# Homologous RNA recombination allows efficient introduction of site-specific mutations into the genome of coronavirus MHV-A59 via synthetic co-replicating RNAs

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# ABSTRACT

We describe a novel strategy to site-specifically mutagenize the genome of an RNA virus by exploiting homologous RNA recombination between synthetic defective interfering (DI) RNA and the viral RNA. The construction of a full-length cDNA clone, pMIDI, of a DI RNA of coronavirus MHV strain A59 was reported previously (R.G. Van der Most, P.J. Bredenbeek, and W.J.M. Spaan (1991), J. Virol. 65, 3219-3226), RNA transcribed from this construct, is replicated efficiently in MHV-infected cells. Marker mutations introduced in MIDI RNA were replaced by the wild-type residues during replication. More importantly, however, these genetic markers were introduced into the viral genome: even in the absence of positive selection MHV recombinants could be isolated. This finding provides new prospects for the study of coronavirus replication using recombinant DNA techniques. As a first application, we describe the rescue of the temperature sensitive mutant MHV Albany-4 using DI-directed mutagenesis. Possibilities and limitations of this strategy are discussed.

# INTRODUCTION

Coronaviruses are a group of enveloped viruses that cause important diseases in domestic animals (for reviews see 1-3). The viral genome is a single-stranded, (+)-sense RNA molecule of exceptional length, e.g. the mouse hepatitis virus (MHV) has a genome of 32 kb (4,5). Coronaviruses have attracted attention because of their unusual replication strategy, which involves discontinuous RNA synthesis (6,7), subgenomic-size replicative forms (8,9) and homologous RNA recombination (10,11).

During mixed infection with different MHV strains, RNA recombination occurs at a remarkably high frequency, both in tissue culture and in infected mice (10-14). Moreover, there is evidence for recombination of infectious bronchitis virus genomes *in vivo* (15). Although the mechanism is still unknown, homologous RNA recombination in coronavirus-infected cells presumably occurs via template switching ('copy-choice'). As proposed for picornavirus RNA recombination (16,17),

polymerase complexes containing nascent RNA are thought to dissociate from their original template and anneal to another, after which RNA synthesis proceeds (11).

We are interested in genetic manipulation of coronaviruses. For several other RNA viruses full-length cDNA clones have been constructed from which infectious RNA can be transcribed in vitro or in vivo (18-24). The use of synthetic, infectious RNA allows detailed studies on viral replication and protein function by means of defined genetic changes. Clearly, a full-length cDNA clone would provide the most powerful and elegant tool to study the molecular genetics of coronaviruses. However, the extreme length of the genome poses an obvious technical problem. Here, we describe an alternative strategy to site-specifically introduce mutations into the MHV genome. This strategy exploits the highfrequency RNA recombination that occurs in MHV-infected cells (10,11) and involves the use of synthetic defective interfering (DI) RNAs. As delineated by Huang and Baltimore (25,26), DI RNAs are truncated genomes that have retained the replication signals, but depend on proteins encoded by the standard virus for replication and packaging. DI RNAs are readily generated during high m.o.i. passaging of MHV (27-29). Recently, we reported the construction of a full-length cDNA clone of an MHV DI RNA. RNA, transcribed from this construct in vitro, is efficiently replicated in infected cells (29). We now provide evidence for homologous RNA recombination between DI RNA and the standard virus genome. Marker mutations introduced into the synthetic DI RNA were replaced by the wild-type residues during replication in MHV-infected cells. More importantly however, these marker mutations were found to be incorporated into the genome of MHV-A59. DI-directed mutagenesis provides exciting new prospects for molecular genetic studies on coronaviruses, as was demonstrated by the rescue of the temperature-sensitive mutant Albany-4.

# MATERIALS AND METHODS

# Cells and viruses

Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) containing 10% fetal calf serum (FCS). MHV strains A59 and Albany-4 (kindly provided by Dr. P. Masters)

were grown in Sac<sup>-</sup>- and 17CII-cells, respectively. Concentrated virus stocks were prepared by PEG 6000 precipitation of tissue culture supernatants as described (30). Transfection experiments and undiluted passages of progeny virus were performed in L-cells. Stocks of recombinant vaccinia virus vTF7-3 (obtained from dr. B. Moss; 31) were prepared in rabbit kidney (RK13) cells.

