Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

VIROLOGY 209, 428–436 (1995)

A *cis*-Acting Viral Protein Is Not Required for the Replication of a Coronavirus Defective-Interfering RNA

CHING-LEN LIAO* and MICHAEL M. C. LAI*†[1]

†*Howard Hughes Medical Institute and* **Department of Molecular Microbiology and Immunology, University of Southern California, School of Medicine, Los Angeles, California 90033-1054*

Received February 2, 1995; accepted March 15, 1995

Mouse hepatitis virus (MHV), a coronavirus, generates defective-interfering (DI) RNAs of different sizes during passages at high multiplicities of infection. All MHV DI RNAs characterized so far contain an open reading frame (ORF) encoding a fused viral protein; in addition, DI RNAs with a long ORF have a competitive advantage over those with a shorter ORF. These findings suggest that DI RNA replication may require an ORF encoding a *cis*-acting viral protein. In this study, we used a naturally occurring DI RNA and inserted a 12-nucleotide (nt) amber-mutation linker at various positions to truncate the ORF. Most of the mutants replicated as well as the wild-type DI RNA, irrespective of the presence or absence and the length of the ORF in the RNA. Sequence analysis showed that all of the mutants retained the insertional mutations even after two viral passages in tissue culture, establishing that the mutant DI RNAs replicated. We have further introduced two 3-nucleotide substitutions of the first two AUG codons of the ORF, thus completely closing the ORF. This DI RNA replicated as well as the wild-type DI, but, after a single passage, the majority of the mutant RNAs was replaced by recombinant RNAs which contain a restored functional ORF. However, an additional insertion of a 12-nt amber-mutation linker downstream of the AUG substitutions prevented recombination, and the DI RNA still replicated. These data indicate that DI RNA replication does not require a DI-specific ORF encoding *cis*-acting viral proteins and that a 12-nucleotide insertion could prevent or delay the occurrence of RNA recombination, suggesting the importance of direct or indirect RNA alignment in homologous RNA recombination.

INTRODUCTION

Mouse hepatitis virus (MHV), the prototype of coronaviruses, contains a single-stranded, positive-sense RNA genome of approximately 31 kb, which is the largest RNA virus genome known so far (Pachuk *et al.*, 1989; Lee *et al.*, 1991). The MHV genome contains seven to eight open reading frames (ORFs); each ORF is preceded by a short stretch of consensus intergenic sequences, which serves as the initiation signal for mRNA transcription (Lai, 1990). In MHV-infected cells, seven to eight virus-specific mRNAs, each with a 3′-coterminal nested-set structure, are synthesized (Lai *et al.*, 1981). All MHV mRNAs contain a leader sequence of approximately 70 nucleotides, which is derived from the 5′ end of the genome (Spaan *et al.*, 1983; Lai *et al.*, 1984). The leader RNA regulates the transcription of MHV mRNAs in both *trans*- and *cis*-acting manners (Jeong and Makino, 1994; Liao and Lai, 1994; Zhang *et al.*, 1994). Similar to other RNA viruses, defective-interfering (DI) RNAs were often generated when MHV was passaged in tissue culture at a high multiplicity of infection (m.o.i.) (Makino *et al.*, 1985). Characteristically, DI RNAs retain both the 5′ and 3′ termini and scattered portions of the internal sequences of the wild-type viral genome (Makino *et al.*, 1985, 1988b, 1990; van der Most *et al.*, 1991). Since these DI RNAs replicate efficiently, they must have preserved the essential *cis*-acting replication signals. However, because of deletions in parts of viral genes, most DI RNAs need wild-type virus as a helper to provide the viral gene products essential for RNA replication *in trans*.

Different DI RNAs retain different fractions of the wild-type genomic RNA. The longest DI RNA identified thus far, i.e., DIssA of the JHM strain of MHV, retains most of the coding capacity of gene 1, and thus is capable of autonomous replication even in the absence of a helper virus (Makino *et al.*, 1988a). Other DI RNAs are considerably shorter; however, curiously, all naturally occurring MHV DIs contain an ORF that consists of several noncontiguous regions derived from gene 1 and other downstream genes, which are fused in-frame (Makino *et al.*, 1988b, 1990; van der Most *et al.*, 1991). Some of the proteins encoded by the DI ORFs have been detected in the virus-infected cells (Makino *et al.*, 1988b, 1990; de Groot *et al.*, 1992). Intriguingly, all of the fusion proteins contain the N-terminal portion, corresponding to p28, of the gene 1 polyprotein (Baker *et al.*, 1989). The biological functions of the DI-encoded fusion proteins remain unclear.

[1] To whom correspondence and reprint requests should be addressed at Dept. of Molecular Microbiology and Immunology, University of Southern California, School of Medicine, 2011 Zonal Avenue, HMR-503C, Los Angeles, CA 90033-1054. Fax: (213) 342-9555. E-mail: michlai@hjsc.usc.edu.

0042-6822/95 $6.00

Similar to MHV DI RNAs, DI RNAs of poliovirus and clover yellow mosaic virus (CYMV) also contain a functional fusion ORF. It has been demonstrated that the fusion ORF is required for the replication of poliovirus DI RNA, which needs the *cis*-acting function of certain gene product(s) of the fusion ORFs (Hagino-Yamagishi and Nomoto, 1989; Collis *et al.*, 1992). The ORFs of all DI RNAs of CYMV are also translatable (White *et al.*, 1991); however, it appears that the translatability of DI RNA, rather than the specific viral gene product(s) from its fusion ORF, is crucial for CYMV DI RNA replication, suggesting that the translation process is a prerequisite for, or coupled to, CYMV DI RNA replication (White *et al.*, 1992). On the other hand, the DI RNAs of vesicular stomatitis virus, influenza virus, and Sindbis virus do not always have an ORF (Akkina *et al.*, 1984; Monroe and Schlesinger, 1984; Nayak *et al.*, 1989). Thus, the role of ORF in DI RNA replication is variable from virus to virus.

