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Translation but Not the Encoded Sequence Is Essential for the Efficient Propagation of the Defective Interfering RNAs of the Coronavirus Mouse Hepatitis Virus

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The defective interfering (DI) RNA MIDI of mouse hepatitis virus strain A59 (MHV-A59) contains a large open reading frame (ORF) spanning almost its entire genome. This ORF consists of sequences derived from ORF1a, ORF1b, and the nucleocapsid gene. We have previously demonstrated that mutations that disrupt the ORF decrease the fitness of MIDI and its derivatives (R. J. de Groot, R. G. van der Most, and W. J. M. Spaan, J. Virol. 66:5898–5905, 1992). To determine whether translation of the ORF per se is required or whether the encoded polypeptide or a specific sequence is involved, we analyzed sets of related DI RNAs containing different ORFs. After partial deletion of ORF1b and nucleocapsid gene sequences, disruption of the remaining ORF is still lethal; translation of the entire ORF is not essential, however. When a large fragment of the MHV-A59 spike gene, which is not present in any of the MHV-A59 DI RNAs identified so far, was inserted in-frame into a MIDI derivative, translation across this sequence was vital to DI RNA survival. Thus, the translated sequence is irrelevant, indicating that translation per se plays a crucial role in DI virus propagation. Next, it was examined during which step of the viral life cycle translation plays its role. Since the requirement for translation also exists in DI RNA-transfected and MHV-infected cells, it follows that either the synthesis or degradation of DI RNAs is affected by translation.

Defective interfering (DI) viruses rely for their propagation on the proteins encoded by the nondefective standard virus (7–9, 33). The truncated, and in some cases rearranged, genomes of DI viruses have lost most of their protein-encoding sequences but have retained the essential *cis*-acting replication and encapsidation signals and are therefore excellent tools to study these signals. For many animal and plant RNA viruses, DI viruses have been isolated and characterized (7, 33). The construction of full-length cDNA clones of the RNA genomes of DI viruses has allowed a mutational analysis of in vitro-transcribed DI RNAs and has provided important new insights into the nature of the *cis*-acting signals that mediate RNA packaging and replication (14, 29, 44). Such studies have also revealed that there are in some cases additional requirements for replication, e.g., Calain and Roux (2) reported that a Sendai virus DI RNA is only a suitable template for replication when its total genome length is a multiple of six nucleotides (nt). This requirement is supposedly related to RNA encapsidation.

For coronaviruses, a group of enveloped viruses containing a single-stranded, positive-sense RNA genome of approximately 32 kb (13, 36), several DI RNAs have been described (18, 24, 39). Three DI RNAs of the coronavirus mouse hepatitis virus (MHV) were used as templates to construct full-length cDNA clones from which DI RNAs can be transcribed in vitro (21, 24, 39) and in vivo (42). These synthetic DI RNAs have been used to map the sequences that are involved in RNA packaging (4, 24, 39), subgenomic mRNA synthesis (19, 40), and RNA replication (11, 15). A typical feature of the MHV DI RNAs is that they contain large open reading frames (ORFs) (23, 24, 39). The MHV strain A59 (MHV-A59) DI MIDI that we have described previously consists of three noncontiguous fragments of the viral genome, i.e., the 5′-terminal 3,889 nt containing sequences from the first ORF of the polymerase gene (ORF1a), 799 nt derived from the second ORF of the polymerase gene (ORF1b), and the 3′-terminal 806 nt comprising the 3′ end of the nucleocapsid gene (N) (39). These fragments have been fused in frame, creating a full-length ORF for a 184-kDa polypeptide (3, 39). We have shown previously that translation of this large fusion ORF is essential for efficient propagation of MIDI and its derivatives (3): when two similar DI RNAs with different-sized ORFs were compared in a competition experiment, the DI RNA with the largest ORF invariably prevailed. In addition, propagation of DI RNAs that contained a truncated ORF resulted in the appearance of escape mutants carrying a full-length ORF. Although these experiments clearly established the necessity of translating a large ORF for DI RNA fitness, they did not reveal why translation is required. Explanations include a role of translation in mRNA stability (3) or RNA replication, packaging, or uncoating. To resolve this issue, it is important to know (i) whether translation per se is necessary or the encoded polypeptide or a specific amino acid sequence is involved and (ii) whether translation is required in DI RNA-transfected and MHV-infected cells or only after passaging. In this study, we have addressed both questions by analyzing three sets of related DI RNAs that contain different ORFs. First, we provide evidence that the requirement for translation is not sequence specific: unrelated sequences inserted into the DI virus RNA genome must also be translated for the DI virus to be propagated. Second, we show that translation of DI RNAs is important in DI RNA-transfected and virus-infected cells. Possible roles of translation in DI virus propagation are discussed.

