Fig. 3, when HE-deficient strains were used as helper viruses (lanes 5 through 12), the engineered HE/flag proteins were detected in the 25HE/flag-transfected cells (lanes 7, 8, 11, and 12). The HE/flag protein could be precipitated by both anti-JHM(2) and M2 antibodies. Interestingly, when the HE-producing strain, JHM(2), was used as a helper virus, both native HE and engineered HE/flag proteins were detected (Fig. 3, lanes 3 and 4). The size of the engineered HE protein was slightly larger than the native HE because of the extra 8 aa. These results show that native and recombinant HE/flag proteins can be synthesized together in infected cells. The M2 monoclonal antibody precipitated only proteins which had a flag epitope (Fig. 3, lanes 4, 8, and 12), in contrast to the anti-JHM(2) antibodies, which precipitated both native HE and engineered HE/flag proteins (Fig. 3, lanes 1, 3, 7, and 11); thus, these two proteins can be distinguished. These results also suggest that

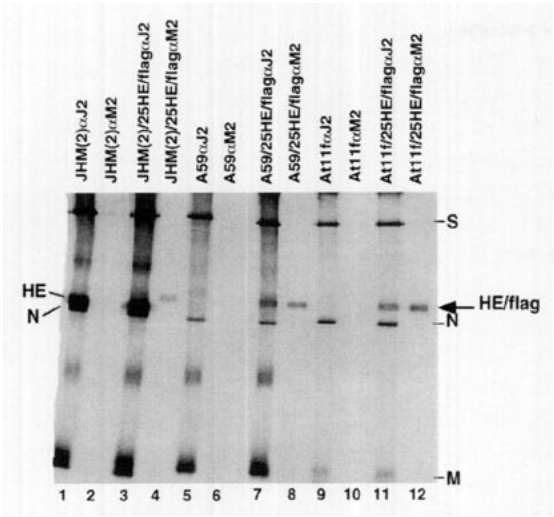

FIG. 3. The expression of HE/flag in the cells. [^{35}S]methionine-labeled cell lysates were immunoprecipitated with anti-JHM(2) antibodies or M2 monoclonal antibody. The precipitated proteins were analyzed by SDS–PAGE on 7.5 to 15% gradient polyacrylamide gel. Lanes 1 to 4 are from JHM(2)-infected cells. Lanes 5 to 8 are from A59-infected cells, and lanes 9 to 12 are from At11f-infected cells. αJ2, immunoprecipitated with anti-JHM(2) antibodies; αM2, immunoprecipitated with anti-flag M2 monoclonal antibody.

the addition of the 8-aa tag to the C-terminus of HE protein did not interfere with its expression or post-translational modifications or alter its antigenic properties.

HE-expressing DI RNA can be passaged and the HE protein expressed during serial virus passages

We next determined whether the HE-expressing DI RNA could be packaged into virions, and the engineered HE protein expressed during serial virus passages. It should be noted that this DI RNA does not contain the previously characterized RNA packaging signals of MHV (Makino *et al.*, 1990; Van der Most *et al.*, 1991; Fosmire *et al.*, 1992); yet, this DI RNA can be carried over for at least several virus passages, probably because of the high efficiency of its replication and nonspecific packaging of RNA into virions (Makino *et al.*, 1990; Lin and Lai, 1993; Liao and Lai, 1994). For this experiment, virus from 25HE-RNA-transfected and A59-infected cells was harvested and used to infect fresh DBT cells; the cellular lysate prepared from this infection was called passage 1 (P1). Virus released from this infection was then used to infect another fresh DBT cell culture, and the cellular lysate made from this infection was termed P2, and so on. As shown in Fig. 4, HE protein was detected in lysates P1 through P3. The amount of HE protein gradually decreased and finally disappeared in P4. This result indicates that the HE-expressing DI RNA can be passaged and the HE protein expressed for at least three virus passages, even though this DI RNA does not contain a specific packaging signal (Makino *et al.*, 1990; Van der Most *et al.*, 1991; Fosmire *et al.*, 1992).