Recent studies on MHV DI RNAs (de Groot *et al.*, 1992; Kim *et al.*, 1993b) have revealed an interesting relationship between the size of the DI-specific ORF and the relative rate of DI RNA accumulation. DI RNAs with a large ORF appear to have a selective advantage over a nearly identical DI RNA which has a premature termination mutation in the ORF. As a result, the revertant or recombinant DI RNAs which have a restored wild-type ORF rapidly became a predominant population after the mutant DI RNAs were passaged in tissue culture (de Groot *et al.*, 1992; Kim *et al.*, 1993b). These observations were interpreted to support the postulated requirement of the fusion ORF for MHV DI RNA replication and accumulation (de Groot *et al.*, 1992; Kim *et al.*, 1993b). However, this requirement has not been directly demonstrated. In this paper, we have experimentally addressed this issue. If MHV DI RNA replication requires a functional fusion ORF, it will suggest either that the gene product(s) encoded by this ORF has an essential *cis*-acting function for DI RNA replication, or that DI RNA replication is coupled to translation. The answers to this issue have been equivocal because the frequent occurrence of recombination between DI RNA and helper viral RNA made it difficult to maintain the structure of input DI RNAs. We therefore developed a strategy to close or truncate the DI ORF in such a way that recombinational restoration of the ORF was prevented or delayed, allowing the direct examination of the effects of mutations on DI RNA replication. Here we showed that mutant DI RNAs without a functional virus-specific ORF were able to replicate, unambiguously demonstrating that a DI-specific ORF encoding a *cis*-acting viral protein is not required for DI RNA replication. We also showed that a 12-nucleotide (nt) insertion prevented or delayed the occurrence of RNA recombination, thus ensuring the genetic stability of DI RNAs. This observation reveals interesting insights into the mechanism of RNA recombination.

MATERIALS AND METHODS

Viruses and cells

The plaque-cloned A59 strain (Manaker *et al.*, 1961) of MHV was used throughout this study. Viruses were propagated in DBT cells (Hirano *et al.*, 1974), a mouse astrocytoma cell line, at an m.o.i. of 0.5. DBT cells also were used as recipient cells for RNA transfection experiments.

Plasmid construction

Plasmid DE25 (pDE25) was derived from DIssE RNA, which is the smallest DI RNA derived from the JHM strain of MHV (Makino *et al.*, 1984). This construct (Makino and Lai, 1989) contains the complete DIssE sequence placed downstream of the T7 polymerase promoter. pDE25 was used as the starting material to construct a series of mutants in which a reading frame termination linker was introduced into the ORF of DIssE at different restriction sites to truncate the ORF. This 12-nt linker, amber-stop *Nhe*I (5′-pdCTAGCTAGCTAG, Pharmacia), contains amber termination codons in all three different reading frames and also an *Nhe*I restriction site. To construct p25Stu/Nhe (Fig. 1A), in which the amber-mutation linker was inserted into the *Stu*I site of pDE25, the *Stu*I-restricted pDE25 fragment was first blunted by T4 DNA polymerase and then ligated with phosphorylated amber-mutation linker by T4 DNA ligase. Similarly, P25AfI/Nhe and p25Spe/Nhe (Fig. 1A) were made by inserting the amber-mutation linker into the *Afl*II site and the *Spe*I site of pDE25, respectively. To construct the closed-ORF mutant p1aM (Fig. 1A), polymerase chain reaction (PCR) cloning was performed using p25Stu/Nhe as template. Briefly, primers 625 (5′-ATACCCCAAAGCCCGGCAAATACGGTCTC-3′) and 629 (5′-TACTATCAAATCTCTTTAGAC-3′, Fig. 1A) were used to generate a 1300-bp DNA fragment by PCR, and primers −40 (5′-GTTTTCCCAGTCACGAC-3′) and 599 (5′-GCCGGGCTTTGCGGGTATGCAACC-3′, Fig. 1A), were used to PCR-synthesize a 300-bp DNA fragment. Both DNA fragments were isolated from low-melting-agarose gel and mixed together to carry out another PCR-amplification, using primers −40 and 629. The resulting DNA fragment of 1600 bp in length was digested with restriction enzymes *Cla*I and *Spe*I; the restricted fragment was isolated from low-melting agarose and inserted into the *Cla*I–*Spe*I site of p25Stu/Nhe, giving rise to p1aM, in which the first two AUGs of the ORF were converted into CCCs, and a 12-nt, amber-mutation linker was inserted at the *Stu*I site. Similarly, to construct p1aMNhe, PCR cloning was carried out using p1aM as the template. For this purpose, primers 678 (5′-AAGCCCCTAGCTAGCTAGGGCAAATACGGTCTC-3′, Fig. 1A) and 629 were first used to amplify a 1300-bp DNA fragment, and primers 679 (5′-TTTGCCCTAGCTAGCTAGGGGCTTTGCGGGTAT-3′, Fig. 1A) and −40 were used to amplify

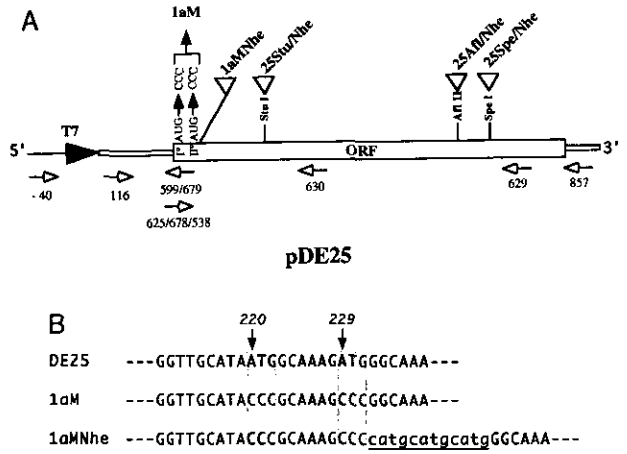

FIG. 1. The schematic diagram of the truncated- and closed-ORF-DI mutants used in this study. (A) A 12-nt amber-mutation linker was inserted into different restriction sites to truncate the ORF of DIssE in *pDE25 (Makino et al., 1984, 1988b), which contains a T7 promoter* sequence upstream of the DissE sequence. Open triangles indicate insertions of the 12-nt amber-mutation linker, which includes an *Nhe*I site. Open arrows indicate the positions of primers used to perform PCR cloning or sequencing. Mutant 1aM was derived from 25Stu/Nhe, in which the first and second AUG codons were converted into CCC codons. Mutant 1aMNhe was derived from 1aM, in which a 12-nt amber-mutation linker was inserted into the position immediately downstream of the second CCC codon in 1aM ORF. Diagram is not drawn to scale. *Primer 630 (5'-GCGGAAATGGCCGGATGTCAA-3') is complementary* to nucleotides 811–831 of DE25, according to the published sequence (Makino *et al.*, 1988b). The remaining primers are described in the text. (B) Sequences of 1aM and 1aMNhe at the substitution sites. The numbers above the two arrows indicate the nucleotide positions of the first two ATGs in the ORF of DE25 (Makino *et al.*, 1988b). The substituted nucleotides are highlighted by shaded boxes. The inserted nucleotides (12-nt linker) in 1aMNhe are in lowercase letters and underlined.

a 300-bp DNA fragment. Both DNA fragments were mixed together and another PCR amplification was performed using primers −40 and 629, resulting in a DNA fragment of approximately 1600 bp in length. This fragment was digested with restriction enzymes *Cla*I and *Spe*I and ligated with the *Cla*I–*Spe*I-restricted fragment of p1aM, yielding p1aMNhe, in which an amber-mutation linker was inserted immediately after the second CCC codon. To obtain RNA transcripts from individual mutants, plasmids were linearized with *Xba*I, and *in vitro* transcription was carried out using T7 RNA polymerase in the presence of a cap analog $m^7G(5')ppp(5')G$ (New England Biolab) (Soe *et al.*, 1987).