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TABLE 1. Sequences and characteristics of the oligonucleotides used in this study

Oligonucleotide	Sequence	Polarity[a]	Binding site in:		
			MIDI	DISP3	ΔH-*out*
48	5′ GTGATTCTTCCAATTGGCCATG 3′	−	5472–5494	5619–5641	3407–3429
c006	5′ CGGAATTCCGACGCGT(T/A)AGATAATGTAAGC 3′	+	5050–5063		
c037	5′ CTGGACGTCG(T/C)AAC 3′	+	Cloning cassette		
c038	5′ (A/G)CGACGTCCAGGTT 3′	−	Cloning cassette		
24	5′ ATTATGTCCAGCACAAGTGTG 3′	+	1743–1764	1743–1764	1743–1764
42	5′ AATCCACCCGACTTTA 3′	+		4817–4833	
c025	5′ TACGCCATTCAAATCGG 3′	−	4122–4139		2057–2074
116	5′ CGTCACTGGCAGAGAACG 3′	−	152–170	152–170	152–170
c112	5′ CCTC(A/T)GAGGTGCA 3′	+/−	Cloning cassette		

[a] −, antisense; +, sense; +/−, both.

MATERIALS AND METHODS

Cells and viruses. Cells were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum. MHV-A59 was grown in 17Cl1 cells to prepare high-titered virus-stocks (35). Mouse L cells were used for transfection and passaging experiments. Infection of L cells and passage experiments were performed as described previously (3, 37).

Recombinant DNA techniques. Standard procedures were used for recombinant DNA techniques (34). DNA sequence analysis was performed with T7 DNA polymerase (Pharmacia) and [α-^{33}P]dATP according to the instructions of the manufacturer.

Construction of plasmids. (i) pDIF. A new ORF1b-N junction was created in pΔH-*in* and pΔH-*out* by PCR mutagenesis, using oligonucleotide primers 48 and c006 and pMIDI (39) as a template. Oligonucleotide c006 contains an adenine or thymidine residue at position 17 (Table 1). The resulting 461-nt PCR product was digested with *Mlu*I and *Sac*I and cloned into *Mlu*I-*Sac*I-cut pUC21. Inserts of recombinant plasmids were sequenced (Pharmacia). Selected plasmids were digested with *Mlu*I and *Sac*I, and the inserts were used to replace the 374-nt *Mlu*I-*Sac*I fragments from pΔH-*in* and pΔH-*out* (3), yielding a set of four pDIF plasmids (Fig. 1).

(ii) pDISP. A double-stranded cassette consisting of oligonucleotides c037 and c038 (Table 1) was introduced into the unique *Dra*III site of pDIF-85T. This cassette contains an *Aat*II site and either a cytidine or thymidine residue at position 11 (c037 [Table 1]). The resulting plasmids were designated pDIF-C and pDIF-T. A 3.6-kb *Eco*RI-*Aat*II fragment from the MHV-A59 spike gene (17) was obtained from plasmid pTUG31MS, which is a derivative of pDGE2 (43), and was used to replace the *Eco*RI-*Aat*II fragments from pDIF-C and pDIF-T. Constructs pDISP3Eco and pDISP6Eco were digested with *Eco*RI, treated with mung bean nuclease, and religated, generating pDISP3 and pDISP6, respectively.

(iii) pΔP-*130* and pΔP-*30*. pMIDI-C (42) was digested with *Pst*I, yielding a 1.5-kb insert and a 6.6-kb fragment containing DI RNA and vector sequences. A double-stranded cassette (oligonucleotide c112 [Table 1]) was inserted into the *Pst*I site of the 6.6-kb fragment. The cassette contains *Pst*I-compatible sticky ends and either an adenine or thymidine residue at position 5. The resulting plasmids were denoted pΔP-*130* and pΔP-*30*.