Engineered HE/flag proteins can be incorporated into virions, resulting in pseudotyped virus

To determine whether engineered HE/flag protein can be incorporated into virions, virus particles were purified from culture media by sucrose gradient sedimentation and examined for the presence of recombinant HE proteins. Figure 5 shows that the recombinant HE protein was present together with the remaining viral structural proteins (S, N, and M) in the same sucrose gradient fractions (sucrose density 1.17–1.19 g/ml) containing virus particles. No free HE protein was detected at the bottom or top of the sucrose gradient, indicating that all of the engineered HE protein released was incorporated into virus particles, and that HE protein was not secreted by itself. Therefore, in the remaining experiments, virus particles in the media were purified by a single step of sedimentation through a 30% sucrose solution.

We then examined the incorporation of the recombinant HE protein into different types of viruses. HE protein was detected from the virus particles by immunoprecipitation with either anti-JHM(2) antibodies or anti-flag monoclonal antibody M2. Figure 6 shows that the engineered HE/flag protein was incorporated into the HE-deficient helper viruses, At11f (Fig. 6A, lanes 5 through 8) and A59 (Fig. 6B, lanes 1 through 4). A protein slightly larger than the engineered HE protein was seen in some of the virus preparations (Fig. 6A, lanes 5 and 7; Fig. 6B, lanes 1 and 3); it was not precipitated by M2 antibody. The nature of this protein is not clear. When the HE-producing strain, JHM(2), was used as a helper virus (Fig. 6A, lanes 1 through 4), both native HE and engineered HE/flag proteins were incorporated into the virion (Fig. 6A, lane 3), suggesting that the engineered HE/flag pro-

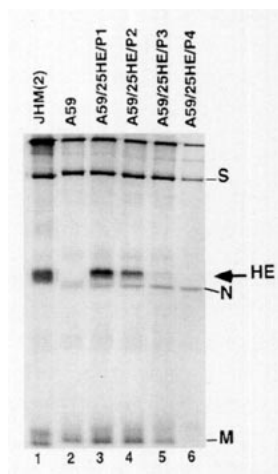

FIG. 4. The expression of HE protein from DI RNA during serial virus passages. [^{35}S]Methionine-labeled cell lysates were prepared from each passage and immunoprecipitated with anti-JHM(2) antibodies. The precipitated proteins were analyzed by SDS–PAGE on 7.5 to 15% gradient polyacrylamide gel. Lane 1, JHM(2)-infected cells; lane 2, A59-infected cells. Lanes 3 to 6 are the cell lysates derived from passages 1 (P1) through 4 (P4) of A59-infected and 25HE-RNA-transfected cells.

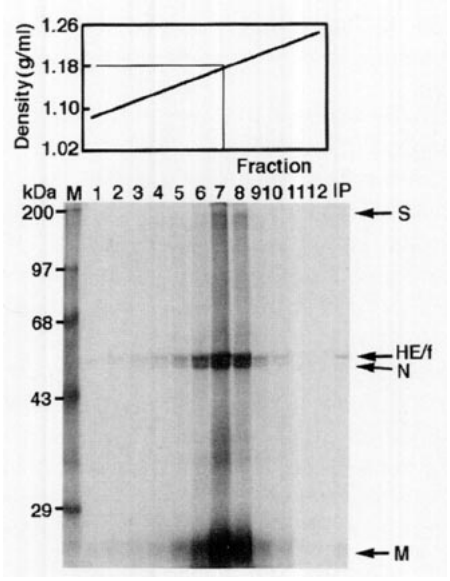

FIG. 5. The detection of HE/flag protein in virions. Media from [^{35}S]-methionine-labeled, A59-infected, and 25HE/flag RNA-transfected cells were purified through discontinuous and step-gradients of sucrose (see Materials and Methods). Equal fractions (lanes 1 through 12) were collected from top to bottom of the sucrose gradients and analyzed by SDS–PAGE on 10% polyacrylamide gel. The viral structural proteins (S, N, and M) and the DI-expressed HE/flag protein are indicated on the right with arrows. Molecular size markers (in kDa) are shown on the left. Lane IP, virus particles from fraction 7 immunoprecipitated with a mixture of monoclonal antibodies specific for HE protein (3B4) and the flag epitope (M2). At the top is the diagram showing the density of each fraction of the sucrose gradient (thick line). Two thin lines identify the density of the peak fraction containing virus particles.

tein can be incorporated together with the native HE protein into virions. These results taken together suggest that engineered HE/flag protein can be expressed in virus-infected cells and incorporated into virions, regardless of whether the helper virus is an HE-deficient or HE-producing virus strain. Incorporation of engineered HE/flag proteins into HE-carrying virus particles would give rise to pseudorecombinant virus particles that contain both engineered and native forms of HE protein.

