Assembled Coronavirus from Complementation of Two Defective Interfering RNAs

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In the presence of an RNA⁻ temperature-sensitive (*ts*) mutant helper virus, two coronavirus mouse hepatitis virus (MHV) defective interfering (DI) RNAs complemented each other, resulting in the assembly of MHV particles; we used this ability to complement as a means to study coronavirus assembly. One of the two DI RNAs was DIssA, a naturally occurring self-replicating DI RNA encoding N protein and the gene 1 proteins that encode RNA polymerase function; DIssA supports the replication and transcription of other non-self-replicating DI RNAs. The other DI was a genetically engineered DI RNA that encoded sM and M proteins. Coinfection of these two DIs at the nonpermissive temperature for the *ts* helper virus resulted in replication and transcription of both DI RNAs but not in synthesis of the helper virus RNAs. MHV particles containing DI RNAs, N protein, and M protein, all of which were exclusively derived from the two DI RNAs, were released from the coinfected cells; the amount of sM protein was below the limits of detection. Analyses of DI RNAs with mutations in the two envelope protein genes demonstrated that M and sM proteins appeared to be required for assembly and release of MHV particles that contained DI RNAs and N protein, while S protein was not required for assembly and release of MHV particles.

Coronavirus is a large enveloped virus with a single-stranded, positive-sense RNA genome. Coronaviruses generally have three envelope proteins, M, S, and sM. M protein, a triple-spanning transmembrane protein (1), is the most abundant glycoprotein. Coronavirus S protein, which forms characteristic peplomers, binds to the coronavirus-specific receptor to initiate infection and induces cell fusion (6, 9, 40). A minute amount of sM protein associates with the coronavirus envelope (10, 23, 42, 48). Some coronaviruses contain a fourth envelope glycoprotein, HE protein (36), which is not essential for coronavirus replication (36).

Recently, Risco et al. showed that within its envelope, the coronavirus transmissible gastroenteritis virus (TGEV) contains an internal core that harbors helical nucleocapsid (ribonucleoprotein consisting of viral RNA and N protein) (33). The authors claimed that the shell of the TGEV core structure that encloses the ribonucleoprotein consists of mostly M protein and a lesser amount of N protein (33). The core appears to be an icosahedral structure that is spatially separated from the lipid envelope that surrounds it. The internal core structure is probably a universal attribute of coronaviruses, because electron microscopy also reveals an internal core structure in mouse hepatitis virus (MHV) (33).

MHV, a prototype coronavirus, contains a 31-kb genomic RNA that encodes seven to eight genes (18, 32). MHV genes are expressed through a genome-sized virus-specific mRNA and six or seven species of virus-specific subgenomic mRNAs with a 3′-coterminal nested set structure (17, 20). These mRNAs are numbered 1 to 7, in order of decreasing size (17, 20), and have a common leader sequence of approximately 72 to 77 nucleotides at the 5′ end (16, 38). Most of the MHV mRNAs are functionally monocistronic; except for the sM protein, which is translated from the second open reading frame (ORF) (gene 5b) of mRNA 5 (5, 19, 37), the 5′-most

ORF on each mRNA is used for translation. The protein product from the 5′-most ORF (gene 5a) in mRNA 5 is not identified in MHV-infected cells. The 5′-most gene, gene 1, is 22 kb and probably encodes proteins for viral RNA synthesis, including RNA polymerase, proteinases, and helicase (18). Genes 3, 6, and 7 encode structural proteins S, M, and N, respectively (15). Other MHV genes, including genes 2, 2-1 (the gene for HE protein), 4, and 5a are not essential for MHV replication, at least in tissue culture (35, 47). The expression of genes 1 and 7 is sufficient for MHV RNA synthesis (13).

MHV assembly takes place at the budding compartment, the smooth membrane of the intermediate compartment between the endoplasmic reticulum and the Golgi complex (14, 41). M protein itself does not determine the budding site; when M protein is expressed in the absence of other viral proteins, it migrates beyond the budding compartment and localizes in the late Golgi complex (14). This indicates that an unidentified viral factor(s) restricts the migration of M protein at the budding compartment. S protein interacts with M protein in the absence of other MHV proteins, and this interaction is probably important for incorporation of S protein into the virion (31). Tunicamycin-treated MHV-infected cells release MHV particles lacking S protein (12, 34), indicating that S protein probably is not necessary for coronavirus assembly. Klumperman et al. (14), however, discussed the possibility that MHV particles released from tunicamycin-treated cells may contain some part of the membrane anchors and the cytoplasmic tails of S proteins; in the presence of tunicamycin, MHV particles containing S protein may be produced initially, but later S proteins lacking N-linked oligosaccharides that directed the protein's conformation may be subsequently degraded. All coronaviruses sequenced so far maintain the gene encoding sM protein (10, 23, 37, 42), indicating that this protein may be required for replication. The sM protein is not necessary for MHV RNA synthesis (13).

Coexpression of cloned MHV envelope proteins by using T7 polymerase-expressing recombinant vaccinia virus showed that both M and sM proteins, but not S protein, are needed for MHV envelope formation. Vennema et al. directly demon-

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strated that virus-like particles (VLPs) are assembled and re-leased from MHV-uninfected cells that express both sM and M proteins (44). VLPs produced from the cells coexpressing N protein and other MHV envelope proteins do not contain N protein (44), indicating that neither the spherical core shell nor the helical nucleocapsid is incorporated into VLPs. Essentially the same procedure was used by Bos et al. (3) to explore the requirement of MHV envelope proteins for MHV assembly. Their data indirectly demonstrated that both sM protein and M protein, but not S protein, are necessary for MHV assembly.

The mechanism of MHV RNA packaging into MHV parti-cles is poorly understood. Analyses of MHV defective inter-fering (DI) RNAs have identified a packaging signal (8, 43) that is necessary for the packaging of MHV DI RNA into virus particles. The packaging signal is sufficient for RNA packaging into MHV particles (45), yet how the packaging signal facili-tates specific packaging of viral RNA remains to be studied. Bos et al. (3) presented indirect evidence that infectious MHV DI particles can be produced by coexpressing a cloned MHV DI RNA, N, sM, M, and S proteins, suggesting that expression of MHV structural proteins and DI RNA are sufficient for MHV DI particle formation and DI RNA packaging.

The MHV envelope proteins are most likely involved in the mechanism of membrane envelopment of the viral core. Gra-dient-purified MHV nucleocapsid (ribonucleoprotein), but not free N protein, can interact with M protein in vitro (39), so M protein probably interacts specifically with nucleocapsid. This interaction may be important for incorporation of virus nu-cleocapsid into virus particles, yet a definite requirement of M protein for MHV nucleocapsid packaging has not been estab-lished. Whether S protein is required for envelopment of the viral core is not known. MHV particles released from tunica-mycin-treated cells lack S protein but contain N protein (12, 34), suggesting that the core, the core shell, or helical nucleo-capsid is present in these MHV particles. Some part of the S protein's membrane anchors and cytoplasmic tails may be present in MHV particles that are released from the tunica-mycin-treated cells (14); therefore, S protein may be involved in packaging of the viral core.