RNA transfection

A59-infected DBT cells were used as recipient cells for RNA transfection. Briefly, monolayers of DBT cells at approximately 80% confluence in 6-cm petri dishes were infected with A59 at an m.o.i. of 5. At 1 hr postinfection (p.i.), the virus-infected cells were washed once with serum-free Eagle's minimal essential medium (MEM) and then covered with 4 ml of prewarmed MEM containing 1% newborn calf serum. Ten micrograms of *in vitro*-transcribed RNAs were dissolved in a final volume of 200 μl 10% (v/v) DOTAP (Boehringer-Mannheim Biochemicals) mixture. The RNA–DOTAP mixtures were incubated at 25° for 10 min and then added slowly to cell cultures. After incubation for 4 hr at 37°, the inoculum was removed, and cells were washed twice with prewarmed serum-free MEM and further incubated for 9 hr (for the extraction of cytoplasmic RNA) or 12 hr (for the collection of released virus) in MEM containing 1% newborn calf serum. Culture fluid was harvested, clarified of cell debris, and stored at −80° for further use as the source of virus.

In vitro translation and immunoprecipitation

In vitro-transcribed RNAs were translated in an mRNA-dependent rabbit reticulocyte lysate system (Promega Biotec) optimized for MHV RNA translation (Denison and Perlman, 1986). Translation was performed in 25-μl mixtures containing [^{35}S]methionine (2400 μCi/ml; New England Nuclear). Immunoprecipitation was carried out as described elsewhere (Baker *et al.*, 1989). Briefly, *in vitro*-translated products were diluted to 1 ml in RIPA buffer (50 m*M* Tris–HCl, pH 7.4, 0.3 *M* NaCl, 4 m*M* EDTA, 0.5% Triton X-100, 0.1% SDS) and incubated with 3 μl of anti-p28 antibody (Baker *et al.*, 1989) and anti-nucleocapsid (N) antibodies for 2 hr at 4°. Antibody–antigen complexes were collected by incubating with 100 μl of a 10% (wt/vol) solution of *Staphylococcus aureus* cells (Pansorbin; Calbiochem-Behring, La Jolla, CA) for 1 hr at 4° and subsequently were washed three times with 1 ml of RIPA buffer. The immunoprecipitated proteins were then eluted from bacteria by boiling for 2 min in electrophoresis sample buffer (0.1 *M* β-mercaptoethanol, 1% SDS, 0.08 *M* Tris–HCl, pH 6.8, 10% glycerol). Bacteria were removed by centrifugation, and proteins were analyzed by electrophoresis on 5 to 15% polyacrylamide-gradient gels containing SDS (Laemmli, 1970).

Metabolic labeling and preparation of intracellular viral RNA

Confluent DBT cells in 60-mm plates were washed once with serum-free MEM medium and incubated with 400 μl undiluted culture fluid containing released virus particles (m.o.i. of approximately 10). Following 1 hr of virus adsorption, inoculum was removed and replaced with 3 ml of MEM containing 1% newborn calf serum and 2.5 μg/ml of actinomycin D. At 5.5 hr p.i., 100 μCi/ml of [^{3}H]uridine was added to each plate, and cells were further incubated at 37° for 1.5 hr. ^{3}H-labeled MHV-specific intracellular RNA was isolated according to published procedures (Yokomori *et al.*, 1992). RNA from each plate was redissolved in 30 μl of H_2O, and 3 μl of each sample was analyzed by agarose gel electrophoresis as described previously (Makino *et al.*, 1984). After electropho-

resis, gels were processed for fluorography and exposed to X-ray films.

Direct sequencing of PCR products

PCR products were separated by electrophoresis on 1% low-melting-agarose gel. The products of correct size were purified and subjected to direct sequencing using the thermal cycle dideoxy sequencing reaction system (New England Biolab). The dideoxy-terminated chains were labeled with ^{35}S-dATP. Twenty thermal cycles were carried out, each cycle consisting of incubations at 95°, 55°, and 72° for 20 sec each.

RESULTS

Replication of mutant DI RNAs with a truncated ORF in the absence of RNA recombination

Previous studies have demonstrated that MHV DI RNAs with a truncated ORF were rapidly replaced by ones with a longer ORF during viral passages, which usually resulted from recombination between DI RNA and helper virus RNAs or nucleotide insertion caused by polymerase errors (de Groot *et al.*, 1992; Kim *et al.*, 1993b), suggesting that DI RNAs with a long ORF have evolutionary advantages. This "repair" process was seen with DI RNAs which have either single-nucleotide substitutions or frameshift point mutations (de Groot *et al.*, 1992; Kim *et al.*, 1993b). This finding suggested that a long ORF provides a competitive advantage for RNA replication or accumulation; however, it did not address whether ORF is required for RNA replication. To critically address this question, we constructed a new type of mutant DI by inserting a tandem of termination codons at various locations within the ORF of DI RNA. The rationale is that nucleotide insertions (instead of substitutions) may prevent RNA recombination, allowing the unambiguous determination of the role of the ORF in DI RNA replication in the absence of recombination. For this purpose, we used the smallest DI RNA, DIssE RNA (Makino *et al.*, 1988b), for mutagenesis to minimize the possible effects from other RNA sequences or structures. DIssE contains an ORF spanning more than 75% of the entire RNA (Makino *et al.*, 1984, 1988b): the first 218 amino acids correspond to the N terminus of gene 1a protein; the following 250 amino acids are derived from the region of gene 1a at 3.3 to 4 kb from the 5′ end of the viral genome; and the last 101 amino acids represent the 3′ end of the N protein (gene 7) (Makino *et al.*, 1988b). The gene product(s) of this ORF has been detected in infected cells and in *in vitro* translation (Makino *et al.*, 1988b). Several truncation mutants of this ORF were made by inserting at different sites a 12-nt linker (see Materials and Methods), which provides TAG-stop codons in all three reading frames, generating three mutants, p25Stu/Nhe, p25Afl/Nhe, and p25Spe/Nhe, with

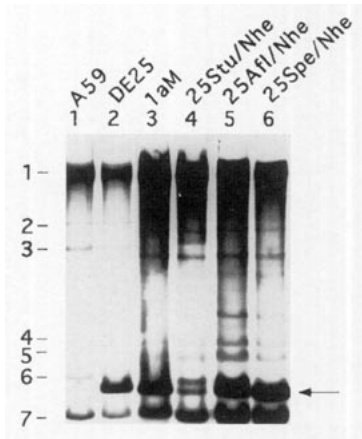

FIG. 2. RNA synthesis of various mutant DIs. [^{3}H]Uridine-labeled intracellular RNAs from passage 1 virus-infected DBT cells were denatured by glyoxal and analyzed by electrophoresis on 1% agarose gel. The seven MHV-A59-specific mRNAs are numbered (lane 1). Arrow indicates the DI RNAs.