## **Recombinant DNA techniques**

Standard procedures were used for recombinant DNA techniques (32,33). DNA sequence analysis was performed using T7 DNA polymerase (Pharmacia) according to the instructions of the manufacturer.

## Construction of pMIDI-derivatives

Marker mutations A (G-A; position 1778) and B (T-C; position 2297) were introduced in pMIDI (29) by PCRmutagenesis using oligonucleotide primers VII and VIII (Table I) and plasmid DNA as template. The generated PCR fragment was cut with XhoI and HincII and used to replace the corresponding XhoI-HincII fragment (nucleotides 1770-2298) in pMIDI. In order to eliminate the UAA termination codon in pMIDI (position 3357), the XhoII-SpeI fragment (nucleotides 3237 - 3689) of pMIDI was replaced by the corresponding fragment derived from an independent, DI-derived cDNA clone, pDI02 (29). This fragment contained the wild-type CAA codon at position 3357 and a serendipitous  $T \rightarrow C$  mutation at position 3572 (Marker C), presumably acquired during cDNA synthesis. Sequence analysis of the resulting plasmid pMIDI-C showed that no inadvertent mutations had been acquired during cloning of the DNA fragments and that all fragments were ligated correctly.

A silent  $T \rightarrow A$  substitution, serving as genetic marker, was introduced into the nucleocapsid sequence of pMIDI-C (position 5030) by PCR-mutagenesis using oligonucleotides IX and X (Table I) and pMIDI DNA (29) as template. The 460 bp DNA that was generated by PCR was cut with *AccI* and *SacI* and used to replace the corresponding *AccI-SacI* (nucleotides 5020-5418) fragment of pMIDI-C. The resulting plasmid was called pMIDI-C\*. The presence of the mutation was confirmed by sequence analysis.

### Introduction of synthetic DI RNAs into MHV-infected cells

Two procedures were used to introduce DI RNAs into MHVinfected cells. RNA transfection of mouse L-cells using *in vitro* transcribed RNA and lipofectin (BRL, Life technologies) was performed as described previously (29). As an alternative, we used DNA transfection and *in vivo* transcription of plasmid DNA by recombinant vaccinia virus vTF7-3-encoded T7 polymerase (31). Monolayers of  $2 \times 10^6$  L-cells grown in 35 mm wells were infected with vTF7-3 at an multiplicity of infection (m.o.i) of 5 PFU. At 45 min post infection (pi) the inoculum was replaced by DMEM minus FCS, followed by transfection with pMIDI-C\* DNA at 90 min pi. For each transfection, 10  $\mu$ l lipofectin was diluted in 200  $\mu$ l DMEM, mixed with 5 $\mu$ g of pMIDI-C\* DNA and kept for 10 min at room temperature. Incubation of vTF7-3 infected cells with this transfection mixture was for 10 min at room temperature, after which 800  $\mu$ l of DMEM minus FCS was added. Two hours after transfection the cells were super-infected with Albany-4 at an m.o.i. of 10 PFU.

# Isolation and analysis of viral RNAs

Intracellular RNA and viral genomic RNA was isolated as described by Spaan et al. (30). RNA was separated in formaldehyde-agarose gels (32). The gels were dried and hybridized with 5'-end-labelled oligonucleotide probes as was described by Meinkoth and Wahl (34). RNA sequence analysis was performed according to Fichot and Girard (35). Prior to sequencing, the RNA preparations were confirmed to be devoid of MIDI-C and other DI RNAs by hybridization as described above.

# cDNA synthesis and PCR amplification

To analyze MIDI-C RNA after replication, intracellular RNA from cells infected with passage 2 virus/DI mixture (Fig. 2A) was separated on a non-denaturing, 1% low melting-point agarose gel. The DI RNA was excised and isolated as described (32). First strand cDNA synthesis was performed using RNase H-free Moloney Murine Leukemia virus reverse transcriptase (BRL, Life Technologies) followed by PCR-amplification using *TaqI* polymerase (Promega) according to the instructions of the manufacturers. Oligonucleotides I and III were used. The generated PCR DNA was cut with *Hind*III (positions 1985 and 3782 in pMIDI-C), the 1.8 kb fragment was cloned into *Hind*III digested pUC20, followed by CaCl<sub>2</sub> transformation of *Escherichia coli* strain HB101. To ensure that the resulting bacterial colonies were independent, the cells were recovered for only 30 min at 37°C after heat shocking.

