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The Production of Recombinant Infectious DI-Particles of a Murine Coronavirus in the Absence of Helper Virus

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We have studied the production and release of infectious DI-particles in vaccinia-T7-polymerase recombinant virus-infected L cells that were transfected with five different plasmids expressing the synthetic DI RNA MIDI-HD and the four structural proteins (M, N, S, and E) of the murine coronavirus MHV-A59. The DI cDNA contains the hepatitis delta ribozyme sequences to generate in the transfected cells a defined 3′ end. In EM studies of transfected cells virus-like particles (VLP) were observed in vesicles. Release of the particles into the medium was studied by immunoprecipitations of proteins released into the culture supernatant. Particle release was independent of S or N, but required M and E. Coexpression of E and M was sufficient for particle release. Coexpression of the structural proteins and the MIDI-HD RNA resulted in the production and release of infectious DI-particles. Infectivity of the DI-particles was determined by adding helper virus MHV-A59 to the medium containing the VLPs and using this mixture to infect new L cells. Intracellular RNA of several subsequent undiluted passages was isolated to detect the MIDI-HD RNA. Passage of the MIDI-HD RNA was dependent on the expression of the structural proteins of MHV-A59 in the transfected cells. In the absence of either E or M, MIDI-HD RNA could not be passaged to fresh L cells. We have thus developed a system in which we can produce coronavirus-like particles and an assay to test their infectivity.

INTRODUCTION

Coronaviruses are enveloped viruses that have a positive-stranded RNA genome of 27–32 kb in a helical nucleocapsid form. During replication, a 3′-coterminal nested set of mRNAs is produced, from which the different proteins are translated (reviewed by Spaan *et al.,* 1988; Luytjes, 1995). Coronaviruses bud in the intermediate compartment of the host cell (Krijnse Locker *et al.,* 1994; Tooze *et al.,* 1987), inserting either two or three viral protein species into the membrane: the spike protein (S), the membrane protein (M), and in some coronaviruses the hemagglutinin protein (HE) (reviewed by Spaan *et al.,* 1988). Recently, an additional small membrane protein (sM or E) has been identified in the virions of the pig (TGEV), avian (IBV), and murine coronavirus (MHV) (Tung *et al.,* 1992; Liu and Inglis, 1991; Yu *et al.,* 1994).

During virus assembly the helical nucleocapsid (NC) consisting of the genomic RNA and many N molecules is enveloped, thereby forming an infectious coronavirus particle. We are particularly interested in understanding murine coronavirus nucleocapsid formation and the interaction between the viral membrane proteins and the NC. The protein component of the NC, the N protein, is the only viral structural protein that is not synthesized on membrane bound ribosomes. It binds specifically to the 5′ leader sequence (Baric *et al.,* 1988). Furthermore, a domain located on the genome, at the 3′ end of the polymerase 1B open reading frame most likely interacts with the N protein as it has been demonstrated that this domain is involved in encapsidation of the genome of defective interfering particles (Van der Most *et al.,* 1991; Fosmire *et al.,* 1992). N protein and the NC interact with membranes (Anderson and Wong, 1993) and with M (Sturman *et al.,* 1980).

The MHV S protein is cotranslationally glycosylated resulting in a S precursor protein of 150 kDa that forms homo-oligomers in the ER (Vennema *et al.,* 1990). The homo-oligomers are either inserted into the virions in the intermediate compartment or are transported to the cell surface through the constitutive pathway. In the Golgi stacks the high mannose sugar side-chains are trimmed and modified, giving rise to an almost endo-H-resistant 180-kDa protein (Niemann and Klenk, 1981). A portion of the MHV-A59 S molecules is cleaved in the post-Golgi into two 90-kDa subunits (Sturman *et al.,* 1985). At the cell surface the spike protein can bind to the receptor on neighboring host cells (Dveksler *et al.,* 1991) and induce cell to cell fusion (Vennema *et al.,* 1990), but receptor-independent fusion has also been described (Gallagher *et al.,* 1992). Cleavage of MHV S is not absolutely required for the induction of cell to cell fusion. However, expression of uncleaved S on the cell surface resulted in a delayed syncytium formation (Taguchi, 1993; Stauber *et al.,* 1993; Bos *et al.,* 1995; Gombold *et al.,* 1993).

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The M protein of MHV-A59 (22–26.5 kDa) is an O-glycosylated (Holmes *et al.,* 1981) triple-spanning membrane protein (Armstrong *et al.,* 1984) that forms large aggregates in the Golgi (Krijnse Locker *et al.,* 1995). Data reported by several groups indicate an important role for M in virus assembly (Holmes *et al.,* 1981; Rottier *et al.,* 1981; Holmes *et al.,* 1987). When expressed independently, the protein accumulates beyond the budding compartment in the trans-Golgi network and is not transported to the plasma membrane (Rottier and Rose, 1987; Krijnse-Locker *et al.,* 1992). When S and M are expressed together, both proteins are retained in the trans-Golgi (Opstelten *et al.,* 1995). Interactions, presumably lateral, between S and M in the ER have been established both in infected cells and in cells coexpressing both proteins and it has been suggested that this interaction plays an important role in the inclusion of the S oligomers into budding virions (Opstelten *et al.,* 1994, 1995).