We have been interested in a self-replicating MHV DI RNA, DIssA (13, 24, 27), for studying the mechanism of coro-navirus assembly. DIssA contains intact genes 1 and 7 and most likely contains gene 2 but lacks genes 3 to 6 and a part of gene 2-1 (Fig. 1A) (13). Coinfection of DIssA DI particles containing the JHM strain of MHV (MHV-JHM) with an RNA⁻ MHV-A59 temperature-sensitive (ts) mutant, LA16, and subsequent passage of the produced virus sample at the permissive temperature (32.5°C) eliminates virtually all MHV-JHM (13). Helper viruses were replaced by LA16 while DIssA DI particles were maintained in the passaged virus sample (13). DIssA and DIssA-derived mRNA 7 but not helper virus mRNAs are synthesized in the cells infected with the passaged virus sample at LA16's nonpermissive temperature (39.5°C) (13). Importantly, DIssA supports replication and transcrip-tion of another DI RNA (13).

In the present study, we describe the establishment of a novel complementary DI system that enabled us to study coro-navirus assembly. We made two DI particle preparations, both of which used LA16 as a helper virus. One DI particle prepa-ration contained an engineered DI RNA encoding structural envelope proteins, and the other preparation contained DIssA DI particles. After coinfection of both DI particles at 39.5°C, DIssA supported replication and transcription of the other DI RNA, which in turn provided envelope proteins for the coin-fected cells. All the necessary proteins for MHV replication accumulated, and MHV particles were produced. Using this

system, we determined which MHV envelope structural pro-teins are essential for MHV DI RNA packaging and MHV particle formation.

MATERIALS AND METHODS

Viruses and cells. The MHV-A59 *ts* mutant, LA16 (2, 13), plaque-cloned MHV-JHM, and a 19-fold undiluted serially passaged MHV-JHM (JHM19th) viral preparation (24) were used. Mouse DBT cells were used for RNA trans-fection and propagation of viruses (11). MHV was grown in 88% Eagle's mini-mum essential medium (pH 6.5)–10% tryptose phosphate broth–2% heat-inac-tivated fetal bovine serum.

Plasmid construction. The 1.6-kb reverse transcription (RT)-PCR product that corresponds to the 3′ 0.1-kb region of gene 4, the entire gene 5 (both 5a and 5b), gene 6, and the 5′ 0.18-kb region of gene 7 was synthesized from MHV-JHM genomic RNA. This PCR product was inserted into the *Eco*RV site of DIssF-derived cDNA clone MC136-1 (25), yielding the parental clone, JW2 (Fig. 1B). JW2ΔsM and JW2ΔM were constructed by a recombinant PCR procedure using JW2 as a template. JW2ΔsM contained an insertion of a termination codon and a 2-nucleotide frameshift mutation at the 5′ region of gene 5b (Fig. 1C). JW2ΔM contained an insertion of a termination codon and a 2-nucleotide frameshift mutation at the 5′ end of gene 6 (Fig. 1C). JW2ΔsM and JW2ΔM had disrupted genes 5b and gene 6, respectively. JW2ΔsM+M was constructed by inserting the 5b region of JW2ΔsM into the corresponding region of JW2ΔM; JW2ΔsM+M had both genes 5b and 6 disrupted. For all constructs, we sequenced the inserts that were derived from PCR products to confirm the presence of specific muta-tions and the absence of extraneous mutations.

RNA transcription and transfection. Plasmids were linearized by *Xma*I diges-tion, transcribed in vitro with T7 RNA polymerase (26), and transfected by lipofection, as described previously (7, 25).

Purification of viruses. The supernatant from virus-infected cells was har-vested at 12 h postinfection (hpi) and briefly centrifuged to remove cell debris. Released viruses were partially purified by ultracentrifugation on a discontinuous sucrose gradient consisting of 20 and 60% sucrose (27). After centrifugation at 26,000 rpm for 3 h at 4°C in a Beckman SW28 rotor, virus particles at the interface between the 20 and 60% sucrose were collected and in some cases were further purified by the 20 to 60% continuous sucrose gradient centrifugation at 26,000 rpm for 18 to 24 h at 4°C. Partially purified viruses or purified viruses were pelleted through a 20% sucrose cushion by centrifugation at 38,000 rpm for 3 h at 4°C on a Beckman SW40 rotor.

Preparation of virus-specific RNA and Northern (RNA) blotting. Virus-spe-cific RNAs were extracted from virus-infected cells or purified virus particles as described previously (29). RNAs were denatured and electrophoresed through a 1% agarose gel containing formaldehyde and transferred onto nylon filters (25). Northern blot analysis was performed with a ³²P-labeled random-primed probe as described previously (8).

Labeling of intracellular and virion proteins, immunoprecipitation, and SDS-PAGE. For labeling intracellular virus-specific proteins, actinomycin D (2.5 μg/ml) was added to virus-infected cells at 3 hpi and virus-specific proteins were labeled with [³⁵S]methionine at 7.5 hpi for 20 min or for 2 h from 6.5 to 8.5 hpi and cell lysates were prepared as described previously (25). For labeling of virion proteins, [³⁵S]methionine was added to virus-infected cells at 7.5 hpi and culture fluids were collected at 12 hpi (25). Virus proteins in the partially purified virion and intracellular virus-specific proteins were immunoprecipitated with anti-MHV-JHM serum (24). Immunoprecipitated proteins were incubated at 37°C for 1 h in sample buffer to prevent M protein aggregation (39) and analyzed by SDS-PAGE.

Analysis of sM protein. To examine the synthesis of sM protein from engi-neered DI RNAs, MHV-specific cDNA was first synthesized by incubating in-tracellular RNA and oligonucleotide 1111 (5′-GAGCCAGAAGGCTGCGAT-3′), which binds at 0.39 kb from the 3′ region of MHV mRNA 7, as described previously (28). MHV-specific cDNA was then incubated with oligonucleotide 1111 and oligonucleotide 10217 (5′-TAATACGACTCACTATAGGGCATCTG GAGAGCCACGCAGCTCG-3′), which contains the T7 promoter sequence at the 5′ region and hybridizes with negative-strand MHV genomic RNA at the 3′ end of gene 5a, in PCR buffer (13) at 94°C for 30 s, 42°C for 45 s, and 72°C for 100 s for 25 cycles. The 1.3 kb DI RNA-specific RT-PCR products were approx-imately 1 kb smaller than the helper virus-derived RT-PCR products. Capped transcripts were synthesized in vitro from the gel-purified DI-specific RT-PCR products (26), and proteins were synthesized in vitro with wheat germ extract (Promega). Protein products were immunoprecipitated by using anti-sM protein antibody (19, 48) and analyzed by SDS-PAGE.