ORFs of different sizes. To confirm the coding capacities of the mutants, RNAs were transcribed *in vitro* and translated in rabbit reticulocyte lysates. The translation products were analyzed directly by SDS–PAGE or by immunoprecipitation with anti-p28 antibody, which recognizes the amino-terminus of gene 1 polyprotein (Baker *et al.*, 1989), and anti-N protein antibodies. Sequence analysis predicted that the products of all three mutant RNAs should contain the p28 sequence but not the N gene. The results showed that all mutant DIs indeed encoded truncated proteins of predicted sizes, which were precipitable with the anti-p28 but not anti-N antibodies (data not shown) (see below).

We then examined whether these mutant DI RNAs could replicate in MHV-infected cells. RNAs were transfected into A59-infected DBT cells; the released virus (called passage 0 virus) was harvested at 12 hr p.i. and used to infect fresh DBT cells. The supernatant from this infection (passage 1 virus) and the infected intracellular RNAs [passage 1 (P1) RNA] were analyzed. Figure 2 shows that all three mutant DIs and wild-type DE25 from passage 1 virus replicated, although the 25Stu/Nhe RNA (lane 4) replicated slightly less efficiently than the others. In 25Stu/Nhe- and 25Atl/Nhe-transfected cells, there were two RNA species of DI size. One of these could be a newly generated DI, the nature of which was not further studied. To determine whether these RNAs represent the original mutant DI RNAs or have been replaced by recombinant RNAs with a restored ORF, the passage 1 RNAs from 25Stu/Nhe were amplified by RT–PCR with primers 629 and 538 (Fig. 1A), and 25Afl/Nhe and 25Spe/Nhe were amplified with primers 629 and 116 (Fig. 1A). All DI RNAs yielded RT–PCR products of predicted sizes (data not shown). These RT–PCR products were directly sequenced without cloning. The results showed that all of the mutant RNAs retained the 12-

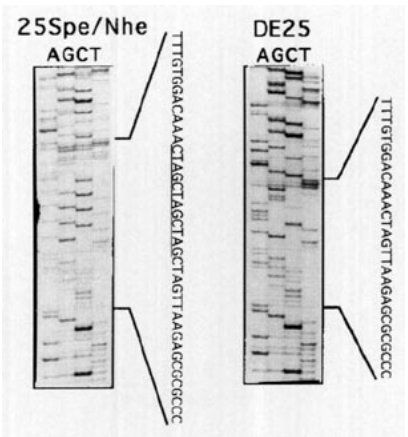

FIG. 3. Direct sequencing of RT–PCR products from passage 1 RNAs of mutant DIs. 25Spe/Nhe and DE25 P1 RNAs were amplified by RT–PCR using primers 629 (5′-ATATACAACATCTGTTGCATT-3′) and 116 (5′-TGGCGTCCGTACGTACCTAA-3′) (Fig. 1A), and the PCR products were subjected to direct sequencing using primer 629. The 12-nt amber-mutation linker is underlined for 25Spe/Nhe.

nt insertions, suggesting that the truncated ORFs were preserved and no recombination had occurred to remove these insertions, in contrast to the previously published DIs which contained point mutations and underwent recombination very rapidly (de Groot *et al.*, 1992; Kim *et al.*, 1993b). Representative sequencing gels are shown in Fig. 3. This result clearly indicated that these DI RNAs, which have a truncated ORF, still replicated efficiently. Restriction-digestion analysis of RT–PCR products from passage 2 intracellular RNAs demonstrated that *Nhe*I completely digested the RT–PCR products of all three mutant RNAs into two predicted products (Fig. 4, compare lanes 1 to 3 with lanes 4 to 6), further suggesting that the original *Nhe*I linker insertion was retained at least until passage 2. It is unlikely that the RT–PCR method preferentially amplified the original mutant RNAs but not recombinant RNA because, when mutant DIs underwent recombination, the same RT–PCR method detected only recombinant RNA but not the original DI RNA (see below). These results indicated that a 12-nt insertion within DI RNAs prevented or delayed the occurrence of recombination between helper and DI RNAs. Since the 12-nt linker contains termination codons in all three reading frames, it is unlikely that any other undetected mutations could have restored the ORF. Therefore, we conclude that DI RNAs with a truncated ORF can replicate efficiently.

To further confirm that the ORFs of mutant DIs remained truncated, we examined the translation capacity of P1 RNAs. We initially attempted to detect DI-specific proteins in DI RNA-transfected cells by metabolic labeling and immunoprecipitation with anti-p28 antibodies. However, no DI-specific proteins were discernible, probably due to low efficiency of DI RNA translation. Thus, we used an alternative approach to examine the translatability of P1 DI RNA *in vitro*. For this purpose, RT–PCR products encompassing the entire ORF of these mutants were generated from P1 intracellular RNAs using primers 116 and 857 (Fig. 1A), and cloned into the *Eco*RV site of vector pBluescript(SK); the resulting recombinant plasmids were transcribed and translated *in vitro*. This pair of primers should be able to detect the original mutant RNAs and any potential recombinant RNAs which have a restored ORF. The translation products were immunoprecipitated with anti-p28 or anti-N antibodies. Figure 5 shows that all three mutant DIs derived from P1 RNAs made truncated proteins of predicted sizes, which were precipitated by anti-p28 antibody (lanes 1 to 4) but not anti-N antibodies (lanes 6 to 9). A total of six independently isolated clones for 25Spe/Nhe, six clones for 25Afl/Nhe, and four clones for 25Stu/Nhe were examined, and all of them gave identical results (data not shown). These results demonstrated that at least most, if not all, of the mutant RNAs retained the original insertions. Taken together, these results clearly indicate that DI RNAs containing a truncated ORF can replicate.