Not much is known yet about the function of the E protein (9.6 kDa). The E protein of TGEV is expressed at the cell surface (Tung *et al.,* 1992), the IBV E protein is part of the viral envelope (Liu *et al.,* 1991), and the E protein of MHV-A59 is acylated and was detected in virions albeit in very low amounts (Yu *et al.,* 1994).

Although several interactions between the structural proteins of MHV have been investigated, it is not known which of these are required for assembly and budding of infectious virions. There is no reverse genetics approach available to study virus assembly. An infectious cDNA clone of MHV-A59 has yet to be constructed and targeted recombination has so far only been successful at the 5′ and 3′ ends of the genomic RNA (Van der Most *et al.,* 1992; Masters *et al.,* 1994; Makino and Lai, 1989; Chang *et al.,* 1994; Peng *et al.,* 1995). A full-length cDNA clone of a naturally occurring defective interfering (DI) RNA of MHV-A59 has been extensively characterized (Van der Most *et al.,* 1991). MIDI contains the signals for replication and packaging, but is dependent on helper virus MHV-A59 for its propagation.

Assembly and budding requirements for other enveloped viruses have been studied with the use of virus-like particles (VLP; Hobman *et al.,* 1994; Qiu *et al.,* 1994; Mebatsion *et al.,* 1995; Suomalainen *et al.,* 1992). In these systems domains in the structural proteins that are important for assembly can be located by insertion of mutated proteins into VLPs.

We describe in this paper the assembly of virus-like particles of MHV-A59 by coexpressing the structural proteins using the vaccinia virus T7 system. Further, we show that a DI-genome can be packaged into these particles. Finally, we show that the DI particles are infectious.

MATERIALS AND METHODS

Cells and viruses

Mouse L cells were grown in Dulbecco's modified Eagle medium (DMEM; Gibco) containing 10% fetal calf serum. MHV-A59 stocks were grown as described (Spaan *et al.,* 1981). Vaccinia virus vTF7.3 stocks (kindly provided by Dr. B. Moss) were grown on RK13 cells.

Construction of plasmids

Standard DNA recombination procedures were used (Sambrook *et al.,* 1989). pMIDI-HD: The hepatitis delta ribozyme and the T7 terminator were introduced at the 3′-end of pMIDI (Van der Most *et al.,* 1991) just downstream of the poly(A)-tail. The unique *Nhe*I site of pMIDI was filled in with the Klenow fragment of DNA polymerase I. Vector (2.0) (Pattnaik *et al.,* 1992; kindly provided by Dr. L. A. Ball) was digested with *Sma*I and *Xba*I to obtain the 250-bp fragment that contains the hepatitis delta ribozyme and the T7 terminator sequence. The fragment was cloned into the Klenow-treated *Nhe*I site of pMIDI. pTUM-M: The construction of pTUM-M was described by Opstelten *et al.* (1993). pTUM-N: was described by Vennema *et al.* (1991). pTUM-S: A *Bam*HI MHV-S containing fragment was cloned into the *Bam*HI site of pTUG3 (Vennema *et al.,* 1991). pIRES-E: The sequence encoding the E gene (155 nt) was amplified by PCR from a cDNA clone of MHV-A59 mRNA5, pRG68 (Bredenbeek, 1990) using oligo's c093 (containing an *Nco*I site at the AUG codon of E: 5′CATGCCATGGCCTTTAATTTATTCCTTAC3′) and c094 (containing the stopcodon and an *Xba*I site downstream of it: 5′ CTAGTCTAGATTAGATATCATCCAC 3′). The amplified fragment was isolated from gel, digested with *Nco*I and *Xba*I, and inserted into the *Nco*I–*Xba*I-digested vector pIRES (Den Boon *et al.,* 1995), containing the encephalomyocarditis virus internal ribosomal entry site.

MHV-A59 infection

Confluent monolayers of L cells were infected with MHV-A59 in PBS-DEAE, supplemented with 3% FCS at a multiplicity of infection (m.o.i.) of 10. After absorption for 1 hr at 37°, virus was removed and cells were cultured in DMEM supplemented with 3% FCS. Undiluted passage was performed as described before (Van der Most *et al.,* 1991).

DNA transfection in the vaccinia T7 system

L cells (1×10^6) were seeded in 35-mm dishes. Sixteen hours later the cells were infected with the T7 RNA polymerase expressing vaccinia virus recombinant (vTF7.3) at a m.o.i. of 5. At 1 hr postinfection the cells were transfected with lipofectin containing the appropriate plasmids as recommended by GibcoBRL.

MIDI replication by MHV

Three hours after DNA transfection, the cells were infected with MHV-A59 at an m.o.i. of 10. Actinomycine D

(20 μg/ml) was added to the medium at 4 hr post MHV infection.