# Isolation of MHV recombinants

Passage 2 virus/DI mixture was plaqued as described (30). Virus from well-isolated plaques was amplified by infecting a monolayer of  $2 \times 10^5$  L-cells. Intracellular RNA was isolated

Table I.				
Oligo	sequence	polarity	Binding site in MIDI-C	ригрозе
I	5' ATTATGTCCAGCACAAGTGTG 3'	+	1744 - 1764	PCR
Π	5' ACCGACATAGAGTCGATC 3'	-	2330-2347	PCR/RNA sequencing
ш	5' ACGTGACTCCAGCACACGTC 3'	-	3894 - 3914	PCR/RNA sequencing
IV	5' TGTCAACGAAATTCT 3'	_	2290-2304	Diff. Hybridization
V	5' AGACTATAGTCACCCCTATT 3'	-	1827 - 1846	RNA sequencing
VI	5' TTCCTGAGCCTGTCTACG 3'	-	5019-5036	Hybridization
VII	5' TGGCAATCTCGAGCAAAGAGCTATC 3'	+	1763 1787	Mutagenesis
VШ	5' AACGAAATTCTTGACAAGCTC 3'	-	2280-2300	Mutagenesis
IX	5' GGGCGTAGACAGGCACAGGAAAAGAAAG 3'	+	5016-5043	Mutagenesis
Х	5' TTTTGTGATTCTTCCAATTGGCCAT 3'	-	5475 - 5498	PCR
XI	5' GGGCTTTGCAACGCTTAC 3'	_	5058-5075	RNA Sequencing
XII	5' GCTCTTAACTAGTTTGTCCACAAAG 3'		3677-3701	RNA Sequencing

and subjected to cDNA synthesis and PCR amplification using oligonucleotides I and II as described above. The PCR DNA was separated on 1% agarose- $0.5 \times TAE$  gels. Hybridization in dried gels (34) was carried out for 16 hrs at 35°C using 5' end-labelled oligonucleotide IV as a probe in  $5 \times SSPE$  (0.9 M NaCl, 0.05 M NaPO<sub>4</sub>, pH 7.7, 0.005 M EDTA) containing  $5 \times$  Denhardt's reagent, 0.05% SDS and 100 µg/ml alkali-degraded yeast tRNA. The gels were washed  $3 \times 20'$  at 35°C in  $5 \times SSPE$ , 0.05% SDS followed by autoradiography.

## RESULTS

#### Construction of pMIDI-C

We previously reported the construction of pMIDI, a full-length cDNA clone of an MHV DI RNA. pMIDI consists of three noncontiguous regions of the MHV genome, namely the 5' most 3889 nucleotides, the 3' most 806 nucleotides and in between, 799 nucleotides derived from ORF1b of the polymerase gene (Fig. 1). This latter segment contains the encapsidation signal (29,36). The three segments are joined in-frame, generating a 'full-length' reading frame for a 184K polypeptide. However, in pMIDI the reading frame is interrupted by a UAA termination codon at position 3357. To study recombination between MHV DI RNAs and the MHV genome, we constructed a pMIDI-derivative, pMIDI-C, in which three silent point mutations were introduced as genetic markers at positions 1778, 2297 and 3572 (mutations A, B and C, respectively; Fig. 1). In addition, the termination codon at position 3357 was replaced by the wild-type CAA codon.

# Point mutations in MIDI-C are replaced by the wild-type sequence during replication in MHV-A59 infected cells

RNA transcribed from pMIDI-C was used for transfection of MHV-infected mouse L-cells. At 12 hrs after transfection the tissue-culture supernatant, containing virus and DI particles, was harvested and passaged twice on fresh L-cells. An outline of the experiment is shown in Fig. 2a. Total intracellular RNA was isolated from passages 0, 1 and 2 (for nomenclature see Fig. 2a) and analyzed by hybridization using (-) sense oligonucleotide probe VI (Table I). This probe is complementary to the 3' end of the genome and hybridizes to MIDI-C and all seven MHV RNAs. As shown in Fig. 2b, MIDI-C RNA was replicated in MHV-infected cells and strongly interfered with viral mRNA

synthesis in cells infected with passage 1 (p1) virus/DI mixture.