Replication of a DI RNA with an ORF closed by substitutions of the initiation codons

We next examined whether DI RNA still replicated when the ORF was completely closed. For this purpose, we constructed a mutant DI (named 1aM) in which the first two AUG codons of ORF were converted into CCC (Fig. 1B). The closure of the ORF was confirmed by *in vitro* transcription and translation; the result showed that no protein products immunoprecipitable with anti-p28 or anti-N antibodies were synthesized (data not shown). The

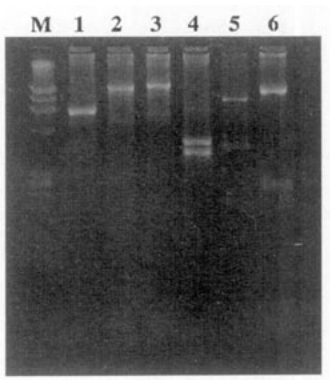

FIG. 4. Restriction analysis of PCR products from passage 2 RNAs of mutant DIs with truncated ORFs. The passage 2 intracellular RNAs from 25Stu/Nhe were amplified by RT–PCR using primers 630 and 116 (Fig. 1A), 25Afl/Nhe was amplified using primers 629 and 538 (5′-GCAGATCTATGGCAAAGATGGGCA-3′) (Fig. 1A), and 25Spe/Nhe was amplified using primers 629 and 116 (Fig. 1A). As controls, DE25 passage 2 RNA was also amplified using primers 630 and 116 (lane 1), 629 and 538 (lane 2), and 629 and 116 (lane 3), respectively. All RT–PCR products were digested by *Nhe*I, separated by electrophoresis on 1% agarose gel and stained with ethidium bromide. Lane 4, 25Stu/Nhe; lane 5, 25Afl/Nhe; lane 6, 25Spe/Nhe. Lane M is ϕX174 DNA digested with *Hae*III.

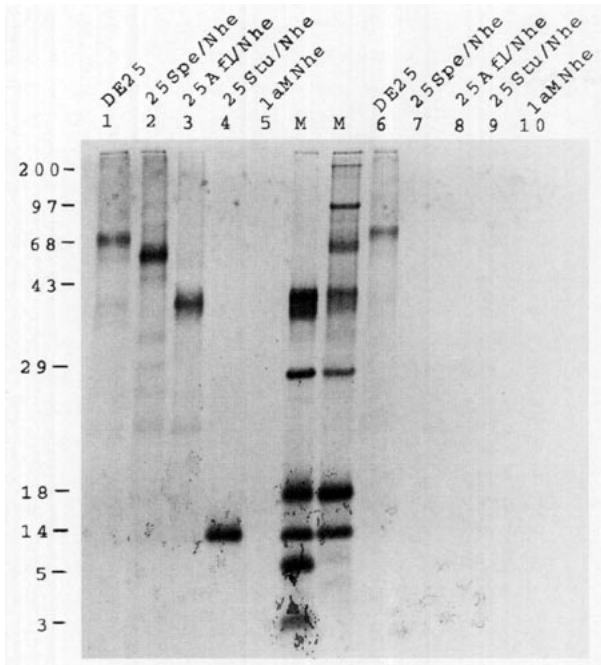

FIG. 5. Determination of coding capacity of mutant DI RNAs. The passage 1 intracellular RNAs of DE25, 25Stu/Nhe, 25Afl/Nhe, 25Spe/Nhe, and 1aMNhe were amplified by RT–PCR using primers 116 and 857 (TTCTCTTTACACATTAGAG) (Fig. 1A). The PCR products were cloned into the *Eco*RV site of pBluescript(SK) and *in vitro* transcribed into mRNAs using bacteriophage T3 or T7 RNA polymerase. [^{35}S]-Methionine-labeled proteins were *in vitro* synthesized using the mRNA-dependent rabbit reticulocyte lysate. The resulting proteins were immunoprecipitated and analyzed by SDS–PAGE on 5 to 20% gradient polyacrylamide gel. Lanes 1 to 5 were immunoprecipitated with anti-p28 antibody, and lanes 6 to 10 were immunoprecipitated with anti-N antibodies. Lanes M are ^{14}C-labeled size markers and the numbers on the left side are molecular sizes in kiloDaltons.

RNA was transfected into MHV-infected DBT cells, and the ^{3}H-labeled passage 1 RNAs were analyzed. As shown in Fig. 2, lane 3, 1aM RNA replicated as efficiently as other mutant and wild-type RNAs. The identity of the RNA was examined by direct sequencing of RT–PCR product made by using primers 630 and 116 (Fig. 1A). Surprisingly, sequence data showed that the ORF of the 1aM RNA in P1 had been restored, i.e., both CCCs were replaced with wild-type AUGs, and the sequences upstream of the ORF were changed to that of helper A59 (data not shown), indicating that homologous recombination between A59 viral RNA and 1aM RNA had occurred. The point of recombination was not determined. We have also cloned RT–PCR products and sequenced individual cDNA clones. Results showed that in all 21 clones examined, both CCCs have been converted to AUGs (data not shown). This result confirmed the high frequency of recombination between DI RNA and helper viral RNA and the competitive advantage of DI RNA with a long ORF (de Groot *et al.*, 1992; Kim *et al.*, 1993b). Thus, in contrast to the 12-nt insertions, the 6-nt substitution did not prevent RNA recombination. It is noteworthy that 1aM RNA did contain a 12-nt insertion at the *StuI* site, which is about 460 nt downstream of the AUG. Interestingly, all 21 cDNA clones retained this 12-nt insertion, as indicated by *Nhe*I digestion (data not shown). This result further suggested that 12-nt insertions indeed prevented recombination, but only in its immediate neighborhood. Because of recombination, we could not determine whether 1aM RNA itself replicated.

Replication of a closed-ORF DI mutant with both point mutations and amber-mutation-linker insertion

To address the true replicating ability of DI RNAs without an ORF, we inserted a 12-nt amber-mutation linker immediately downstream of the substituted initiation codons of the 1aM ORF to attempt to prevent RNA recombination. The RNA (1aMNhe) (Figs. 1A and 1B) was transfected into MHV-infected cells, and the [^{3}H]uridine-labeled passage 1 and passage 2 RNAs were analyzed. Figure 6A shows that 1aMNhe RNA replicated as well as the wild-type DI RNA at both passages. The identities of these RNAs were determined by direct sequencing of the RT–PCR products using primers 630 and 116 (Fig. 1A). The sequence data (Fig. 6B) demonstrated that the majority, if not all, of the PCR products derived from 1aMNhe DI RNA retained both the CCC codons and the amber-mutation linker at the initiation region of the ORF. To further confirm that the ORF of 1aMNhe DI RNA was not restored by recombination or mutation at other sites, the PCR product from P1 intracellular RNAs encompassing the entire ORF of 1aMNhe was analyzed for its coding capacities by *in vitro* transcription and translation. The results showed that no protein was detected by immunoprecipitation with either anti-p28 or anti-N antibodies, indicating that no viral protein was made (Fig. 5, lanes 5 and 10). We examined two different PCR clones, and both gave identical results. These findings demonstrated that MHV DI RNAs without a virus-specific ORF could replicate. We conclude that a *cis*-acting, virus-specific gene product encoded from the DI-specific fusion ORF is not required for MHV DI RNA replication and that a 12-nt insertion prevented or delayed the occurrence of RNA recombination, thus maintaining the integrity of the mutant DI during viral passages.