RESULTS

Strategy for establishment of the complementary DI system. We aimed to establish a complementary DI system in which two DI RNAs complemented each other to produce MHV particles. In this system, two MHV preparations, each of which contained a different type of DI particle, were used. One MHV
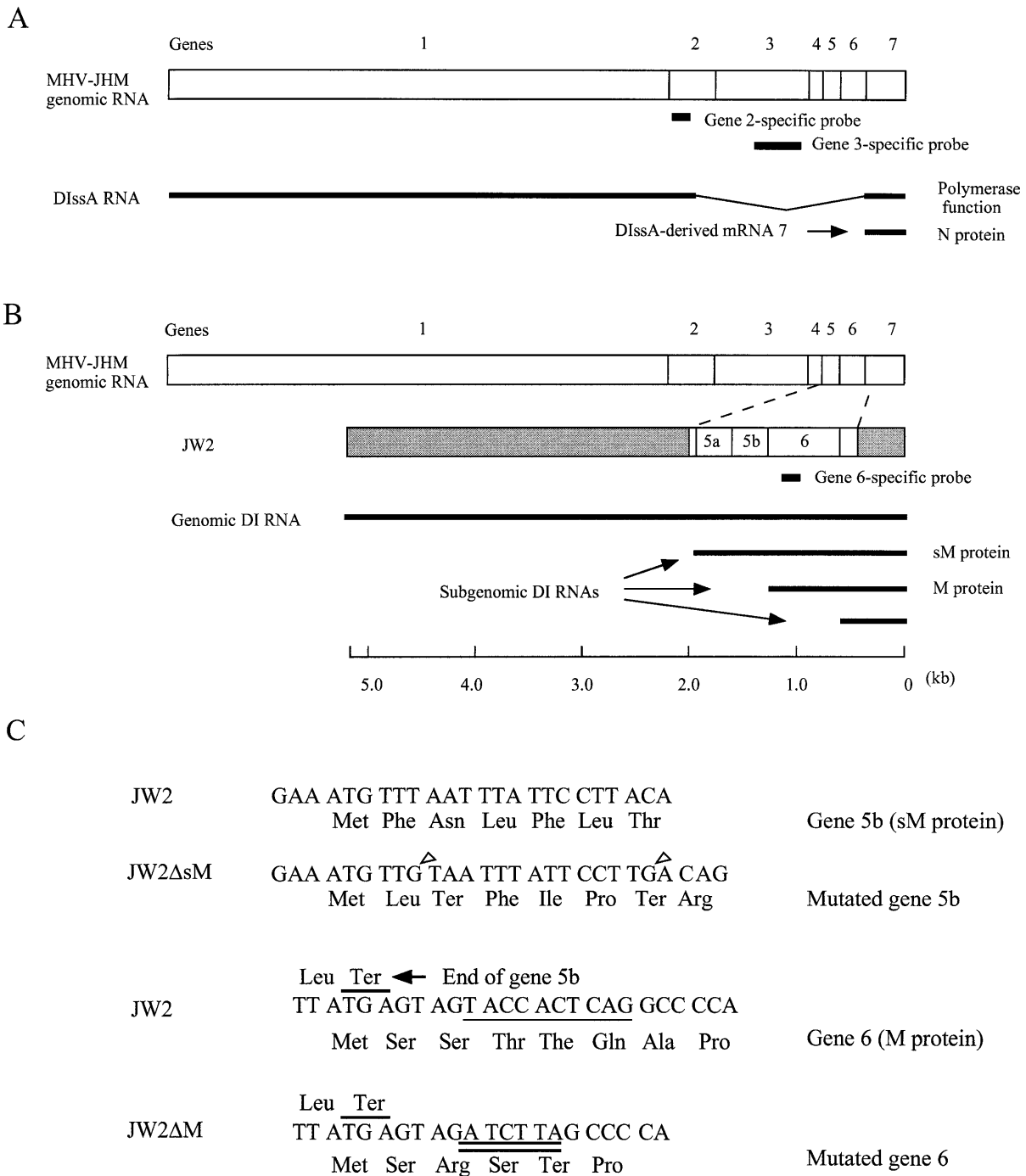
A

B

C

JW2	GAA ATG TTT AAT TTA TTC CTT ACA Met Phe Asn Leu Phe Leu Thr	Gene 5b (sM protein)
JW2ΔsM	GAA ATG TTG TAA TTT ATT CCT TGA CAG Met Leu Ter Phe Ile Pro Ter Arg	Mutated gene 5b
JW2	Leu Ter ◄— End of gene 5b TT ATG AGT AGT ACC ACT CAG GCC CCA Met Ser Ser Thr The Gln Ala Pro	Gene 6 (M protein)
JW2ΔM	Leu Ter TT ATG AGT AGA TCT TAG CCC CA Met Ser Arg Ser Ter Pro	Mutated gene 6

FIG. 1. Diagrams of the structures of DIssA (A), JW2 (B), and the nucleotide and deduced amino acid sequences of the 5′ regions of gene 5b and gene 6 (C). (A) Comparison of the structure of DIssA RNA and wild-type MHV-JHM genomic RNA. The thin line represents the deleted region in DIssA (13). The two bold lines represent two major mRNAs, DIssA, and N protein-encoding mRNA 7, which are synthesized in DIssA-replicating cells (13). The locations of a gene 2-specific probe and a gene 3-specific probe used for Northern blot analysis (see below) are also shown. (B) Structure of JW2 and its expected subgenomic DI RNAs. The location of the inserted 1.6 kb-long sequence in JW2 is shown. The shaded regions represent the parental MC136-1 sequence, which is almost identical to DIssF (25). The largest subgenomic DI RNA and the medium-sized subgenomic DI RNA encode sM protein and M protein, respectively. The location of a gene 6-specific probe used for Northern blot analysis (Fig. 2A and 3A) is also shown. (C) Mutations present in JW2-derived mutants. The two upper sequences and the two lower sequences represent the 5′ regions of gene 5b and gene 6, respectively. The two G nucleotide insertions in JW2ΔsM are shown by open arrowheads. The underlined JW2 gene 6 nucleotide sequence was mutated into the sequence shown by the double underlines in JW2ΔM. JW2ΔsM+M contained both the mutations introduced in JW2ΔsM and JW2ΔM.

sample contained the self-replicating DIssA DI particles (13, 24, 27) that provide polymerase function and N protein (pol/N supplier) (Fig. 1A). The other sample contained DI particles, each carrying an engineered DI RNA which subgenomically

expresses virus envelope proteins (env supplier). To exclude infectious helper MHV in the complementary DI system, an RNA⁻ MHV-A59 *ts* mutant, LA16 (2, 13), was used as a helper virus for both the pol/N supplier and the env supplier.

The pol/N supplier was prepared after coinfection of LA16 and JHM19th, which is an MHV-JHM sample containing DIssA DI particles, at 32.5°C and passaging them three times at 32.5°C (13); most of the MHV-JHM virions were easily removed and replaced by LA16, whereas the DIssA DI particles were maintained in this preparation (13). In agreement with a previous study (13), the ratio of viral infectivity in the pol/N supplier preparations at 39.5°C to that at 32.5°C was on average 8.0×10^{-3}. Also, DIssA RNA and mRNA 7 but not DIssE RNA, which is present in JHM19th, were efficiently synthesized in the pol/N supplier-infected cells at 39.5°C (data not shown). For the preparation of the env supplier, in vitro-synthesized DI RNA transcripts were transfected into LA16-infected cells and cultures were incubated at 32.5°C. Released viruses were passaged once at 32.5°C and used as the env supplier. For complementation experiments, cells were coinfected with the pol/N supplier and the env supplier, and incubated at 39.5°C. We expected that both DI RNAs but not LA16 would replicate, transcribe, and express all of the MHV proteins necessary for MHV replication. We assumed that MHV would be released from coinfected cells.