To determine whether recombination had occurred between the MHV genome and the synthetic DI RNA, the sequence of p2 MIDI-C RNA was examined. For this purpose, the DI RNA was subjected to cDNA synthesis followed by PCR amplification (RT-PCR) using the oligonucleotide primers I and III (Fig. 1: Table I). To prevent in vitro recombination of genomic and DI sequences during RT-PCR, the MIDI-C RNA used as a template was gel-purified. It should be noted that genomic RNA was already present in very low amounts in unfractionated p2 RNA (Fig. 2b). RT-PCR produced a single, DI-specific DNA species with an expected length of 1.9 kb. This DNA was digested with HindIII (positions 1985 and 3782 in pMIDI-C) and the resulting 1.8 kb fragment was cloned into pUC20. Sequence analysis of 64 independent clones showed that mutations B and C had been replaced by the wild-type sequence in 14 (22%) and 4 clones (6%) respectively. Mutation A (position 1778) is not located on the cloned HindIII fragment and was thus not analyzed.

Since (i) the mutations were exclusively replaced by the wildtype sequence and (ii) the frequency with which the mutations were replaced depended on their distance from the artificial junction of the 1a and 1b segment, reversion by point mutation seems very unlikely. In fact, these results are best explained by homologous RNA recombination between MIDI-C RNA and the genome of the standard virus.

#### Isolation of MHV recombinants

RNA recombination between MIDI-C RNA and the viral genome could, in principle, also yield recombinant viruses carrying the marker mutations. To study this possibility, p2 virus was plaqued and 150 well-isolated plaques were used to inoculate mouse L-cells. Total cellular RNA, isolated from the infected cells, was subjected to RT-PCR using oligonucleotides I and II (Fig. 1; Table I). In all cases a DNA fragment with the expected length of 0.6 kb was generated. An additional, faster migrating DNA species was observed consistently and probably represented single-stranded material. Evidently, the 0.6 kb fragment would also be produced with MIDI-C RNA as template (Fig. 1). However, MHV DIs are rapidly lost by infecting at a low m.o.i. (27,29) and the obtained PCR fragments were therefore expected to be genome-specific.

The PCR-DNAs were screened for the presence of marker mutation B by differential hybridization using the 15-mer



Fig. 1. Schematic representation of the structure of MIDI-C RNA and the MHV-A59 genome. The different parts of MIDI-C, derived from the 5'end (ORF1a), ORF1b and 3' end of the MHV-A59 genome (N) are indicated. The locations are shown of marker mutations A, B and C, and of the oligonucleotides I, II and III, used for RT-PCR. The orientations of these oligonucleotides are indicated by arrowheads.



Fig. 2. Transfection and passage of MIDI-C RNA in tissue culture cells. A. Schematic outline of the experiment and explanation of the nomenclature used for virus stocks and intracellular RNA preparations. B. Analysis of intracellular RNAs. MHV-A59-infected L-cells were either transfected with MIDI-C RNA or mock-transfected with phosphate-buffered saline (PBS). The resulting virus stocks were passaged on fresh L-cells. RNA from transfected cells (p0), passage 1 (p1) RNA and passage 2 (p2) RNA were separated in formaldehyde-agarose gels and hybridized to 5' end labelled oligonucleotide VI. The viral mRNAs and mIDI-C RNA on passage 2. RNA obtained after initial mock-transfection endogenous MHV DIs accumulate.



