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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., 1994; 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).
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 NheI 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 Smal and XbaI 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 NheI 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 BamHI MHV-S containing fragment was cloned into the BamHI 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 oligos c093 (containing an NcoI site at the AUG codon of E: 5’CATGCCATGGCCTTTAATTTATTCCTTAC3’ and c094 (containing the stopcodon and an XbaI site downstream of it: 5’CTAGTCAGATTAGATATCCAC3’). The amplified fragment was isolated from gel, digested with Ncol and Xbal, and inserted into the Ncol–Xbal-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°C, 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 x 10⁶) 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 days after DNA transfection, the cells were infected with MHV-A59 at an m.o.i. of 10. Actinomycine D
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′ GTGATTTCTCCAATGGCCATG 3′), which binds to the 3′ end of the genome, and oligo c122 (5′ ATGCCCATGCCGACCCCT 3′), which binds to the region between the hepatitis delta ribozyme and the T7 terminator, were used for hybridization. Oligonucleotides were labeled using [γ-32P]dATP (NEN-Dupont) and T4 polynucleotide kinase.

Electron microscopy

Cells were fixed and embedded in dry gelatin/epoxy resin (Lueders, 1988) and polymerized at 60°C. Ultrathin sections (60 nm) were examined with a Philips EM-410LS electron microscope at 80 kV.

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 independently 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 membranous particles.
Likewise, the 180-kDa forms of the spike protein depend 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 a marker for release of particles.

MHV-A59 structural proteins were expressed in vTF7-infected cells and labeled with [35S]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
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 coinfected 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 setup 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
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-
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 viron 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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