HE/flag proteins with deletions in the external domain failed to be incorporated into virions

We have previously demonstrated that most of the viruses recovered from JHM(2)-infected mice with subacute and chronic demyelination did not synthesize any intact or truncated HE protein despite the fact that they retained at least part of the HE ORF (Yokomori *et al.*, 1993), suggesting that the C-terminus-truncated HE proteins are not stable. In order to evaluate the sequence requirement of HE for its incorporation into virions, we, therefore, made two constructs containing a deletion in the external domain of the HE/flag protein but retaining the C-terminal internal domain and the flag epitope: 25HE/flagΔBal had a deletion of 165 aa (from aa 214 to 379), and 25HE/flagΔNco had a deletion of 231 aa (from aa 115 to 346). The predicted sizes of the nonglycosylated 25HE/flagΔBal and 25HE/flagΔNco proteins are 31 and 24 kDa, respectively. Figure 7 shows that the amounts of both truncated proteins (lanes 1 through 6) were comparable to that of the full-length HE/flag protein (lanes 7 through 9) in the A59-infected cells, indicating that deletion of the outside domain of the HE protein does not interfere with its stability. The sizes of the expressed 25HE/flagΔBal and 25HE/flagΔNco proteins were approximately 35 (Fig. 7, lanes 5 and 6) and 28 kDa (Fig. 7, lane 3), respectively, which are larger than the predicted sizes of the primary translation products, suggesting that both had undergone post-translational modifications. Interestingly, 25HE/flagΔBal protein was precipitated by all three antibodies; in contrast, 25HE/flagΔNco protein was poorly recognized by polyclonal anti-JHM(2) antibodies (Fig. 7, lane 1), and was not recognized at all by monoclonal antibody 3B4 (Fig. 7, lane 2), suggesting that the epitope for the 3B4 antibody resides within aa 115–214 of the HE protein.

To determine whether these mutant HE/flag proteins could be incorporated into virions, we examined the released virus particles for the presence of HE/flag protein. As shown in Fig. 8, the wild-type HE/flag protein (lanes 5 and 6), but not the mutant proteins (lanes 1 through 4), was detected in virions. Even after longer exposures, no mutant proteins were detected (data not shown), suggesting the importance of the outside domains of the HE protein for its incorporation into virus particles.

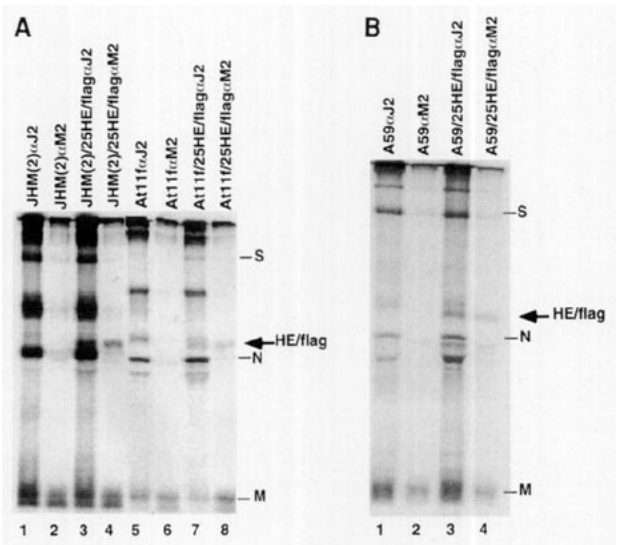

FIG. 6. The incorporation of HE/flag proteins into various virions. [^{35}S]Methionine-labeled virus particles from various virus-infected and RNA-transfected cells were concentrated by sedimentation through discontinuous sucrose fractions, lysed in RIPA buffer, and immunoprecipitated with either anti-JHM(2) antibodies (αJ2) or M2 monoclonal antibody (αM2). The precipitated proteins were analyzed by SDS–PAGE on 7.5 to 15% gradient polyacrylamide gel. (A) Lanes 1 through 4 were the virions released from the JHM(2)-infected cells. Lanes 5 through 8 were the virions released from the At11f-infected cells. (B) Lanes 1 through 4 were the virions released from the A59-infected cells.