Fig. 3. Differential hybridization of RT-PCR DNA. The p2 virus/DI mixture was plaqued. Intracellular RNAs isolated from cells infected with virus of 150 randomly-chosen plaques was subjected to RT-PCR using oligonucleotides I and II. Equal amounts of PCR-DNAs were separated in 1% agarose gels and hybridized to oligonucleotide IV. The PCR-fragments generated for plaques 1 through 8 are shown. The 752 bp *Xhol-PstI* (nucleotides 1771 – 2323) fragments derived from pMIDI-C (+) and pMIDI (-) were used as positive and negative controls, respectively.

oligonucleotide probe IV (5' TGTCAACGAAATTCT 3', the guanine residue complementary to the introduced cytidine is underlined). PCR-DNA derived from two plaques, 4 and 138, specifically hybridized with this probe (Fig. 3 and not shown). The RNA preparations that had been used for RT-PCR were confirmed to be devoid of MIDI-C RNA by Northern blot analysis (not shown). Therefore, the hybridization with oligo IV indicated that marker B had been incorporated in the viral genomes.

To determine whether viruses 4 and 138 were true recombinants, four consecutive plaque purifications were performed. Of each plaque generation, three to five well-isolated plaques were analyzed by differential hybridization of PCR-amplified cDNA. Wild-type MHV, treated identically, served as a negative control. In all cases, the progeny of viruses 4 and 138 contained mutation B (not shown), confirming that this genetic marker had been introduced into the viral genome and strongly arguing against MIDI-C contamination.

As a final control, we performed direct RNA sequencing. Stocks of viruses 4 and 138, that had been plaque-purified twice, were used to infect L-cells at an m.o.i. of 10. Intracellular RNAs were harvested and used for RNA sequence analysis using the oligonucleotide primers II and V (Figs. 1, 4; Table I). In the case of virus 4, sequence analysis was also performed on RNA isolated from sucrose-gradient purified virus. In addition to mutation B, the RNAs of viruses 4 and 138 contained mutation A (Fig. 4). Analysis of virus 4 RNA using oligonucleotide primer XII (Table I) showed that mutation C was absent. By using oligonucleotide III as a primer, we could distinguish between MIDI-C RNA and genomic RNA: as shown in Fig. 1, this oligonucleotide is derived from the 3' end of ORF1b, but in MIDI-C binds immediately downstream of the artificial ORF1a/1b junction. Priming on DI RNA would thus yield an ORF1a sequence. However, for both intracellular RNA preparations the ORF1b sequence was obtained; consequently, priming by oligonucleotides II, III, V and XII had occurred on genomic RNA.

On the basis of our combined data, we concluded viruses 4 and 138 to be MHV mutants, generated by homologous RNA recombination between the synthetic MIDI-C RNA and the MHV-A59 genome.

# Rescue of the MHV Albany-4 ts-mutant by homologous recombination

Having demonstrated that sequences of synthetic DI RNAs can be introduced into the viral genome via RNA recombination, we explored 'DI-directed mutagenesis' as a means to identify and localize mutations that result in a conditionally lethal phenotype. For this purpose, we used MHV strain Albany-4, a temperaturesensitive (*ts*-) mutant of MHV-A59. The *ts*-phenotype of this virus is thought to be the result of an 87 nucleotides deletion in the nucleocapsid (N) gene (nt 1138–1224 of the N-ORF). At 39°C, virus growth is impaired. Also, incubation of Albany-4 virions at 39°C abolishes infectivity (37).

pMIDI-C contains the 3' terminal 510 nucleotides of the wildtype N-ORF including the 87 nucleotides that are deleted in Albany-4 (Fig. 5). Incorporation of the N-sequences of MIDI-C RNA into the Albany-4 genome via RNA recombination should eliminate the *ts*- defect and generate wild-type MHV. To distinguish rescued Albany-4 recombinants from MHV-A59 contaminants, a silent  $T \rightarrow A$  substitution was introduced into the N sequence of pMIDI-C (nucleotide 1200 of the N-ORF) as a genetic marker (Fig. 5). The resulting plasmid was called pMIDI-C\*.

In this set of experiments, we used DNA transfection and *in vivo* transcription by vaccinia virus-expressed T7 polymerase as an alternative to RNA transfection to introduce MIDI-C\* into MHV-infected cells. Mouse L-cells, infected with recombinant vaccinia virus vTF7-3 (31), were either transfected with pMIDI-C\* DNA or mock-transfected with PBS. Subsequently, these cells were super-infected with MHV Albany-4. After a 25 hr



XO Y Y PLAQUES 1 2 3 1 MIDHC\* 4 5 6 7

Fig. 6. Intracellular RNAs of MHV Albany-4 recombinants. Stocks of MHV Albany-4 (p2, grown at 39°C) obtained after an initial transfection with pMIDI-C\* or an initial mock-transfection with PBS, were used to infect fresh L-cells at 37°C to isolate intracellular RNAs (lanes 'MOCK' and 'MIDI-C\*'). Also, the p2 virus/MIDI-C\* mixture was plaqued at 39°C. Virus from four well-isolated, randomly-chosen plaques was propagated and intracellular RNAs were isolated (lanes 1-4). The RNA preparations were separated in formaldehyde-agarose gels and hybridized to 5'-end labelled oligonucleotide probe VI.