DISCUSSION

In this paper we have demonstrated that MHV mutant DI RNAs with a truncated or closed ORF replicated as well as the natural DI RNAs, suggesting that MHV DI RNA replication does not require the *cis*-acting functions of virus-specific fusion proteins. This study was made possible by our unique mutagenesis strategy, which prevented or delayed the occurrence of RNA recombination between the mutant DI and helper viral RNAs; therefore, DI RNA replication could be studied independently of RNA recombination. Our data suggest that MHV DI is

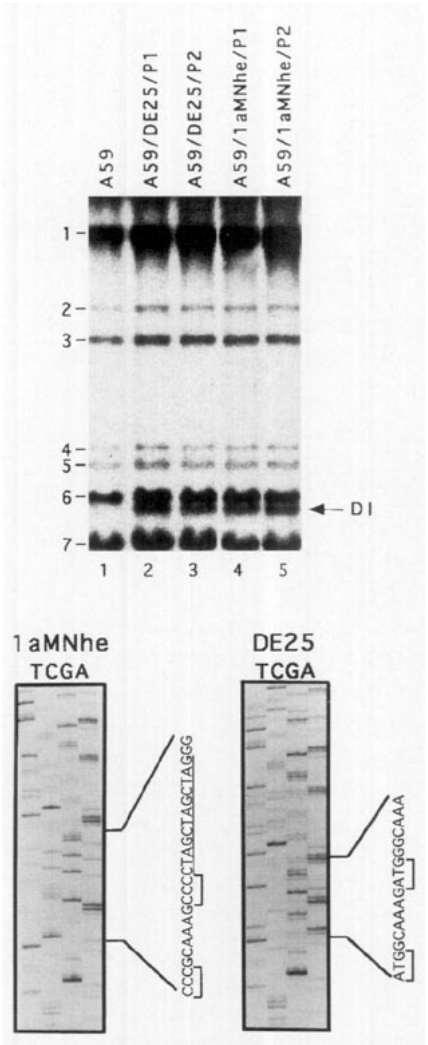

FIG. 6. Passage 1 and 2 RNAs of mutant 1aMNhe. (A) [^{3}H]Uridine-labeled intracellular RNAs from 1aMNhe-RNA-transfected and A59-infected DBT cells were denatured by glyoxal and analyzed by 1% agarose gel electrophoresis. Lane 1, A59 virus infection alone. Lanes 2 and 3, DE25 passage 1 and 2 RNAs, respectively. Lanes 4 and 5, 1aMNhe passage 1 and 2 RNAs, respectively. Arrow indicates the DI RNAs. (B) Direct sequencing of RT–PCR products from P1 RNA. 1aMNhe and DE25 P1 RNAs were amplified by RT–PCR using primers 629 (5′TACTATCAAATCTCTTTAGAC3′) and 116 (Fig. 1A), and the PCR products were subjected to direct sequencing using the same primer. The 12-nt amber-mutation linker is underlined, and CCC substitutions are indicated for 1aMNhe.

distinct from DI RNAs of poliovirus (Kuge *et al.*, 1986; Kaplan and Racaniello, 1988; Hagino-Yamagishi and Nomoto, 1989; Collis *et al.*, 1992) and clover yellow mosaic virus (White *et al.*, 1991, 1992), both of which require an ORF in DI RNAs to replicate. This conclusion raised several puzzling questions: First, why do all of the naturally occurring MHV DIs have a long ORF encoding at least part of gene 1a (Makino *et al.*, 1988a, 1990; van der Most *et al.*, 1991)? One possibility is that this fact is the fortuitous result of the nature of the *cis*-acting signal for MHV RNA replication, which includes part of the gene 1a-coding sequence (Lin and Lai, 1993; Kim *et al.*, 1993a). Second, why, as shown previously (de Groot *et al.*, 1992; Kim *et al.*, 1993b) and confirmed here, does a DI RNA with a longer ORF have a competitive advantage over a DI containing a shorter or no ORF at all, thus allowing the former DI RNA to accumulate? It has been demonstrated that DI RNAs containing ORFs of different sizes replicated equally well when they replicated separately; however, when they replicated together, the ones with a longer ORF rapidly predominated (Kim *et al.*, 1993b). Thus, the advantage of a long ORF was seen only when two types of RNA were in direct competition. One possible mechanism is that the binding and sliding of ribosomes along the DI RNA molecules increases the stability of RNA or removes RNA secondary structures so that the replication efficiency of DIs increases. The role of ribosomal or translational factors in removing RNA secondary structures to facilitate replication has been shown *in Qβ phage RNA replication* (Kamen, 1975; Weber and Weissmann, 1981). These factors may be present in low amounts, which are barely sufficient for the replication of one DI RNA species. When two DI RNAs are in competition, the longer ORF may capture these factors more effectively. All capped RNAs are expected to be scanned by ribosomes; the longer ORF may allow ribosomes and translation factors to stay with the RNA longer. Another potential indirect mechanism is that proteins from the translational machinery may stay bound to DI RNA after RNA replication and facilitate viral RNA packaging or uncoating in the next round of infection. Regardless, these postulated factors would facilitate, but are not absolutely required for, DI RNA replication. It should be noted that although our data showed that neither *cis*-acting viral protein nor DI-specific ORF are required for MHV DI RNA replication, we cannot rigorously rule out the possibility that translation per se is linked to RNA replication because the DI RNA mutants may still encode short and nonspecific proteins and that translation per se from these short ORFs may be linked to RNA replication. Nevertheless, our data clearly showed that an ORF encoding a *cis*-acting, virus-specific protein is not inherently required for RNA replication.

It should also be noted that the DIssE used in this study is the smallest DI RNA currently known. The short RNA may contain a minimum number of RNA secondary structures, and it is, thus, less dependent on cellular or viral factors for conformational alteration. Therefore, we cannot rule out the possibility that replication of MHV genomic RNAs may require ribosomal factors or *cis*-acting viral proteins for replication.