In vitro transcription and transfection. Plasmid DNA was linearized with *Nhe*I and transcribed with T7 RNA polymerase as described previously (39). RNA transfection of mouse L cells with lipofectin (Bethesda Research Laboratories) was carried out as described previously (39).

Labelling of viral RNAs with [^{3}H]uridine. Cells were infected with MHV-A59 and transfected at 1 h postinfection. Three hours postinfection, the culture fluid was replaced by 1 ml of Dulbecco's modified Eagle's medium containing 3% fetal calf serum and supplemented with 10 μg of actinomycin D per ml. Thirty minutes later, [^{3}H]uridine (200 μCi/ml) (NEN) was added.

Isolation and analysis of viral RNAs. Intracellular RNAs were isolated as described by Spaan et al. (37). ^{3}H-labelled intracellular RNAs were isolated at 8 h postinfection with TRIzol reagent according to the instructions of the manufacturer (Gibco BRL). RNA samples were separated in 1% agarose–2.2 M formaldehyde gels (25). Hybridization was done in dried gels with 5′-end-labelled oligonucleotide 116 (Table 1) (25). Oligonucleotides were labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase. ^{3}H-labelled RNAs were visualized by fluorography using 2,5-diphenyloxazole (34).

cDNA synthesis and PCR amplification (RT-PCR). First-strand cDNA synthesis was carried out with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories). PCR amplification was done with *Taq* polymerase (Promega). For the amplification of ΔH-*out*-specific cDNA, we used oligonucleotide primers 48 and 24. Reverse transcription PCR (RT-PCR) of DISP derivatives was performed with oligonucleotide primers 48 and 42. Direct sequencing of RT-PCR-generated DNA was carried out by the method of Gyllensten (6) with oligonucleotide primers c025 (ΔH-*out*) and 42 (DISP).

In vitro translation and polyacrylamide gel electrophoresis. In vitro translation was performed in a 10-μl reaction mixture containing nuclease-treated, methionine-depleted rabbit reticulocyte lysate (Promega), supplemented with 10 μCi of ^{35}S-labelled amino acids (^{35}S-Express [NEN]; 1,203 Ci/mmol), 20 μM unlabelled amino acid mixture lacking methionine (Promega), and 10 to 50 ng of in vitro-transcribed RNA or cytoplasmic RNA from the equivalent of 10^5 infected cells. The labelled proteins were either immunoprecipitated as described previously (3) or analyzed directly by electrophoresis in sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gels.

Oligonucleotides. Oligonucleotides were synthesized on an Applied BioSystems 391 PCR-MATE oligonucleotide synthesizer. The sequences of the oligonucleotides used in this study are listed in Table 1.

RESULTS

Translation of N sequences is not essential for propagation of MHV DI RNAs. The MHV-A59 DI RNA MIDI and its derivatives consist of sequences derived from ORF1a, ORF1b, and N, which have been fused in-frame (3, 39, 42). Full translation of the fusion ORFs is required for efficient propagation of the DI RNAs (3). To determine whether this requirement of translation is determined by sequences of the C-terminal part of the ORF, that is, ORF1b and N sequences, we designed four different DI RNAs on the basis of the MIDI derivatives ΔH-*in* and ΔH-*out* (3), and containing different ORFs (Fig. 1). A 600-nt deletion, comprising 250 nt from the ORF1b segment and 350 nt derived from the N segment, was made (Fig. 1). In addition, we introduced either a UAA stop codon or a UUA Leu codon at the new ORF1b-N junction of the ΔH derivatives. The ΔH-*in* derivative DIF-85T carries a full-length ORF for a 85-kDa polypeptide, consisting of 1,775 nt derived from ORF1a, 392 nt from ORF1b, and 143 nt from the N ORF. DIF-80A contains the UAA termination codon at the ORF1b-N junction and encodes a 80-kDa ORF1a-ORF1b fusion polypeptide. The ΔH-*out* derivative DIF-66T is identical to DIF-85T except for a 1-nt deletion at the ORF1a-ORF1b junction, resulting in a truncated ORF for a 66-kDa ORF1a polypeptide. DIF-66A contains both the 1-nt deletion at the ORF1a-ORF1b junction and the termination codon at the ORF1b-N junction. In vitro translation of RNA transcripts confirmed that the DI RNAs encoded the expected polypeptides (Fig. 2a, left panel).