Fig. 4. Sequence analysis of intracellular RNA isolated from cells infected with MHV recombinant 4. Mutations A and B were analyzed using oligonucleotides V and II, respectively. Sequences are presented as (-)strand RNA. Arrowheads indicate the introduced mutations. Identical results were obtained for recombinant 138 (not shown).



Fig. 5. Rescue of the Albany-4 ts-lesion via RNA recombination between the viral genome and MIDI-C\* RNA. MIDI-C\* and the genomic RNAs of MHV-A59 and Albany-4 are shown schematically. Nucleocapsid sequences (N) are depicted as boxes; the shaded boxes represent the 87 nucleotides that are deleted in Albany-4 (nucleotides 1138-1224 of the nucleocapsid ORF). A silent  $U \rightarrow A$  substitution that was introduced in MIDI-C\* as a genetic marker is indicated by an asterisk. The locations of the oligonucleotides VI and XI are also shown.

incubation at 33°C, the tissue culture supernatants were harvested and passaged once at 33°C in fresh L-cells, followed by a single passage at 39°C. Vaccinia virus remains cell-associated, and is therefore lost during passage. The 39°C/p2 virus stocks were used to infect monolayers of L-cells at 37°C. Intracellular RNAs were separated in formaldehyde-agarose gels and hybridized to 5'-end labelled oligonucleotide VI. This probe binds to the



Fig. 7. RNA sequence analysis of recombinant Albany-4 viruses. Intracellular RNA of four plaque-purified recombinant Albany-4 viruses, described in Fig. 6, was subjected to sequence analysis using oligonucleotide XI as a primer. Intracellular RNA from Albany-4- and MHV-A59-infected cells served as controls. Sequences are presented as (-)strand RNA. An arrowhead indicates the introduced marker mutation.

sequence, that has been deleted in Albany-4 (Table 1; Fig. 5) and is therefore specific for the wild-type N-gene. Strikingly, probe VI not only detected MIDI-C\* RNA but also the nested set of MHV mRNAs (Fig. 6, lanes 'MOCK' and 'MIDI-C\*'), indicating that during passage, viruses had accumulated that had incorporated MIDI-C\* sequences into their genomes.

To obtain direct evidence for this, the p2 virus/DI mixture was plaqued at 39°C and virus from 20 randomly-chosen plaques was propagated in L-cell monolayers. For 19 of these plaque-purified viruses, the intracellular RNAs hybridized to probe VI in a dotblot assay (not shown). Four RNA preparations were analyzed in more detail. Gel-hybridization using oligonucleotide probe VI yielded the nested set of MHV RNAs; MIDI-C\* RNA was not detected (Fig. 6, lanes 1–4). Sequence analysis showed that the wild-type N-sequence had been restored and that the U $\rightarrow$ A marker mutation at nucleotide 1200 of the N-ORF was present (Fig. 7), providing formal evidence that the isolated viruses were generated by recombination between MIDI-C\* RNA and the Albany-4 genomic RNA.