Preparation and characterization of the env suppliers. We constructed a DI cDNA that provided virus envelope proteins in the complementary DI system. Initially, DI RNA containing a region corresponding to all of MHV-JHM genes 3, 4, 5, and 6 was examined for its ability to replicate in the MHV-infected cells, but this DI RNA failed to replicate (data not shown). Since MHV particles lacking S protein are released from tunicamycin-treated cells (12, 34), we assumed that S protein is not necessary for MHV assembly. Therefore we constructed another DI cDNA clone, JW2, that contained genes 5 and 6 and the 5′ region of gene 7 (Fig. 1B). To examine replication and transcription of JW2 DI RNA, in vitro-synthesized JW2 RNA was transfected into LA16-infected cells and incubated for 20 h at 32.5°C. The supernatant was collected and then passaged once at 32.5°C to prepare passage 1 virus sample. This sample was named JW2 env supplier. The ratio of virus infectivity at 39.5°C to that at 32.5°C of the JW2 env supplier was 4.0×10^{-4}. DBT cells were infected with the JW2 env supplier and incubated at 32.5 or 39.5°C. Intracellular RNA was extracted at 9 hpi, and JW2 RNA synthesis was examined by Northern blot analysis (Fig. 2A) using a gene 6-specific probe (Fig. 1B). JW2 replicated and transcribed two subgenomic DI RNAs, each of which encoded genes 5 and 6 at 32.5°C but not at 39.5°C. As expected, Northern blot analysis using a probe that hybridized at the 3′ end of the MHV genome revealed synthesis of the smallest subgenomic DI RNA encoding the 5′ region of gene 7 (see Fig. 1B) at 32.5°C but not at 39.5°C (data not shown).

To study the role of sM protein and M protein in MHV assembly, we constructed three JW2-derived mutant DI cDNAs. JW2ΔsM and JW2ΔM had a mutation at the beginning of gene 5b and gene 6, respectively (Fig. 1C); the ORF of gene 5b and the ORF of gene 6 were disrupted in JW2ΔsM and JW2ΔM, respectively. JW2ΔsM+M had both mutations which are present in JW2ΔsM and JW2ΔM; accordingly, JW2ΔsM+M had disrupted ORFs of genes 5b and 6. RNA transcripts were synthesized in vitro from these clones and transfected into LA16-infected cells. Passage 1 virus samples from cells transfected with JW2ΔM, JW2ΔsM, and JW2ΔsM+M were named JW2ΔM env supplier, JW2ΔsM env supplier, and JW2ΔsM+M env supplier, respectively. Northern blot analysis of intracellular RNA species in the passage 1 virus-infected cells showed that all three mutant DI RNAs replicated and transcribed subgenomic DI RNAs with efficiencies similar to those of JW2 (Fig. 2A). DI RNAs were synthesized at 32.5°C but not at
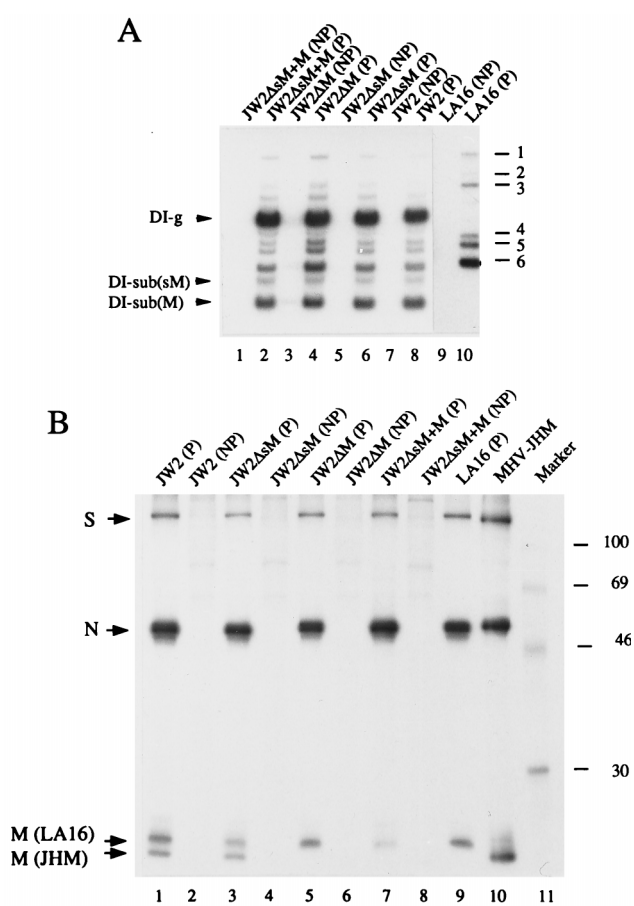

FIG. 2. Virus-specific RNA synthesis (A) and protein synthesis (B) in the cells infected with env suppliers. (A) RNA replication and transcription of JW2 and its mutants. DBT cells were infected with env suppliers at 39.5°C (NP) or 32.5°C (P). Intracellular RNAs were extracted at 9 hpi and analyzed by Northern blot analysis using a gene 6-specific probe (Fig. 1B). The numbers to the right of the gel indicate LA16-specific mRNAs. DI-g, DI-sub(sM), and DI-sub(M) represent genomic DI RNA, sM protein-encoding subgenomic DI RNA, and M protein-encoding subgenomic DI RNA, respectively. (B) Analysis of the virus-specific proteins in env supplier-infected cells. DBT cells were infected with env suppliers at 39.5°C (NP) or 32.5°C (P). MHV-JHM-infected cells (lane 10) and LA16-infected cells (lane 9) were incubated at 37 and 32.5°C, respectively. At 7.5 hpi, cultures were pulse-labeled with [^{35}S]methionine, and cytoplasmic lysates were prepared. Virus-specific proteins were immunoprecipitated with anti-MHV-JHM serum and electrophoresed on SDS–10% polyacrylamide gels. Positions of MHV S, N, and M proteins are shown on the left. M (LA16) and M (JHM) represent LA16 M protein and MHV-JHM M protein, respectively. The lane labeled Marker contains a ^{14}C-labeled protein molecular mass marker (in kilodaltons).

39.5°C. We also found that JW2 and its mutants replicated with similar efficiency in the DI RNA-transfected, LA16-infected cells at 32.5°C (data not shown).

To know whether M protein encoded by subgenomic DI RNA was translated in DI RNA-replicating cells, infected DBT cells were labeled for 20 min at 7.5 hpi with [^{35}S]methionine and cell extracts were prepared. MHV-specific proteins were immunoprecipitated with an anti-MHV serum (24) and analyzed by SDS-PAGE (Fig. 2B). As expected, virus-specific proteins were synthesized at 32.5°C but not at 39.5°C in the cells infected with env suppliers (Fig. 2B). Two M proteins with different electrophoretic mobilities were detected in the cells infected with the JW2 env supplier or JW2ΔsM env supplier. Because MHV-JHM M protein migrated slightly faster than LA16 M protein (Fig. 2B, lanes 9 and 10), the faster-migrating

M proteins must have been translated from the M protein-encoding subgenomic DI RNA. MHV-JHM-derived M protein was not detected in the cells infected with the JW2ΔM env supplier or the JW2ΔsM+M env supplier. These results were consistent with the structure of these DI RNAs.