Isolation and analysis of viral RNA

Intracellular RNA was isolated from infected and transfected L cells 8 hr postinfection or transfection as described previously (Spaan *et al.,* 1981). RNAs were separated on 1% agarose/2.2 *M* formaldehyde gels (Meinkoth and Wahl, 1984), and hybridization was done in dried gels using 5′ end-labeled probes (Meinkoth and Wahl, 1984). Oligo 48 (5′GTGATTCTTCCAATTGGCCATG 3′), which binds to the 3′ end of the genome, and oligo c122 (5′ATGCCATGCCGACCCCT 3′), which binds to the region between the hepatitis delta ribozyme and the T7 terminator, were used for hybridization. Oligonucleotides were labeled using [γ-^{32}P]ATP (NEN-Dupont) and T4 polynucleotide kinase.

Electron microscopy

Cells were fixed in 1.5% glutaraldehyde in 0.1 *M* cacodylaatbuffer, pH 7.3, 300 mOsmol for 60 min at room temperature and were subsequently washed twice in phosphate-buffered Ringer solution. Postfixation was done for 30 min, 4°, in 1% OsO_4 in 0.1 *M* cacodylaatbuffer with 0.01 *M* potassium hexacyaniferrate (III) and 0.01 *M* $CaCl_2$. The cells were washed twice in Ringer solution and subsequently flat embedded in epoxy resin LX-112 and polymerized at 60°. Ultrathin sections (60 nm) were stained with uracyl acetate followed by lead hydroxide and examined with a Philips EM-410LS electron microscope at 80 kV.

Metabolic labeling of proteins and lysis of cells

Cells were metabolically labeled with 100 μCi ^{35}S-labeled methionine and cysteine (Tran^{35}S label, ICN Biomedicals) in medium lacking methionine from 4 to 8 hr posttransfection. The labeling medium was subsequently replaced with chase medium, containing four times the normal concentration of methionine and cysteine. At 12 hr posttransfection the medium was collected. Cells were lysed in RIPA buffer (150 m*M* NaCl, 1.0% NP-40, 0.5% DOC, 0.1% SDS, 50 m*M* Tris, pH 8.0) and 2 m*M* PMSF. The lysate was centrifuged at 4° for 10 min at 13,000 rpm to remove nuclei and cell debris. The medium was cleared by a 4-min centrifugation (4,000 rpm) and one-fifth volume of a 5× concentrated RIPA buffer containing 10 m*M* PMSF was added. Immunoprecipitations were performed on the supernatant using rabbit polyclonal MHV-A59 antiserum k134. After an overnight incubation at 4°, 50 μl Pansorbin cells (Calbiochem, La Jolla) and KCl to a final concentration of 0.5 *M* were added, followed by an incubation for 1 hr at 4°. After washing the samples three times in RIPA, they were boiled in Laemmli sample buffer for 2 min (Laemmli, 1970). Samples were analyzed by SDS–PAGE on 12.5% gels.

RESULTS

Coronavirus-like particles are detected in L-cells

First, we studied whether virus-like particles were produced in the vTF7.3-infected L-cells that were cotransfected with four DNA constructs encoding the known structural proteins S, M, N, and E of MHV-A59. vTF7.3-infected cells that were mock transfected or transfected with all structural proteins were fixed at 10 hr after transfection. MHV-infected L-cells were fixed at 6 hr postinfection and prepared for electron microscopy analysis.

In L-cells endogenous retrovirus type A particles containing a clear double membrane (reviewed by Kuff and Lueders, 1988) were observed in vesicles (Fig. 1). MHV virions were detected in collecting or budding vesicles in the MHV-A59-infected cells. Virions were heterogeneous in size, but could easily be distinguished from the retroviruses as the latter have a distinct morphology.

In vaccinia virus-infected cells that were not transfected, the retroviruses were also detected, together with several forms of maturing vaccinia virions (Fig. 1; Joklik and Becker, 1964).

In cells expressing the recombinant structural proteins of MHV, VLPs, that were similar in size to the MHV-A59 virions, were observed along with the retrovirus type A particles and the vaccinia virions. The virus-like particles were less electron dense than the retro- and coronavirus particles and did not have the typical retrovirus type A particle double membrane. The coronavirus VLPs were absent in cells that did not express the recombinant structural proteins of MHV (Fig. 1).

Virus-like particles are released from cells expressing the structural proteins of MHV-A59

Next, we determined whether virus-like particles were released by studying which proteins and which forms of the proteins were detected into the medium of transfected cells. MHV-A59 virions contain the M, N, E proteins and the 180- and 90-kDa cleaved forms (S1 and S2) of the spike protein (Spaan *et al.,* 1988; Yu *et al.,* 1994). However, not all of these proteins can be used as markers for virion release into the medium of infected cells. The N protein is detected in the medium independent of virion formation. The S1 subunit of the spike protein is found in the medium even when S alone is expressed in the cells, since it is not stably associated to the membrane bound S2 subunit (Sturman *et al.,* 1990). Other viral membrane proteins like the 150-kDa S precursor protein can reach the medium only on membrane fragments when cells start to lyse. These fragments, however, are cleared by centrifugation. M is the only membrane protein that is known to be retained in the *trans*-Golgi network when expressed alone (Krijnse Locker *et al.,* 1992), release of M into the medium is taken as proof for release of membraneous particles.