## DISCUSSION

Homologous RNA recombination has been described for a number of positive-stranded RNA viruses (16,17,38,39). For the coronavirus MHV in particular, recombination of genomic RNAs has been well documented (10-13,40). Here, we extend these observations by providing evidence for recombination between DI RNAs and the MHV-A59 genome. The synthetic DI RNA used in this study, MIDI-C, consisted of three non-contiguous parts of the MHV genome: the 5'-most 3889 nucleotides containing the 5' non-translated region and part of ORF1a. a 799 nucleotide middle segment derived from ORF1b and 806 nucleotides encompassing the 3' end of the nucleocapsid (N) gene and the 3' non-translated region (29). We have shown that silent mutations introduced into the ORF1a sequence of MIDI-C were exclusively replaced by the wild-type residues during DI replication in MHV-infected cells. These marker mutations, however, were not replaced at an equal rate: markers B and C, located 1500 and 300 nucleotides upstream of the artificial ORF1a/1b border in MIDI-C, were replaced in 22% and 6% of the passage 2 DI RNAs, respectively. Apparently, the frequency of replacement correlated with the distance between the mutation and the ORF1a/1b border, i.e. the region in which template-switching has to occur in order to remove the mutation while maintaining the original MIDI-C structure. These finding are in accordance with the current model for coronavirus RNA recombination, in which template-switching by the viral polymerase occurs randomly (11,41). In this model, the probability of recombination in a defined region of an RNA molecule is proportional to the length of this region.

Most convincingly, recombination between MIDI-C RNA and the MHV genomic RNA was demonstrated by the identification of viruses that had incorporated the genetic markers A and B into their genomes. It should be emphasized that these viruses were isolated in the absence of selection. These findings lead to the important conclusion that synthetic DI RNAs can be used to site-specifically alter the MHV genome by exploiting RNA recombination. The potential of DI-directed mutagenesis is illustrated by the rescue of the conditionally lethal mutant Albany-4. The *ts*-phenotype of this mutant is caused by a deletion at the 3' end of the N-gene (37). Viruses that had restored the N-gene by recombination with MIDI-C\* RNA, were selected for by passaging the virus/DI mixture at the restrictive temperature.

The fact that synthetic DI RNAs can be used to modify the MHV genome may have important implications. For other RNA viruses, e.g. alphaviruses and picornaviruses, the use of infectious RNA transcripts derived from full-length cDNA clones has profoundly advanced the study of replication and pathogenesis (42-50). In the case of MHV however, the construction of a full-length cDNA clone is hampered by the extreme length of the viral genome. Until such a clone becomes available, the only means to generate defined genetic changes will be through homologous RNA recombination. In principle, our mutagenesis strategy, which is reminiscent of that used for large DNA viruses (51,52), should be applicable to other coronaviruses as well, provided that these viruses support DI replication and exhibit high frequency RNA recombination. As shown by recent studies, these properties are not unique to MHV (15,53).

Several applications of DI-directed mutagenesis come to mind. Firstly, it should be possible to map other mutations that result in a conditionally lethal phenotype; as for Sindbis virus (42-45),

the characterization of MHV *ts*-mutants that exhibit impaired RNA synthesis at the restrictive temperature (54-56), will further our understanding of the coronavirus RNA polymerase. Secondly, it should be possible to introduce mutations that change the biological properties of coronaviruses. In addition to the genes encoding the polymerase and the structural proteins, coronaviruses contain several accessory genes that are not required for growth in tissue culture (57-61). By mutating these genes, their role during virus replication in the natural host can be assessed. Finally, foreign sequences, e.g heterologous genes under the control of a transcription-initiation signal, may be introduced at the 3' end of the MHV genome. The isolation of such recombinants will be facilitated by selecting for repair of the Albany-4 *ts*-lesion.

Clearly, there will be limitations in this system: thus far, we have introduced mutations only in the 5' and 3' terminal regions of the MHV genome. It remains to be determined whether mutations can be introduced efficiently into more internal regions, since this would require double recombination events. Also, in the case of mutations causing reduced replication *in vitro*, the screening for recombinant viruses will be difficult. Presumably, such problems can be solved by improving screening procedures and by applying selection, e.g. via rescue of *ts*-markers (this paper) or by using neutralizing antibodies (40). Studies to address these issues are currently in progress.

While this manuscript was being completed, a publication by Koetzner et al. (62) appeared describing the rescue of MHV Albany-4 by targeted RNA recombination using a synthetic mRNA 7 homologue. However, as stated by these authors the observed recombination frequency using this method was too low to allow direct identification of recombinants without selection. In fact, it was anticipated that a more general applicability of targeted RNA recombination would require finding conditions that favour higher rates of recombination of exogenous RNA. The results described in the present paper show that by using synthetic co-replicating DI RNAs site-specific mutations can be introduced efficiently into the MHV genome.

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