We examined production of intracellular sM protein by immunoprecipitation of [^{35}S]methionine-labeled sM protein with anti-sM protein antibody, and we failed to detect sM protein synthesis in the cells infected with env suppliers (data not shown). This was because only a minute amount of sM protein is synthesized (19, 48). Even if we had detected sM protein in the env supplier-infected cells, we would not have known whether the detected sM protein was derived from LA16 or from subgenomic DI RNA, because the molecular weights of the MHV-JHM sM protein and the LA16 sM protein are presumably very similar. Thus, we indirectly examined the synthesis of sM protein in env supplier-infected cells as described in Materials and Methods. The sM protein was synthesized in vitro from the RT-PCR products of the 5b gene of JW2 and JW2ΔM but not from those of JW2ΔsM and JW2ΔsM+M (data not shown). This result was consistent with the structure of DI RNAs and indicated that subgenomic DI RNA-derived sM protein was expressed only in the cells infected with the JW2 env supplier and the JW2ΔM env supplier but not the JW2ΔsM env supplier and the JW2ΔsM+M env supplier.

Characterization of intracellular RNAs and virion RNAs in the cells coinfected with the pol/N supplier and env supplier. We examined whether coinfection of the pol/N supplier and each of the env suppliers at 39.5°C produced MHV particles. To increase the infectivity of the env supplier, passage 1 virus sample was concentrated approximately 10 times by using Centriprep 100 (Amicon) and used as inoculum. DBT cells were coinfected with the pol/N supplier and each of the env suppliers at 39.5°C, and intracellular RNAs were extracted at 8 hpi. For the analysis of virion RNAs, culture fluids were collected at 12 hpi and virus particles were partially purified by discontinuous sucrose gradient centrifugation. Virion RNAs from the partially purified virus sample and intracellular RNAs were examined by Northern blot analysis using a gene 6-specific probe (Fig. 3A); this probe hybridizes with env supplier genomic DI RNA and two larger subgenomic DI RNAs but not with DIssA RNA (13) (Fig. 1). All env suppliers replicated and transcribed with similar efficiencies (Fig. 3A, lanes 2 to 5). The small amount of mRNA 6 detected in the cells infected with the pol/N supplier (Fig. 3A, lane 1) probably was synthesized from LA16 revertant viruses and MHV-JHM that was present in the pol/N supplier preparation. A trace amount of RNA signal was detected in the cells infected with only the JW2 env. supplier (Fig. 3A, lane 6), demonstrating that LA16 did not support env suppliers' replication at 39.5°C and that DIssA supported replication and transcription of env suppliers as demonstrated previously (13). Analysis of virion RNAs showed that JW2 genomic RNA was present in the partially purified virus particles (Fig. 3A), whereas only trace amounts of the genomic DI RNAs of other env suppliers were detected. That there was a slightly larger amount of JW2ΔsM genomic RNA than of JW2ΔM and JW2ΔsM+M genomic RNA was due to the presence of slightly larger amounts of LA16 revertants and MHV-JHM in the JW2ΔsM supplier preparation (see below).

Northern blot analysis using a gene 2-specific probe revealed that DIssA-containing virus particles were produced from the cells coinfected with the pol/N supplier and the JW2 env supplier (Fig. 3B, lane 5). A very limited amount of viral particles containing DIssA was produced from the cells infected with the pol/N supplier alone or from those coinfected with other

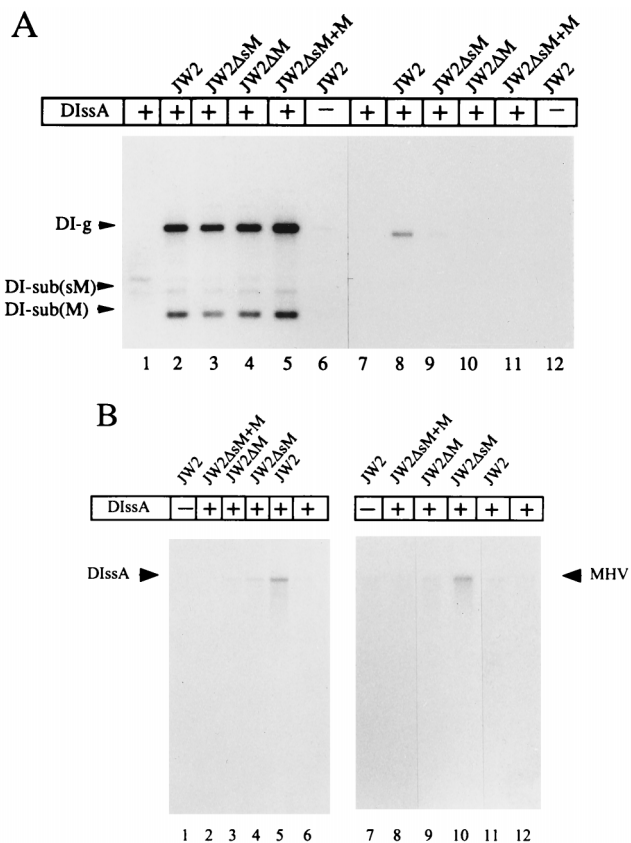

FIG. 3. Northern blot analyses of the env supplier-specific RNAs (A) and DIssA RNA (B). DBT cells were coinfected with the pol/N supplier and the env supplier and cultured at 39.5°C. Intracellular RNA was extracted at 8 hpi, or the supernatant was collected at 12 hpi. Virion RNAs were extracted from the partially purified virus particles. The presence and absence of the pol/N supplier are represented by + and −, respectively. (A) Intracellular env supplier-specific RNAs (lanes 1 to 6) and virion env supplier-specific RNAs (lanes 7 to 12) were examined by Northern blot analysis using a gene 6-specific probe. Only the pol/N supplier was used as the inoculum for lanes 1 and 7. DI-g, DI-sub(sM), and DI-sub(M) represent genomic DI RNA, sM protein-encoding subgenomic DI RNA, and M protein-encoding subgenomic DI RNA, respectively. (B) DIssA and genomic RNAs of LA16 revertant viruses and MHV-JHM in the virion were examined by Northern blot analysis using a gene 2-specific probe (lanes 1 to 6) and a gene 3-specific probe (lanes 7 to 12), respectively (Fig. 1A). Only the pol/N supplier was used as the inoculum for lanes 6 and 12.

env suppliers and the pol/N supplier (Fig. 3B, lanes 1 to 6). To clarify that the majority of signals detected indeed represented DIssA RNA but not genomic RNAs of LA16 revertants and MHV-JHM, the same RNA samples were examined by Northern blot analysis using a gene 3-specific probe (Fig. 3B, lanes 7 to 12). Because DIssA does not contain gene 3 (13) (Fig. 1A), the gene 3 probe should have hybridized only with the genomic RNAs of LA16 revertants and MHV-JHM and not with DIssA RNA. Only faint signals were detected in most of the RNA samples. The virion RNA sample from the cells coinfected with the pol/N supplier and the JW2ΔsM env supplier showed a slightly stronger RNA signal (Fig. 3B, lane 10), indicating the presence of a larger amount of genomic RNAs from possible LA16 revertants or from MHV-JHM contaminating this virus preparation. This may have contributed to the slightly stronger signal seen in Fig. 3B, lane 4; some of the signal probably represented helper virus genomic RNA. These data suggested that MHV particles containing DIssA RNA and env supplier's DI RNA were efficiently produced only from the cells coin-

fected with the pol/N supplier and the JW2 env supplier. Thus, these data indicated that sM and M proteins, but not S protein, were necessary for packaging of DI RNAs as well as for MHV virion assembly and release.