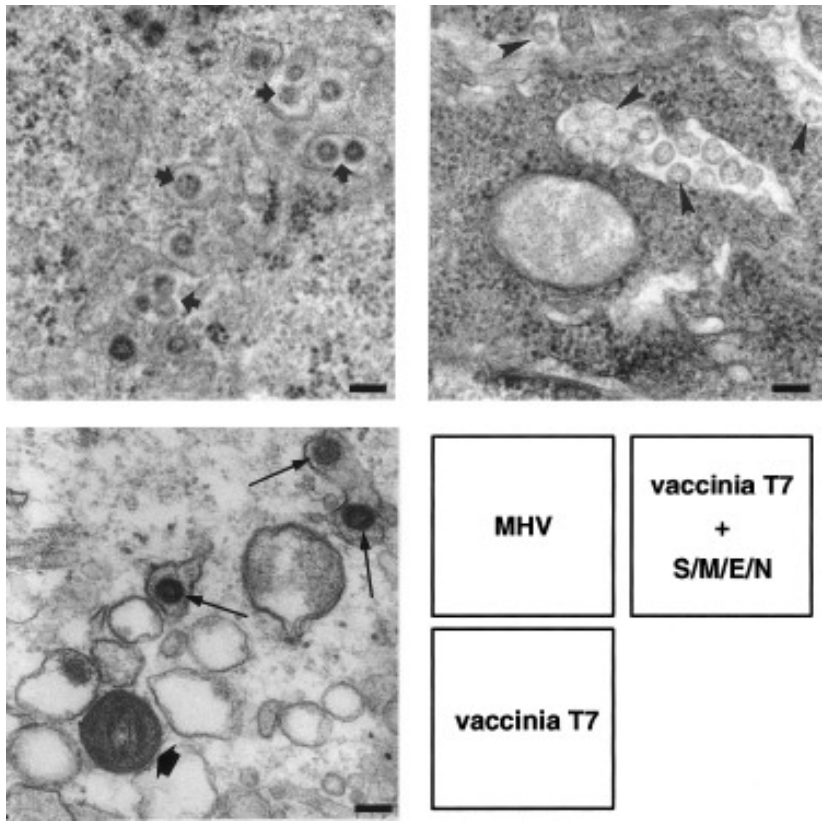

FIG. 1. Electron microscopy analysis of VLPs. L cells infected with MHV-A59 (upper left), vaccinia vTF7.3 (lower left), or vaccinia vTF7.3-infected and DNA-transfected cells (upper right). MHV-infected cells were fixed 6 hr postinfection. Vaccinia-infected and DNA-transfected cells were fixed at 10 hr postinfection. The short fat arrows denote the MHV virions. Arrowheads point to the VLPs. The long arrows indicate the retrovirions, and the large arrow points to the vaccinia virion. The bars are 200 nm.

Likewise, the 180-kDa forms of the spike protein depends on membraneous particles for release out of the cell. Thus, only detection of M or the uncleaved mature spike protein (180 kDa) in the medium can be used as marker for release of particles.

MHV-A59 structural proteins were expressed in vTF7-infected cells and labeled with [^{35}S]methionine from 4 to 8 hr posttransfection and subsequently chased for 4 hr. The medium was cleared by centrifugation to remove cells and cellular debris containing viral membrane proteins. Cell lysates and the medium from the same cells were subjected to immunoprecipitation with the polyclonal rabbit antiserum k134. With this antibody the immature 150-kDa, the mature 180-kDa, and very little of the 90-kDa spike proteins could be detected in the cell lysates, in addition to N and the five forms of M (Fig. 2A, first lane). M and 180-kDa S could also be detected in the medium of the cells, indicating that membraneous particles had been released from the transfected cells. The E protein could not be detected with this antibody. We have not yet succeeded in producing an E-specific antibody. Since the immature ER-restricted 150-kDa form of the spike protein and the M0 and M1 forms of M (Fig. 2B, first lane; Krijnse Locker *et al.,* 1992) were not detected in the medium, clearance of the medium had been successful.

To determine which proteins are required for particle release, we transfected L-cells with different combinations of three plasmids, each encoding a structural protein as indicated above the lanes in Fig. 2. When S or N were not expressed in the cells, the M protein, which is a marker for particle formation, could still be detected in the medium. However, when E was omitted, neither S180 nor M were released into the supernatant. Omission of M also resulted in the absence of S180 in the medium. These data indicated that particle release was dependent on the expression of E and M.

We next tested whether coexpression of E and M alone was sufficient for particle release (Fig. 2C). As indicated by the detection of M in the medium, particles were indeed formed and released, although less efficiently.