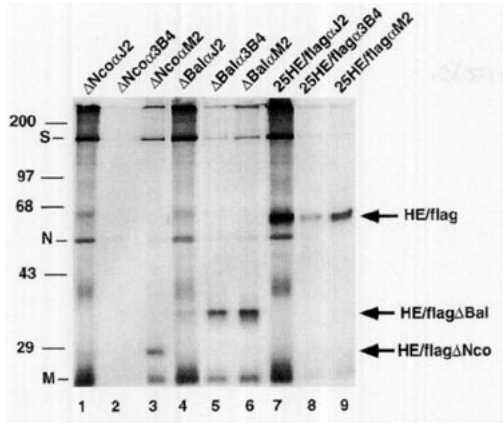

FIG. 7. The expression of mutant HE/flag proteins in the virus-infected cells. [^{35}S]Methionine-labeled cell lysate from A59-infected cells transfected with the partial HE-deletion mutants were immunoprecipitated with anti-JHM(2) antibodies (αJ2) or M2 monoclonal antibody (αM2). The precipitated proteins were analyzed by SDS–PAGE on 7.5 to 15% gradient polyacrylamide gel. Lanes 1 through 3 are the cellular lysates derived from 25HE/flagΔNco RNA transfection. Lanes 4 through 6 are from 25HE/flagΔBal RNA transfection. Lanes 7 through 9 are from 25HE/flag RNA transfection.

DISCUSSION

In this study, we have demonstrated the utility of an MHV DI expression vector for the study of the biology of MHV infection by using the HE protein as a model system. We showed that engineered HE protein could be synthesized in MHV-infected cells and efficiently incorporated into virions. Thus, this system enables the formation of pseudotyped virions, which contain the wild-type viral genome but incorporate the engineered HE glycoproteins derived from an exogenous source. This is the first system that successfully permits the introduction of an exogenous MHV structural protein into virus particles. It is also the first homologous viral expression system for MHV, i.e., both DI-RNA vector and helper virus are from the same virus. We also showed that the engineered and native viral proteins could be coexpressed in both MHV-infected cells and virions, thus generating a phenotypically mixed virus. Although the DI vector used in this study is not ideal for packaging into virions, this DI RNA, nevertheless, could be maintained for at least three serial virus passages. Thus, it will be possible in the future to use a DI RNA containing a packaging signal (e.g., DIssF RNA) (Makino *et al.*, 1990; Van der Most *et al.*, 1991) to engineer a recombinant MHV containing a bipartite RNA genome. This will be similar to the bipartite Sindbis virus constructs previously reported (Bredenbeek *et al.*, 1993). Therefore, this vector system opens up the possibility of exploiting phenotypic mixing, pseudotype virus particles, and pseudorecombinant viruses as genetic tools for studying the molecular biology of MHV genes and the biology of MHV infection.

The genetic manipulation of a viral genome is an important tool to unambiguously determine the roles of any viral gene product in viral infection. It is critical that the engineered viral gene and its normal counterpart be compared under the isogenic background. However, this approach has not been possible so far for coronavirus because of the lack of an infectious MHV genomic cDNA. Thus, the functions of MHV genes could be studied only by using spontaneous or chemically induced viral mutants, such as temperature-sensitive mutants and monoclonal antibody-resistant mutants, as well as natural RNA recombinant viruses. Because of the high frequencies of RNA mutation and recombination, the genetic background of such mutants may be quite different from that of the parental viruses. Thus, the biological properties of any virus mutant could not be linked unequivocally to the identified mutations. The DI vector system described here allows the use of the same helper virus to incorporate either wild-type or mutant gene products; thus, the genetic background of these viruses will be identical. Therefore, this DI system may offer the best alternative to a full-length infectious RNA. Another common approach for studying the biochemical properties of viral proteins is the use of heterologous expression virus vectors, such as vaccinia virus or Sindbis virus. Although these vectors offer many advantages, they are not ideal for generating pseudotyped or phenotypically mixed MHV because of the difficulty in removing background noise derived from the heterologous vector virus. The system described here overcame this problem. It should be mentioned that we have previously used an IRES sequence in place of IG sequence for the expression CAT activity (Lin and Lai, 1993). Conceivably, this system can be used for a similar purpose.