To determine the buoyant density of MHV particles released from cells coinfected with the pol/N supplier and JW2 env supplier, the partially purified virus particles were centrifuged on a continuous 20 to 60% sucrose gradient at 26,000 rpm for 18 to 24 h in a Beckman SW28 rotor. Ten fractions were collected from the bottom of the gradient, and virion RNA was extracted from each fraction. Northern blot analysis of JW2 DI RNA in each fraction showed that a major peak of JW2 DI RNA signal was at a density of approximately 1.18 g/cm^3, while the major peak of genomic RNA of MHV-JHM and that of LA16 were at a density of approximately 1.19 g/cm^3 (data not shown). MHV particles released from cells coinfected with the pol/N supplier and JW2 env supplier were slightly lighter than those of infectious MHV.

Characterization of virus-specific intracellular proteins and virion proteins in the coinfected cells. Northern blot analysis of virion RNAs demonstrated that coinfection of the pol/N supplier and the JW2 env supplier, but not other env suppliers, produced virus particles containing DI RNAs. These data, however, did not exclude the possibility that empty virus particles lacking virion RNAs were produced in certain combinations of the pol/N supplier and the env supplier. We characterized virus-specific intracellular proteins and virion proteins to examine whether MHV particles lacking S protein were being produced in the cells coinfected with the pol/N supplier and the JW2 env supplier and to rule out that empty MHVs were produced in the cells coinfected with other env suppliers and the pol/N supplier. Virus-infected cells were incubated at 39.5°C and virus-specific proteins were labeled with [35S]methionine for 20 min at 7.5 hpi or for 2 h from 6.5 to 8.5 hpi. As a control, LA16-infected cells were maintained at 32.5°C. Virus-specific proteins were immunoprecipitated with an anti-MHV serum and analyzed by SDS-PAGE. Lower-molecular-weight proteins and higher-molecular-weight proteins were separated on 10% gels (Fig. 4A and C) and 7.5% gels (Fig. 4B and D), respectively. DIssA-derived N protein was synthesized in all the pol/N supplier-infected cells. S protein was synthesized in MHV-JHM-infected cells at 37°C or in LA16-infected cells at 32.5°C but not in the pol/N supplier-infected cells, the JW2 env supplier-infected cells, or the coinfected cells (Fig. 4B and D). We found three proteins (Fig. 4D) of unidentified origin in the 2-h labeling samples. Because one of these proteins appeared only in the cells infected with the pol/N supplier, this protein probably was a translation product of a naturally occurring DI RNA(s) present in the pol/N supplier (13). MHV-JHM-specific M protein was synthesized in the pol/N supplier- and JW2 env supplier-coinfected cells and in the pol/N supplier- and JW2ΔsM env supplier-coinfected cells (Fig. 4A, lanes 2 and 3; Fig. 4C, lanes 3 and 4), demonstrating expression of subgenomic DI RNA-encoding M protein in these coinfected cells. These data were consistent with intracellular viral RNA analysis (Fig. 3A) and with the structure of the DI RNAs. In some experiments, we detected a protein band that closely comigrated with LA16-specific M protein (Fig. 4C, lanes 2 to 7). This protein was most likely a host protein; the presence of this host protein in mock-infected cells was apparent after a long exposure of the autoradiogram (Fig. 4C, lane 11). To further confirm that LA16-specific proteins were not produced at the nonpermissive temperature, DBT cells were infected with LA16 virus samples that had been concentrated 10-fold. Virus-specific proteins were labeled with [35S]methionine at 39.5°C from 1 to 5 hpi or from 5 to 10 hpi.

Radiolabeled proteins were then immunoprecipitated by anti-MHV serum and analyzed by SDS-PAGE. We did not detect synthesis of any LA16-specific proteins (data not shown), demonstrating that LA16-specific protein synthesis did not occur at any time during the course of virus infection at 39.5°C.

To examine virion proteins, virus-infected cells were labeled with [35S]methionine at 39.5°C for 8 to 12 hpi. As a control, [35S]methionine-labeled LA16 and MHV-JHM were grown at 32.5 and 37°C, respectively. Released virus particles were partially purified by sucrose gradient centrifugation, because we could not estimate the buoyant density of potential empty particles. In order to eliminate contaminated radiolabeled cellular proteins in the virus preparation, virus-specific proteins were immunoprecipitated with anti-MHV serum and analyzed by SDS-PAGE (Fig. 5A). Three major MHV-specific proteins, the S, N, and M proteins, were detected in partially purified MHV-JHM and LA16 (Fig. 5A, lanes 8 and 9). We detected N protein and MHV-JHM-specific M protein, but not S protein, in the MHV sample from cells coinfected with the pol/N supplier and the JW2 env supplier (Fig. 5A, lane 2), demonstrating assembly and release of MHV particles containing DI RNAs (Fig. 3), N protein, and MHV-JHM-derived M protein. The sM protein is present in very small amounts in MHV (48). Under our experimental conditions, we could not demonstrate the presence of sM protein in the partially purified MHV from cells coinfected with the pol/N supplier and the JW2 env supplier even after immunoprecipitation using anti-sM protein antibody (data not shown). No MHV-specific proteins were detected in the supernatant from other coinfected cells, from the pol/N supplier-infected cells, or from the JW2 env supplier-infected cells (Fig. 5A), demonstrating that MHV particles containing N protein and M protein were not produced from these cells.

To directly examine the viral structural proteins, partially purified virus particles from cells coinfected with the pol/N supplier and the JW2 env supplier were further purified by continuous sucrose gradient centrifugation. The released virus particles showed a single radioactive peak at a buoyant density of approximately 1.18 g/cm^3, which was the same as the virus buoyant density shown by the Northern blot analysis (see above). SDS-PAGE analysis of virus structural proteins showed that virus particles from the cells coinfected with pol/N supplier and JW2 env supplier contained N protein and M protein but not S protein (Fig. 5B); both N protein and M protein comigrated with MHV-JHM N protein and M protein, respectively. These data demonstrated that N protein was the product of DIssA-specific mRNA 7 and M protein was the product of the JW2-specific M protein encoding subgenomic DI RNA. We sometimes detected a high-molecular-weight protein of unknown origin (Fig. 5B, lane 2) in virus preparations. This protein did not comigrate with MHV S protein and was not detected consistently (Fig. 5B, lane 6); this protein may be a protein encoded by some DI RNAs in the virus preparation. Minor proteins found in one virus preparation from the cells coinfected with the pol/N supplier and the JW2 env supplier (Fig. 5B, lane 6) did not comigrate with MHV proteins and probably represent contaminating host proteins.