Assay to determine infectivity of the virus like particles

After having established that virus-like particles were produced in transfected cells and subsequently released into the medium, we next analyzed whether the VLPs

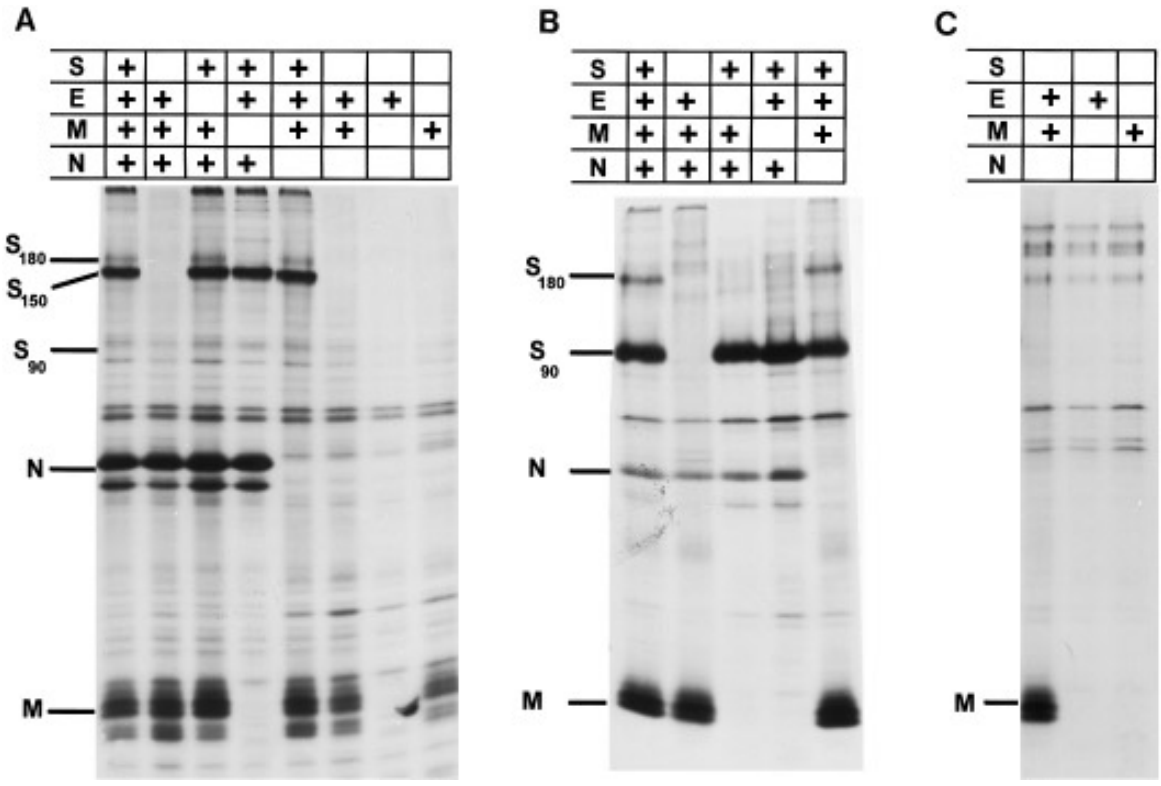

FIG. 2. Analysis of the structural proteins of MHV-A59 in transfected L cells. Cell lysates (A) and lysates of the supernatant (B and C) of the cells analyzed in A were subjected to immunoprecipitation with the polyclonal antibody k134. Which proteins were expressed in the vTF7.3-infected cells is indicated above the lanes. Release of M and 180-kDa S into the medium were used as markers for the presence of VLPs. C was exposed twice as long as B.

were able to package an MHV DI genome (Van der Most *et al.,* 1991).

Following transfection of vTF7.3-infected cells with pMIDI, encoding MIDI RNA under the control of the T7 promoter (Van der Most *et al.,* 1991), a distinct RNA band of 5.4 kb was detected only in cells that had been superinfected with MHV-A59 (Fig. 3A). However, in the absence of MHV the DI RNA could not be detected. This is most likely due to the lack of a T7 terminator sequence on pMIDI: the T7 transcripts that are produced are heterogeneous in length and cannot be detected by hybridization. Since nothing is known about possible 3′ end constraints for RNA packaging we have introduced the *cis*-acting hepatitis delta ribozyme followed by the T7 terminator sequence into the cDNA clone behind the poly(A)-tail in order to generate an RNA that has a 3′ end that resembles the 3′ end of MIDI RNA as much as possible. Only four nonviral nucleotides are present downstream of the poly(A)-tail. The structure of the resulting construct, named pMIDI-HD is shown in Fig. 3B.