In vitro-transcribed RNAs derived from all four DI viruses were transfected into MHV-A59 infected L cells. As controls, MHV-A59-infected cells were transfected with ΔH-*out* RNA or mock transfected with phosphate-buffered saline. The resulting virus stocks (p0 virus) were passaged several times. Passage 1 and 3 intracellular RNAs were separated in denaturing formaldehyde-agarose gels and hybridized to radiolabelled oligonucleotide 116. This probe detects the MHV genomic RNA and the DI RNAs. As shown in Fig. 2b, all DI RNAs replicated, but the ΔH-*in* derivatives DIF-85T and DIF-80A accumulated to much higher levels than those of the

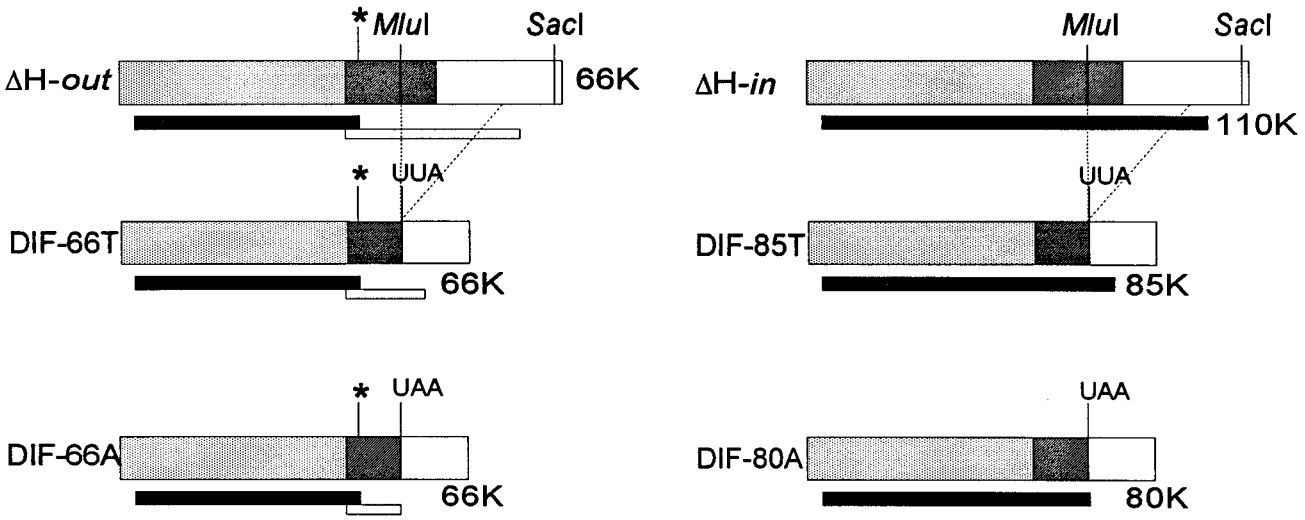

FIG. 1. Construction of the DIF DI RNAs. Sequences derived from the MHV 5′ terminus (stippled boxes), sequences derived from ORF1b (hatched boxes), and sequences derived from the MHV 3′ terminus (open boxes) are shown. Dotted lines indicate the sequences that are deleted from ΔH-*in* and -*out*. The ORFs encoding the 66-, 80-, and 85-kDa (K) DI polypeptides are depicted as black bars. The termination codons of DIF-66T and DIF-66A are indicated by asterisks.

ΔH-*out* derivatives. This accumulation is apparent from the analysis of both p1 and p3 RNA samples. For ΔH-*out*, two RNA species were detected in p3 RNA. Sequence analysis of RT-PCR DNA prepared with p3 ΔH-*out* RNA as a template revealed that the smaller RNA species contained a deletion of 203 nt that restored the full-length ORF (not shown).