These observations and the viral RNA data (Fig. 3) showed that the M and sM proteins were needed jointly for assembly and release of virus particles that contained viral RNA bound to N protein. We could not rigorously exclude the possibility that empty MHV particles consisting of only sM proteins were synthesized in the cells coinfected with the pol/N supplier and JW2ΔM supplier. If such empty particles were formed, they did not package the virus core, which would contain DI RNA and N protein (Fig. 3 and 5). S protein was not synthesized in
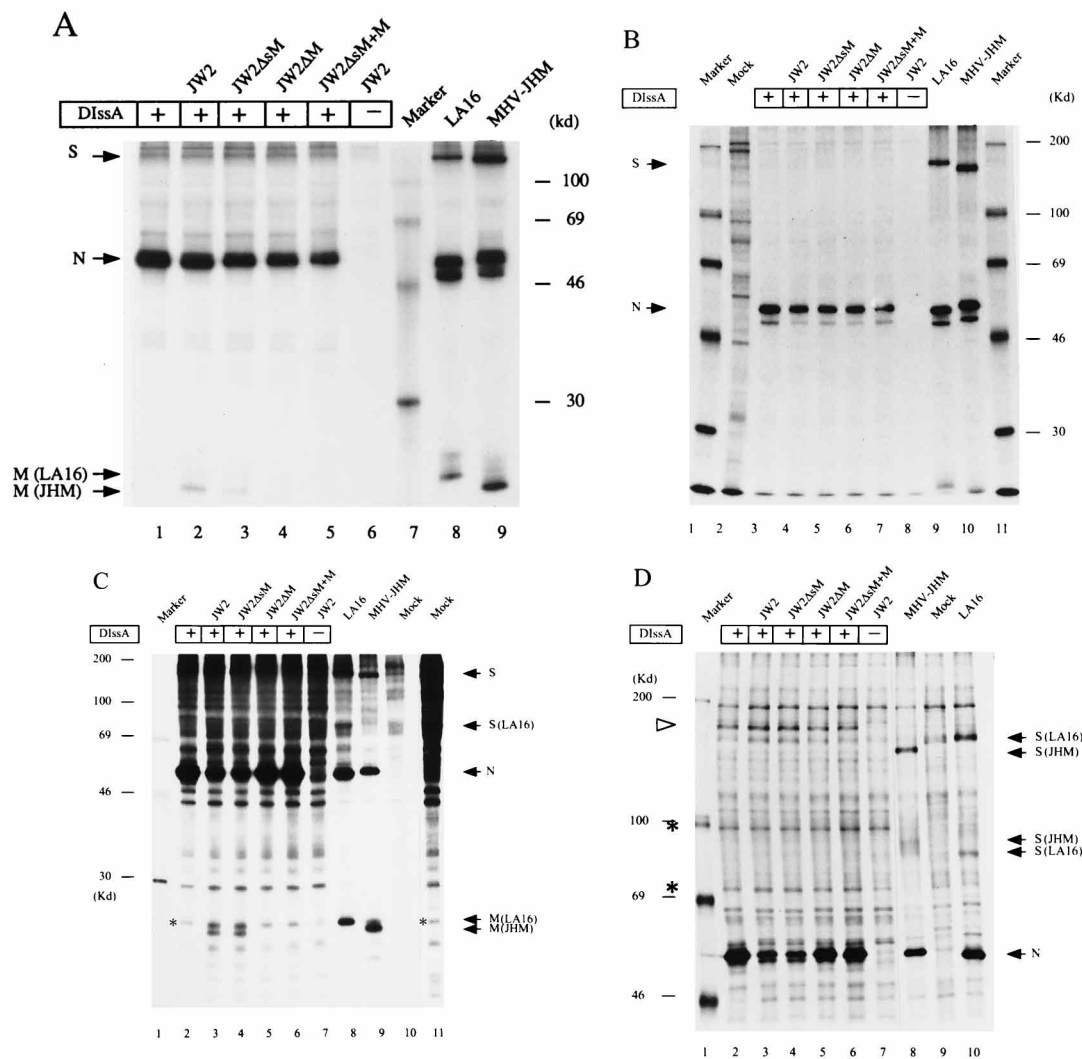
FIG. 4. Analysis of the intracellular virus-specific proteins after coinfection of the pol/N supplier and env supplier. Virus-infected cells were labeled with [³⁵S]methionine for 20 min at 7.5 hpi (A and B) or for 2 h from 6.5 to 8.5 hpi (C and D), and cytoplasmic lysates were prepared. Virus-specific proteins were immunoprecipitated with anti-MHV-JHM serum and electrophoresed on an SDS–10% polyacrylamide gel (A and C) or an SDS–7.5% polyacrylamide gel (B and D). M (LA16), M (JHM), S (LA16), and S (JHM) represent LA16 M protein, MHV-JHM M protein, LA16 S protein, and MHV-JHM S protein, respectively. The lane labeled Marker contains ¹⁴C-labeled protein molecular mass marker. For other lanes, DBT cells were infected as follows (numbers in parentheses are lane numbers for panels A to D, respectively): mock infected at 39.5°C (lanes labeled Mock), infected with the pol/N supplier only at 39.5°C (1, 3, 2, and 2), JW2 env supplier only at 39.5°C (6, 8, 7, and 7), infected with MHV-JHM only at 37°C (9, 10, 9, and 8), infected with LA16 only at 32.5°C (8, 9, 8, and 10), or coinfected with the env supplier and the pol/N supplier at 39.5°C (2 to 5, 4 to 7, 3 to 6, and 3 to 6). (C) Lane 11 shows a longer exposure of the autoradiogram shown in lane 10. The asterisks show a host protein that migrated closely with LA16-specific M protein (see text). (D) Three proteins (marked by an open triangle and two asterisks) of unidentified origin were found in the 2-h labeling samples. Because the protein shown by the open triangle appeared only in the cells infected with the pol/N supplier, this protein probably was a translation product of a naturally occurring DI RNA(s) present in the pol/N supplier (13).

the pol/N supplier-, JW2-env supplier-coinfected cells at 39.5°C, while MHV particles containing viral RNA were released. Thus, S protein was not required for assembly of the viral core, for envelopment of the viral core, or for viral release. We have presented the first direct identification of a combined requirement for MHV envelope proteins M and sM in the envelopment of the viral core.

DISCUSSION

We established a complementary DI system that enabled us to study the effects of deleting entire individual envelope protein genes on MHV assembly. Analysis of virion RNAs and proteins unambiguously demonstrated that MHV particles