When cellular RNA of vTF7-infected, pMIDI-HD DNA-transfected L-cells was isolated 8 hr after transfection, two equally abundant RNA species hybridizing to the MHV-specific 3′ end probe were detected; RNA A and RNA B (Fig. 3A). RNA A comigrates with MIDI RNA and RNA B hybridizes to a probe that is complementary to the sequence between the hepatitis delta ribozyme and the T7 terminator (data not shown). This indicated that both the ribozyme and the termination signal were active *in vivo.*

The pMIDI-HD construct was used in a simple but very sensitive protocol to study the production of infectious VLPs. vTF7-infected L cells were transfected with pMIDI-HD and four different plasmids encoding the structural proteins of MHV (M, N, S, and E), all under the control of the T7 promoter. The production of RNA-containing particles was tested by mixing helper virus MHV-A59 with the medium of the transfected cells and adding this mixture to a new monolayer of L cells. When the VLPs are infectious, they will be able to deliver the packaged MIDI-HD RNA to the cytoplasm of the cells and the DI RNA will subsequently be replicated and packaged by the coinfecting helper virus MHV-A59. The presence of MIDI-HD in the intracellular RNA is thus used as a marker for the infectivity of the particles. The experimental set-up is schematically presented in Fig. 4.

Two plates of L-cells were infected with vTF7.3. In one plate, pMIDI-HD was cotransfected with the plasmids encoding the structural proteins M, S, E, and N. In the other, pMIDI-HD was cotransfected with pUC20 DNA. The amount of DNA for both plates was similar. The medium of the transfected cells was harvested 12 hr

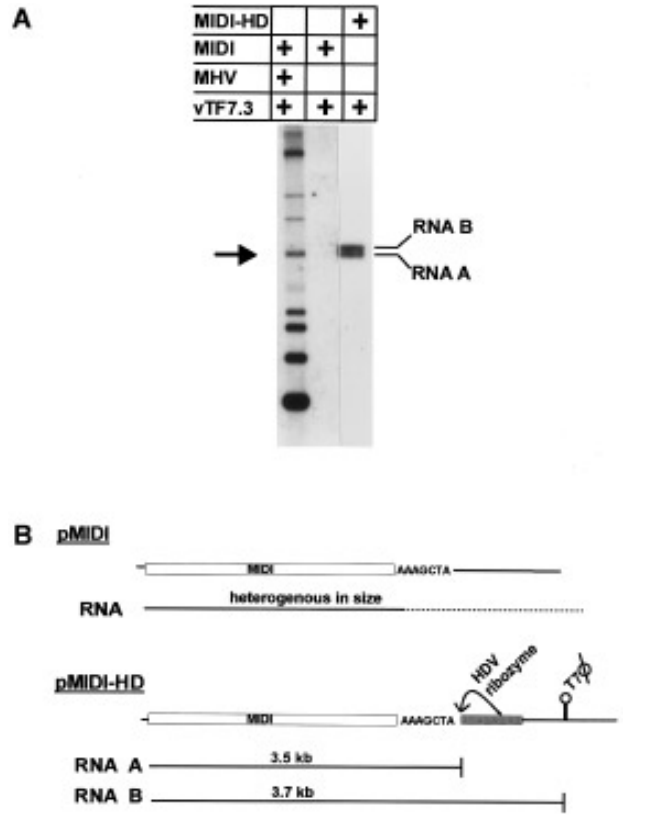

FIG. 3. Production of DI RNA in transfected cells. (A) Hybridization analysis of intracellular RNAs. Intracellular RNAs were isolated from cells that were infected with vaccinia vTF7.3, transfected with pMIDI or pMIDI-HD DNA, and superinfected with MHV-A59 as indicated above the lanes. The arrow points to the DI-RNA. RNA A and RNA B are explained in B. (B) Schematic presentation of the RNAs that are produced by the T7 polymerase in vTF7.3-infected cells after DNA transfection of pMIDI-HD or pMIDI. The predicted size of the RNAs is indicated above the lines.

posttransfection and the latter was mixed with helper virus MHV-A59 (m.o.i. of 10). A fresh monolayer of L-cells was infected with this mixture and serial undiluted passages were performed. Intracellular RNA of P0 cells and of the passages was analyzed in a hybridization assay. Figure 5 shows that MIDI-HD RNA was produced abundantly in transfected cells. After passaging of the material derived from the cells that were transfected with pMIDI-HD and the structural genes, MIDI-HD RNA could be detected in the intracellular RNA of P2, P3, and P4 cells (Fig. 5A). However, no MIDI-HD RNA was observed when pMIDI-HD was cotransfected with pUC20 and subsequently passaged in the presence of helper virus (Fig. 5B). This experiment showed that the virus-like particles can package the MIDI-HD RNA and that they are infectious. To confirm that the DI was indeed MIDI, we hybridized the gels of two independent experiments with a MIDI-specific probe. The oligo bound to the ORF1a–ORF1b junction sequence of MIDI and gave a specific signal in both cases (data not shown). Endogenous DIs do not hybridize to this oligo (data not shown). Since