It has been shown that MHV can undergo type I homologous recombination (Lai, 1992) between two viral RNAs of different MHV strains (Lai *et al.*, 1985; Makino *et al.*, 1986; Keck *et al.*, 1987, 1988a,b), between viral genomic RNA and DI RNA (van der Most *et al.*, 1992; Furuya *et al.*, 1993; Kim *et al.*, 1993b), and even between viral genomic RNA and transfected RNA fragments (Koetzner *et al.*,

1992; Laio and Lai, 1992). The sequence and structural requirements of the parental RNA molecules for recombination are not yet clear. The results from this study provided an insight into the mechanism of RNA recombination. Our studies showed that a 12-nt insertion prevented or delayed the occurrence of homologous recombination in the vicinity of the insertion. This result is consistent with that of a deletion mutant DI studied previously, in which a 2.1-kb deletion prevented recombination repair by the helper virus (de Groot *et al.,* 1992). In contrast, nonsense point mutations and single-nucleotide deletion mutations were readily reverted to wild-type sequence through homologous recombination (de Groot *et al.,* 1992; Kim *et al.,* 1993b). Our results also indicated that even the 6-nt substitution mutant (1aM) underwent recombination. Taken together, these data suggest that homologous recombination readily occurs between DI RNA and helper viral RNAs when sequence differences do not involve extensive deletions or insertions. Conceivably, recombination requires the donor and recipient RNAs to be aligned either directly or indirectly with each other. The presence of a long stretch of insertion or deletion might interfere with the alignment and thus interfere with recombination. It will be of interest to know what extent of homology between two RNA molecules is required for type I homologous RNA recombination to occur. The DI RNA with a closed ORF described in this report may provide a convenient system for studying this issue. In this regard, it is particularly enlightening to note that recombination occurred at the AUG → CCC substitution sites, but not at the site of the 12-nt insertion, which is located 460 nt downstream in 1aM RNA, whereas recombination did not occur or occurred much more slowly at the substitution site in 1aMNhe, which contains the 12-nt insertion immediately adjacent to the substitutions. These observations further confirm the constraints imposed by sequence insertion on RNA recombination.

In conclusion, we have demonstrated that the replication of MHV DI did not require the viral gene product(s) encoded by its ORF, suggesting that no viral protein has *cis*-acting effects on DI RNA replication. Thus, although the length of DI ORF provides a competitive advantage for DI RNA accumulation, the viral gene product from ORF itself is not required for RNA replication. In addition, we found that the 12-nt insertions in DI RNAs delayed or reduced the frequency of homologous recombination between the DI RNA and the wild-type helper virus RNA. However, the occurrence of recombination is likely regulated by multiple factors such as the position and sequence of the insertions. Our system described here will allow further understanding of the mechanism of RNA recombination.

ACKNOWLEDGMENTS

We thank Daphne Shimoda for editorial assistance. This work was supported by Research Grant AI 19244 from the National Institutes of Health. M.M.C.L. is an Investigator of Howard Hughes Medical Institute.

REFERENCES

Akkina, R. K., Chambers, T. M., and Nayak, D. P. (1984). Expression of defective-interfering influenza virus-specific transcripts and polypeptides in infected cells. *J. Virol.* **51,** 395–440.

Baker, S. C., Shieh, C.-K., Soe, L. H., Chang, M.-F., Vannier, D. M., and Lai, M. M. C. (1989). Identification of a domain required for autoproteolytic cleavage of murine coronavirus gene A polyprotein. *J. Virol.* **63,** 3693–3699.

Collis, P. S., O'Donnell, B. J., Barton, D. J., Rogers, J. A., and Flanegan, J. B. (1992). Replication of poliovirus RNA and subgenomic RNA transcripts in transfected cells. *J. Virol.* **65,** 6480–6488.

de Groot, R. J., van der Most, R. G., and Spaan, W. J. M. (1992). The fitness of defective interfering murine coronavirus DI-a and its derivatives is decreased by nonsense and frameshift mutations. *J. Virol.* **66,** 5898–5905.

Denison, M. R., and Perlman, S. (1986). Translation and processing of mouse hepatitis virus virion RNA in a cell-free system. *J. Virol.* **60,** 12–18.

Furuya, T., Macnaughton, T. B., La Monica, N., and Lai, M. M. C. (1993). Natural evolution of coronavirus defective-interfering RNA involves RNA recombination. *Virology* **194,** 408–413.

Hagino-Yamagishi, K., and Nomoto, A. (1989). *In vitro* construction of poliovirus defective interfering particles. *J. Virol.* **63,** 5386–5392.

Hirano, N., Fujiwara, K., Hino, S., and Matsumoto, M. (1974). Replication and plaque formation of mouse hepatitis virus (MHV-2) in mouse cell line DBT culture. *Arch. Gesamte Virusforsch.* **44,** 298–302.

Jeong, Y. S., and Makino, S. (1994). Evidence for coronavirus discontinuous transcription. *J. Virol.* **68,** 2615–2623.

Kamen, R. I. (1975). Structure and function of the Qβ RNA replicase. *In* "RNA Phages" (N. D. Zinder, Ed.), pp. 203–234. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Kaplan, G., and Racaniello, V. R. (1988). Construction and characterization of poliovirus subgenomic replicons. *J. Virol.* **62,** 1687–1696.

Keck, J. G., Matsushima, G. A., Makino, S., Fleming, J. O., Vannier, D. M., Stohlman, S. A., and Lai, M. M. C. (1988a). In vivo RNA–RNA recombination of coronavirus in mouse brain. *J. Virol.* **62,** 1810–1813.

Keck, J. G., Soe, L. H., Makino, S., Stohlman, S. A., and Lai, M. M. C. (1988b). RNA recombination of murine coronaviruses: Recombination between fusion-positive mouse hepatitis virus A59 and fusion-negative mouse hepatitis virus 2. *J. Virol.* **62,** 1989–1998.

Keck, J. G., Stohlman, S. A., Soe, L. H., Makino, S., and Lai, M. M. C. (1987). Multiple recombination sites at 5′-end of the murine coronavirus RNA. *Virology* **156,** 331–341.

Kim, Y.-N., Jeong, Y. S., and Makino, S. (1993a). Analysis of *cis*-acting sequences essential for coronavirus defective interfering RNA replication. *Virology* **197,** 53–63.

Kim, Y.-N., Lai, M. M. C., and Makino, S. (1993b). Generation and selection of coronavirus defective interfering RNA with large open reading frame by RNA recombination and possible editing. *Virology* **194,** 244–253.

Koetzner, C. A., Parker, M. M., Ricard, C. S., Sturman, L. S., and Masters, P. S. (1992). Repair and mutagenesis of the genome of a deletion mutant of the coronavirus mouse hepatitis virus by targeted RNA-recombination. *J. Virol.* **66,** 1841–1848.

Kuge, S., Saito, I., and Nomoto, A. (1986). Primary structure of poliovirus defective-interfering particle genomes and possible generation mechanisms of the particles. *J. Mol. Biol.* **192,** 437–487.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Lai, M. M. C. (1990). Coronavirus: Organization, replication and expression of genome. *Annu. Rev. Microbiol.* **44,** 303–333.