were assembled and released from the cells coinfected with the pol/N supplier and the JW2 env supplier. The released MHV particles contained M protein, N protein, and DI RNAs, while the presence of sM protein was not confirmed due to its small amount in the virus. MHV particles released from the cells coinfected with the pol/N supplier and JW2 env supplier had a slightly lighter buoyant density than wild-type MHV; the absence of S protein probably affected the buoyant density of the virus particles. Studies of JW2-derived mutants suggested that both M protein and sM protein were necessary for incorporation of the core (a spherical core shell around a filling of RNA and N protein) into enveloped, released viral particles. The S protein was not required for these processes. This conclusion is consistent with the results of Vennema et al. (44) and Bos et al.
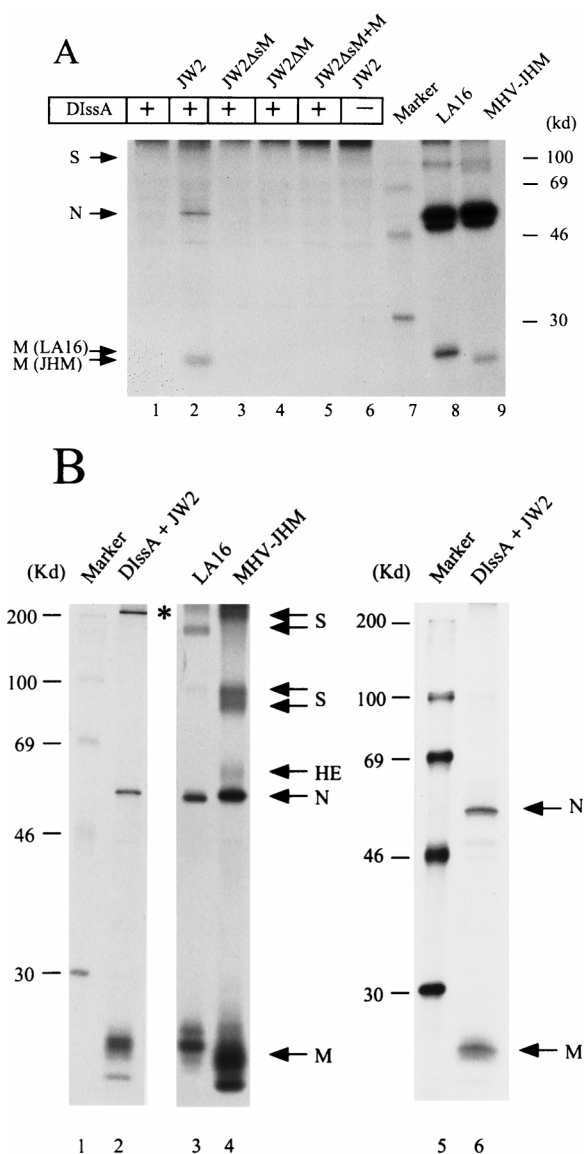
FIG. 5. Analysis of virion proteins after coinfection of the pol/N supplier and env supplier. MHV-JHM and LA16 were grown at 37 and 32.5°C, respectively, while other virus samples (lanes 1 to 6 [A] or lanes 2 and 6 [B]) were grown at 39.5°C. M (LA16) and M (JHM) represent LA16 M protein and MHV-JHM M protein, respectively. The lane labeled Marker contains a ^{14}C-labeled protein molecular mass marker. (A) [^{35}S]methionine-labeled virus was partially purified, and virus-specific proteins were immunoprecipitated with anti-MHV-JHM serum and electrophoresed on an SDS–10% polyacrylamide gel. The presence and absence of the pol/N supplier in the inoculum are represented by + and −, respectively. Only the pol/N supplier was used as the inoculum for lane 1. (B) [^{35}S]methionine-labeled virus was purified and electrophoresed on an SDS–10% polyacrylamide gel. Two different preparations of virus samples from cells coinfected with the pol/N supplier and JW2 env supplier are shown (lanes 2 and 6). The asterisk indicates a large protein of unknown origin (see text); this protein was only detected in some virus preparations from cells coinfected the pol/N supplier and JW2 env supplier. The virus sample applied to lane 2 was the progeny of 10^7 cells, while virus samples from the cells infected with MHV-JHM and LA16 (lanes 3 and 4) were progeny of 5×10^5 cells. S, HE, N, and M represent S protein, HE protein, N protein, and M protein, respectively.

(3); they showed the formation and release of empty VLPs from MHV-uninfected cells expressing M, S, and sM proteins, and concluded that both M and sM protein are required for VLP formation. We further identified viral envelope proteins

that were required for envelopment of the viral core. Our finding that S protein was not required for envelopment of the viral core excluded the possibility that some part of the S protein membrane anchors and/or cytoplasmic tails may play a role in core envelopment.

An electron microscopic study by Risco et al. (33) showed that coronavirus TGEV contains a spherical core structure inside of the viral envelope (33). The authors claimed that the spherical core shell contains mostly M protein and a lesser amount of N protein (33). If M protein is indeed present in the spherical core, then coronavirus M protein is present at two different sites in a virus particle, i.e., once in the envelope and again in the spherical core. They showed that MHV also contains the internal core. However, our biochemical study indicated that the MHV core contained N protein and MHV genomic RNA but not M protein (30). We also found that there was a stable interaction between envelope M protein and the core in the virus particles (30). It is possible that M protein detected in the TGEV spherical core represents envelope M protein that collapsed on the internal core. We speculate that the internal core is the structure that restricts the migration of M protein at the budding compartment (14) through a strong interaction between envelope M protein and the spherical core and that the interaction that leads to the envelopment of the core resulting in virus particles occurs between M protein and the internal core. Hopefully, future studies using the complementary DI system will provide information on the mechanism of coronavirus spherical core formation and the mechanism of M protein restriction in the budding compartment of MHV-infected cells.

As shown in this study, S protein was not necessary for packaging of the internal core. Since expression of both M protein and sM protein is required for virus envelope formation (3, 44), we could not identify whether envelopment of the internal core requires M protein alone, sM protein alone, or a combination of both M protein and sM protein. It is possible that sM may only be involved in the completion of envelope assembly and/or in the release of viral particles from the cells.

Another question about coronavirus assembly is whether gene 1 proteins participate. Bos et al. showed that when helper MHV and the culture fluid from the cells expressing an MHV DI RNA and all MHV structural proteins are coinfected and passaged several times, MHV DI RNA accumulates (3); their data may indicate that gene 1 proteins are not required for MHV assembly. However, it appears that only a small amount of DI particles are produced in their system; no direct evidence was shown for the production of DI particles from the plasmid-transfected cells, and several passages of virus samples were required for DI RNA accumulation (3). The low level of DI particle production in the study of Bos et al. (3) raises questions about whether the internal core was properly formed in the plasmid-transfected cells, and whether the DI RNAs were specifically packaged into VLPs. Comparing the efficiency of DI particle formation in the system used by Bos et al. (3) and that in the complementary DI system is difficult, yet it seems that a larger amount of DI particles were produced in the complementary DI system; without subsequent passage, we directly demonstrated formation of virus particles containing DI RNAs in the complementary DI system. One of the obvious major differences between the complementary DI system and the system used by Bos et al. (3) is involvement of DIssA, which expresses gene 1 proteins. If the amount of DI particles produced from the complementary DI system was indeed significantly higher than that in the system used by Bos et al., this would indicate that some gene 1 protein(s) were involved in viral assembly.

Expression vectors based on RNA viruses other than retroviruses have been developed previously (48). The MHV complementary DI system has a similarity with one of the Sindbis virus expression vector systems, in which two Sindbis transcripts complement each other to produce Sindbis virus carrying a non-Sindbis virus gene (4). One of the transcripts is a self-replicating Sindbis replicon that encodes Sindbis virus nonstructural proteins and subgenomically expresses a non-Sindbis virus foreign gene. Another transcript subgenomically expresses viral structural proteins. Cotransfection of these transcripts results in replication and assembly of Sindbis virus containing the foreign gene (4). Although Sindbis virus expression vector systems are excellent in many aspects, they may be limited by the size of RNAs that they can package; very large RNAs may not be packaged well into alphavirus-based vectors. MHV DI RNAs containing an inserted MHV gene or a non-MHV sequence express these genes in DI RNA-replicating cells (21, 22, 25), indicating that coronaviruses themselves and/or coronavirus DI RNAs may be developed as expression vectors. Coronavirus carries the largest single-stranded RNA genome, and may be particularly suitable for delivery of large RNA molecules as an expression-vector. Coronavirus expression vectors may express multiple genes through expression of multiple subgenomic mRNAs (25). The finding that S protein was not necessary for MHV assembly may potentially confer a unique property on coronavirus expression vector systems. Development of a complementary DI system-based coronavirus vector in which the assembled viruses contain S protein derived from different coronaviruses, from an envelope protein(s) of other enveloped viruses, or even from a cellular membrane protein(s) is an attractive idea. Such vectors may prove to be invaluable for delivering a specific, large gene to a specific target cell. As we have shown in this study, insertion of S protein abolished DI RNA replication; currently we do not know what kind of inserted sequences DI RNA replication will tolerate.

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