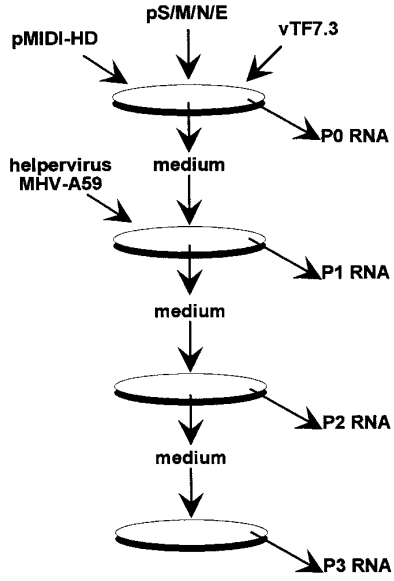

FIG. 4. Schematic representation of the VLP infectivity assay. vTF7.3-infected L cells are transfected with the plasmids encoding the structural proteins of MHV-A59 (M, N, S, and E) and pMIDI-HD. The culture medium of these cells is mixed with helper virus MHV-A59, before infecting P1 cells. Several undiluted passages are performed. The nomenclature used for intracellular RNAs is explained.

both E and M were required for VLP release (Fig. 2), we reasoned that the DI RNA would not be transferred from the transfected cells to the P1 cells when either of these proteins was omitted. Intracellular RNA of the transfected and P1-, P2-, and P3-infected cells was isolated and analyzed by hybridization (Fig. 6). As before, MIDI-HD RNA was passaged when all four structural proteins (S, M, E, and N) were expressed in P0 cells. However, when plasmids encoding either E or M were not included, MIDI-HD RNA could not be detected in the intracellular RNA after passaging with helper virus (Fig. 6). An endog-

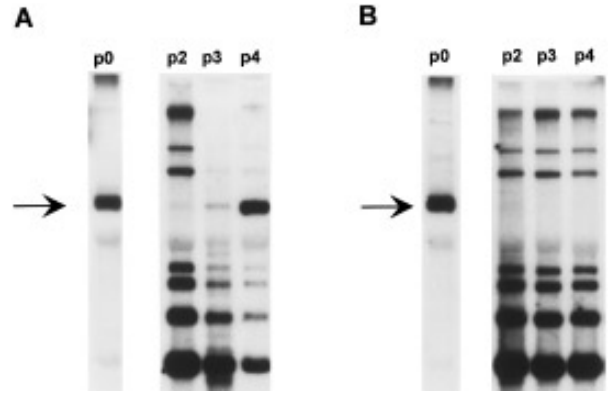

FIG. 5. Infectivity of DI-VLPs. (A) DNA:S/M/E/N and pMIDI-HD. (B) DNA:pUC and pMIDI-HD. L cells were infected with vTF7.3 and subsequently transfected with different plasmids as indicated. The medium was mixed with helper virus MHV-A59; the mixture was used to infect fresh cells. Several undiluted passages were performed. Intracellular RNA from P0, P2, and P3 cells were isolated and analyzed in a hybridization analysis. The arrow points to the MIDI-HD RNA.

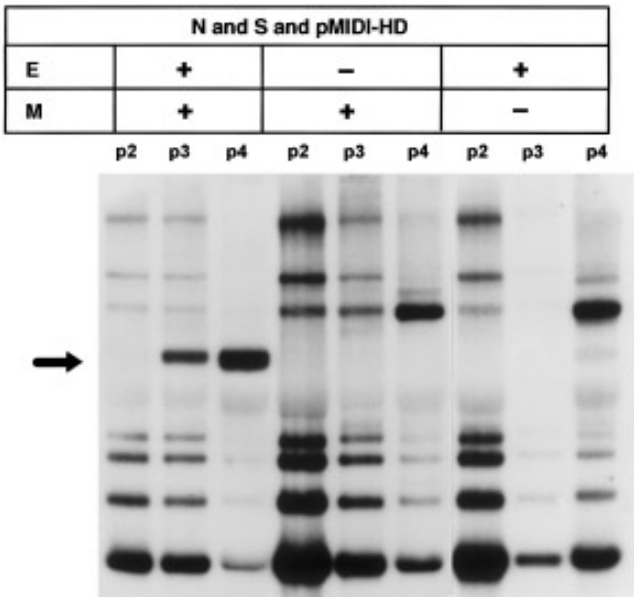

FIG. 6. Dependence of VLP infectivity on structural proteins E and M. L cells were infected with vTF7.3 and subsequently transfected with different plasmids as indicated. The medium was mixed with helper virus MHV-A59; the mixture was used to infect fresh cells. Several undiluted passages were performed. Intracellular RNA from P2, P3, and P4 cells was isolated and analyzed in a hybridization analysis. Above the lanes is indicated whether E or M expressing plasmids were present in P0 cells together with N, S, and pMIDI. The arrow points to the MIDI-HD RNA.

enous DI comigrating with RNA3 that is never observed when MIDI is present in the cells was observed in these RNA samples (Van der Most *et al.,* 1992). Expression of endogenous DI is variable, but it is never seen before passage 4. Presumably, expression of MIDI interferes with appearance of the endogenous DI, as seen in the P4 lane of the E+/M+ experiment in Fig. 6. It is therefore unlikely that the endogenous DI would prevent expression of MIDI in the E−/M+ and E+/M− experiments. Instead, the presence of the endogenous DI would be an extra indication of the absence of MIDI in the controls.