Lai, M. M. C. (1992). RNA recombination in animal and plant viruses. *Microbiol. Rev.* **56,** 61–79.

Lai, M. M. C., Brayton, P. R., Armen, R. C., Patton, C. D., Pugh, C., and Stohlman, S. A. (1981). Mouse hepatitis virus A59: mRNA structure

and genetic localization of the sequence divergence from hepatotropic strain MHV-3. *J. Virol.* **39,** 823–834.

Lai, M. M. C., Baric, R. S., Brayton, P. B., and Stohlman, S. A. (1984). Characterization of leader RNA sequences on the virion and mRNAs of mouse hepatitis virus, a cytoplasmic RNA virus. *Proc. Natl. Acad. Sci. USA* **81,** 3626–3630.

Lai, M. M. C., Baric, R. S., Makino, S., Keck, J. G., Egbert, J., Leibowitz, J. L., and Stohlman, S. A. (1985). Recombination between nonsegmented RNA genomes of murine coronavirus. *J. Virol.* **56,** 449–456.

Lee, H.-J., Shieh, C.-K., Gorbalenya, A. E., Koonin, E. V., La Monica, N., Tuler, J., Bagdzhadzhyan, A., and Lai, M. M. C. (1991). The complete sequence (22 kilobases) of murine coronavirus gene 1 encoding the putative proteases and RNA polymerase. *Virology* **180,** 567–582.

Liao, C.-L., and Lai, M. M. C. (1992). RNA recombination in a coronavirus: Recombination between viral genomic RNA and transfected RNA fragments. *J. Virol.* **66,** 6117–6124.

Liao, C.-L., and Lai, M. M. C. (1994). Requirement of the 5′-end genomic sequence as an upstream *cis*-acting element for coronavirus subgenomic transcription. *J. Virol.* **68,** 4727–4737.

Lin, Y.-J., and Lai, M. M. C. (1993). Deletion mapping of a mouse hepatitis virus defective-interfering RNA reveals the requirement of an internal and discontiguous sequence for replication. *J. Virol.* **67,** 6110–6118.

Makino, S., and Lai, M. M. C. (1989). High-frequency leader sequence switching during coronavirus defective interfering RNA replication. *J. Virol.* **63,** 5285–5292.

Makino, S., Fujioka, N., and Fujiwara, K. (1985). Structure of the intracellular defective viral RNAs of defective interfering particles of mouse hepatitis virus. *J. Virol.* **54,** 329–336.

Makino, S., Keck, J. G., Stohlman, S. A., and Lai, M. M. C. (1986). High-frequency RNA recombination of murine coronaviruses. *J. Virol.* **57,** 729–737.

Makino, S., Shieh, C.-K., Keck, J. G., and Lai, M. M. C. (1988a). Defective-interfering particles of murine coronaviruses: Mechanism of synthesis of defective viral RNAs. *Virology* **163,** 104–111.

Makino, S., Shieh, C.-K., Soe, L. H., Baker, S. C., and Lai, M. M. C. (1988b). Primary structure and translation of a defective interfering RNA of murine coronavirus. *Virology* **166,** 1–11.

Makino, S., Taguchi, F., Hirano, N., and Fujiwara, K. (1984). Analysis of genomic and intracellular viral RNAs of small plaque mutants of mouse hepatitis virus, JHM strain. *Virology* **139,** 138–151.

Makino, S., Yokomori, K., and Lai, M. M. C. (1990). Analysis of efficiently packaged defective interfering RNAs of murine coronavirus: Localization of a possible RNA-packaging signal. *J. Virol.* **64,** 6045–6053.

Manaker, R. A., Piczak, C. V., Miller, A. A., and Stanton, M. F. (1961). A hepatitis virus complicating studies with mouse leukemia. *J. Natl. Cancer Inst.* **27,** 29–51.

Monroe, S. S., and Schlesinger, S. (1984). Common and distinct regions of defective-interfering RNAs of Sindbis virus. *J. Virol.* **49,** 865–872.

Nayak, D. P., Chambers, T. M., and Akkina, R. M. (1989). Structure of defective-interfering RNAs of influenza virus and their role in interference. *In* "The Influenza Viruses" (R. M. Krug, Ed.), pp. 269–317. Plenum Press, New York.

Pachuk, C. J., Bredenbeek, P. J., Zoltick, P. W., Spaan, W. J. M., and Weiss, S. R. (1989). Molecular cloning of the gene encoding the putative polymerase of mouse hepatitis coronavirus strain A59. *Virology* **171,** 141–148.

Soe, L. H., Shieh, C.-K., Baker, S. C., Chang, M.-F., and Lai, M. M. C. (1987). Sequence and translation of the murine coronavirus 5′-end genomic RNA reveals the N-terminal structure of the putative RNA polymerase. *J. Virol.* **61,** 3968–3976.

Spaan, W., Delius, H., Skinner, M., Armstrong, J., Rottier, P., Smeekens, S., van der Zeijst, B. A. M., and Siddell, S. G. (1983). Coronavirus mRNA synthesis involves fusion of noncontiguous sequences. *EMBO J.* **2,** 1839–1844.

van der Most, R. G., Bredenbeek, P. J., and Spaan, W. J. M. (1991). A domain at the 3′ end of the polymerase gene is essential for encapsidation of coronavirus defective interfering RNAs. *J. Virol.* **65,** 3219–3226.

van der Most, R. G., Heijnen, L., Spaan, W. J. M., and de Groot, R. J. (1992). Homologous RNA recombination allows efficient introduction of site-specific mutations into the genome of coronavirus MHV-A59 via synthetic co-replicating RNAs. *Nucleic Acids Res.* **20,** 3375–3381.

Weber, F. H., and Weissmann, C. (1981). Interaction of Qβ replicase with Qβ RNA. *J. Mol. Biol.* **153,** 631–660.

White, K. A., Bancroft, J. B., and Mackie, G. A. (1991). Defective RNAs of clover yellow mosaic virus encode nonstructural/coat protein fusion products. *Virology* **183,** 479–486.

White, K. A., Bancroft, J. B., and Mackie, G. A. (1992). Coding capacity determined in vivo accumulation of a defective RNA of clover yellow mosaic virus. *J. Virol.* **66,** 3069–3076.

Yokomori, K., Banner, L. R., and Lai, M. M. C. (1992). Coronavirus mRNA transcription: UV light transcriptional mapping studies suggest an early requirement for a genomic-length template. *J. Virol.* **66,** 4671–4678.

Zhang, X., Liao, C.-L., and Lai, M. M. C. (1994). Coronavirus leader RNA regulates and initiates both *in trans* and *in cis* subgenomic mRNA transcription. *J. Virol.* **68,** 4738–4746.