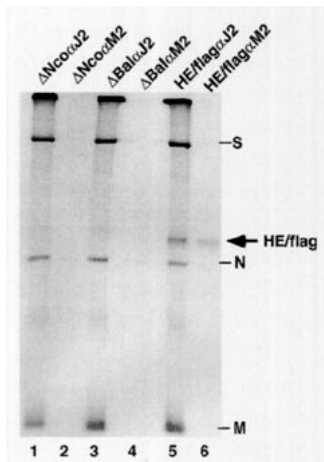

FIG. 8. The detection of mutant HE/flag proteins in the A59 virions. [^{35}S]Methionine-labeled virus particles from the media of the A59-infected and mutant DI RNA-transfected cells were purified, dissolved in RIPA buffer, and immunoprecipitated with either anti-JHM(2) antibodies (αJ2) or anti-flag M2 monoclonal antibody (αM2). The precipitated proteins were analyzed by SDS–PAGE on 7.5 to 15% gradient polyacrylamide gel. Lanes 1 and 2 were the virus particles derived from the 25HE/flagΔNco RNA transfection. Lanes 3 and 4 were from the 25HE/flagΔBal RNA transfection. Lanes 5 and 6 were from the 25HE/flag RNA transfection.

To extend the application of this expression system, we have further demonstrated that, by the addition of an 8-aa tag to the C-terminus, engineered HE protein can be distinguished from native HE protein in infected cells and virions (Figs. 3 and 5). These results suggest that this system is suitable for expressing other viral structural proteins that are essential for MHV assembly; preliminary results showed that N, M, and the gene 5b proteins of MHV-A59 can be expressed in MHV-infected cells and incorporated into the virion (unpublished observations). An additional advantage of this system is its high specificity, because the MHV DI expression vector can express its engineered genes only in cells infected with MHV. Thus, any engineered protein will likely be regulated by viral or cellular genes in the same manner as are native viral proteins. Another potential use of this system is to study the effects of viral nonstructural proteins as well as gene products of nonviral origin, such as cytokines, cytokine antagonists, or other immune modifiers, on MHV infection. These gene products will be concentrated in the MHV-infected cells or their immediate surroundings; thus, the effects of these molecules on MHV infection will be highly specific. Taken together, these data demonstrated that this MHV DI RNA is a versatile and powerful expression system, which makes it possible to unequivocally address the issues that previously have been hampered by the lack of an infectious full-length cDNA clone for the MHV genome.

In this study, we have used this expression system to reveal the sequence requirements for HE protein to be stably expressed in the infected cells and incorporated into virions (Figs. 7 and 8). Our results here (Fig. 7) demonstrated that mutant HE proteins with an intact C-terminal hydrophobic domain can be efficiently and stably expressed in infected cells. These findings, coupled with the previous observation that C-terminally truncated HE proteins were not stably expressed (Yokomori *et al.*, 1993), suggest the importance of the C-terminus of HE protein for its stability in infected cells. The N-terminus of HE protein alone has been demonstrated to have the capacity to be translocated across the endoplasmic reticulum membrane and glycosylated (Yokomori *et al.*, 1993). The two mutant HE proteins, which have retained the C-terminal transmembrane domain but have internal deletions in the outside domain, appeared to be glycosylated, since both of them had larger-than-predicted sizes (Fig. 7, lanes 1 to 6); however, neither were incorporated into virions, despite the fact that both contain the intact C-terminal transmembrane domain. One possible explanation is that the mutant HE proteins were not properly folded and transported to the site of virus assembly. Another possibility is that the outside domain of HE protein needs to interact with other viral proteins for its incorporation into MHV particles, and the deleted region is required for this interaction. In any case, the expression system described here will be useful for further studying the mechanism of virus assembly and the functional significance of the various domains of viral structural proteins in MHV pathogenesis.

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