DISCUSSION

In this paper we describe the production and infectivity of coronavirus-like particles, by coexpressing the structural proteins of MHV-A59 and a DI genome. Infectivity of the VLPs was demonstrated by transfer of a DI genome to fresh cells. We present an assay that might be a powerful tool to study packaging, assembly, and budding by expressing mutated structural proteins and RNAs. Questions concerning the protein–protein and RNA–protein interactions that are required for these processes can now be addressed.

When we expressed the structural proteins of MHV-A59 in L cells with the use of the vaccinia vTF7.3 expression system, we obtained morphological proof for the production of VLPs by electron microscopy (Fig. 1).

Although we have not shown the expression of E by immunoprecipitation using an E-specific antibody, our data clearly suggest that not only M but also the expression of E is required for the production of VLPs. More importantly, coexpression of E and M appeared to be sufficient for the release of virus-like particles. Therefore, E and M must be important factors in virus budding.

Not much is known yet about the small membrane protein E, except that the acylated protein is found in virions in very low amounts (Yu *et al.,* 1994) and that it is expressed at the cell surface (Tung *et al.,* 1992). Other enveloped viruses, like Influenzavirus, alphaviruses, and pestiviruses, also have small membrane proteins that play an important role in the biogenesis of infectious progeny (Pinto *et al.,* 1992; Allison *et al.,* 1995; Loewy *et al.,* 1995). The E protein might have similar functions. Possibly, an interaction between E and M induces the budding process. Both proteins are modified in the intermediate compartment; the M protein acquires GalNac (Tooze *et al.,* 1988; Krijnse Locker *et al.,* 1992) and the E protein becomes acylated (Yu *et al.,* 1994). Whether these modifications are required for the interactions per se, or for budding itself, remains to be determined.

From the present data we cannot exclude that E in the absence of M can induce virus budding (Fig. 2B). The only markers for VLP release are S180 and M and if insertion of S into particles is dependent on an interaction with M (Opstelten *et al.,* 1995), the absence of the spike protein in the medium would not exclude the formation and release of particles from cells expressing solely the E protein.

A function of M in coronavirus budding has been proposed before. When hybridomas producing monoclonal antibodies to the M protein were infected with MHV-A59, no virions were produced (Holmes *et al.,* 1987). In MHV-A59-infected cells treated with tunicamycin and in hybridomas expressing anti-S antibodies, S-deficient virions are produced (Holmes *et al.,* 1981; Rottier *et al.,* 1981; Holmes *et al.,* 1987), indicating that S is not required for virion release from infected cells, but M is. These findings are consistent with the data presented in this paper, in which omission of S does not prevent release of particles into the medium, whereas M is absolutely required. The interaction between M and N (Sturman *et al.,* 1980, Anderson and Wong, 1993) was thought to be important during the budding process in the intermediate compartment. We show here that the nucleocapsid–M interaction is not a prerequisite for budding, since even in the absence of RNA and N, virus-like particles are released into the medium (Fig. 2B). In this aspect, the budding mechanism of MHV-A59 is distinct from that of the alphaviruses where nucleocapsid–envelope protein interactions are the driving force for budding (Suomalainen *et al.,* 1992; Lopez *et al.,* 1994; Strauss and Strauss, 1994).

The viral RNA itself, or its replication, is not directly involved in the budding process, since particles were

released in the absence of RNA. However, in our system, packaging of the DI RNA is not very efficient, since it can only be detected after two passages (Fig. 5). One obvious reason for the inefficiency might be that very few cells were transfected with all five plasmids and which is a prerequisite for the production of infectious VLPs. Since MIDI can be efficiently passaged by MHV-A59 (Van der Most *et al.,* 1991), inefficient packaging into the VLPs is unlikely to be due to defective signals on the DI genome. A more likely alternative explanation for the inefficient packaging is that replication, which does not occur in the transfected cells, might be required for efficient packaging. However, we cannot exclude the possibility that the four additional nonviral nucleotides at the 3′ end downstream of the poly(A)-tail have a negative effect on packaging. Another reason for inefficient packaging might be that RNAs that are produced in the vaccinia expression system are poorly capped (Fuerst and Moss, 1989), whereas genomic RNA and DI RNA of MHV-A59 is. Capping may play a role in packaging. Although packaging was not very efficient, our data show for the first time that DIs can be packaged into the virions in the absence of genomic RNA of MHV-A59.

Binding of the spike protein to the receptor on the host cell is the first step of the infectious cycle (Collins *et al.,* 1982). By inserting mutant spike proteins into the VLPs, the effect of the mutations on infectivity can be studied. When chimeric receptor binding proteins containing the transmembrane region and cytoplasmic tail of MHV-A59 are introduced into the VLPs, they can be targeted to nonmurine cells. The final goal would be to package RNAs unrelated to MHV-A59 into the VLPs and then target the VLPs to specific tissues.

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