To study the DI RNA-encoded polypeptides, p3 intracellular RNAs were isolated and translated in rabbit reticulocyte lysates and directly analyzed by polyacrylamide gel electrophoresis. As shown in Fig. 2a, DIF-85T and DIF-80A produced the 85- and 80-kDa polypeptides, respectively. Translation of DIF-66T yielded a 85-kDa polypeptide and a small amount of a 66-kDa polypeptide, which demonstrates that in-frame escape mutants had been selected for. For DIF-66A, we detected trace amounts of 66- and 80-kDa polypeptides. The identities of the translation products were confirmed by immunoprecipitation using anti-P28 and anti-N sera (not shown). In vitro translation of ΔH-*out* p3 RNA yielded a 110-kDa product and an additional 100-kDa polypeptide, indicating that consistent with previous findings (3), in-frame escape mutants had accumulated during passage of ΔH-*out*. The 100-kDa product is likely to be encoded by the ΔH-*out* deletion mutant.

The poor propagation of the two ΔH-*out* derivatives and the fact that in-frame escape mutants accumulated, in particular during passage of DIF-66T, clearly showed that translation is essential for efficient propagation of these DI viruses. Thus, the deleted ORF1b and N sequences do not determine the requirement for translation. Translation of the remaining N ORF (141 nt) does not play a major role either, since DIF-85T and DIF-80A accumulated to similar levels. This conclusion was confirmed by a competition experiment (as described previously in reference 3), in which DIF-80A and DIF-85T were cotransfected in a 10:1 molar ratio; in vitro translation of p3 RNA showed that DIF-80A was still present in approximately fivefold molar excess (not shown). Hence, only translation of ORF1a and the remaining part of the ORF1b sequences appears to be important for propagation of these DI viruses.

Insertion of the spike gene into DIF-85T: effects of premature termination of translation on DI virus propagation. DIF-85T and DIF-80A both encode fusion polypeptides comprising part of the ORF1b sequence, which contains the RNA packaging signal (4, 39). Therefore, it cannot be excluded that translation of this specific sequence is required for efficient DI virus propagation. In order to determine whether the requirement for translation is sequence specific, we decided to introduce unrelated sequences into DIF-85T, i.e., sequences that do not occur in any of the natural DI RNAs identified so far (18, 22, 39). If translation of such a sequence is also required for efficient DI RNA propagation, it is very unlikely that the sequence of the ORF or the DI RNA-encoded polypeptide itself has an important role. For this purpose, we chose to insert a large fragment of the MHV-A59 spike gene into DIF-85T, upstream of the ORF1b sequence. By using cassette mutagenesis, four different constructs were obtained, each containing a 3.6-kb fragment encompassing nt 80 to 3635 from the MHV-A59 spike gene (17). Our cloning strategy (see Materials and Methods) led to the partial deletion of ORF1a and ORF1b sequences from DIF-85T (positions 1313 to 1983 and 1984 to 2049, respectively). As depicted in Fig. 3, the resulting 5.8-kb DI RNAs, which will be referred to as the DISP DI RNAs, encode different polypeptides. Due to a frameshift at the ORF1a-spike junction, DISP3eco and DISP6eco carry a truncated ORF coding for a 42-kDa ORF1a polypeptide. To obtain in-frame DI RNAs, the unique *Eco*RI sites of pDISP3eco and pDISP6eco were treated with mung bean nuclease, yielding pDISP3 and pDISP6, respectively. DISP3 carries a full-length ORF encoding a 185-kDa product, whereas DISP6 contains a UAA stop codon at the spike-ORF1b junction and encodes a 171-kDa ORF1a-spike fusion polypeptide.

Transcripts derived from all four constructs were translated in a rabbit reticulocyte lysate to confirm that the DI RNAs contain the expected ORFs (Fig. 4a). The fitness of these DI RNAs was studied by transfecting transcripts of the four DISP constructs into MHV-A59-infected cells. For a control, we transfected in vitro-transcribed DIF-85T RNA. Passage 3 intracellular RNA preparations were analyzed by hybridization with oligonucleotide 116. As shown in Fig. 5, the in-frame DIs DISP3 and DISP6 accumulated to similar levels in p3 RNA, whereas DISP3eco and DISP6eco were not detected. Replication of DISP3 and DISP6 was less efficient than DIF-85T (Fig. 5). This is already obvious in p1 RNA where DIF-85T RNA could easily be detected, in contrast to DISP3 and DISP6 (not

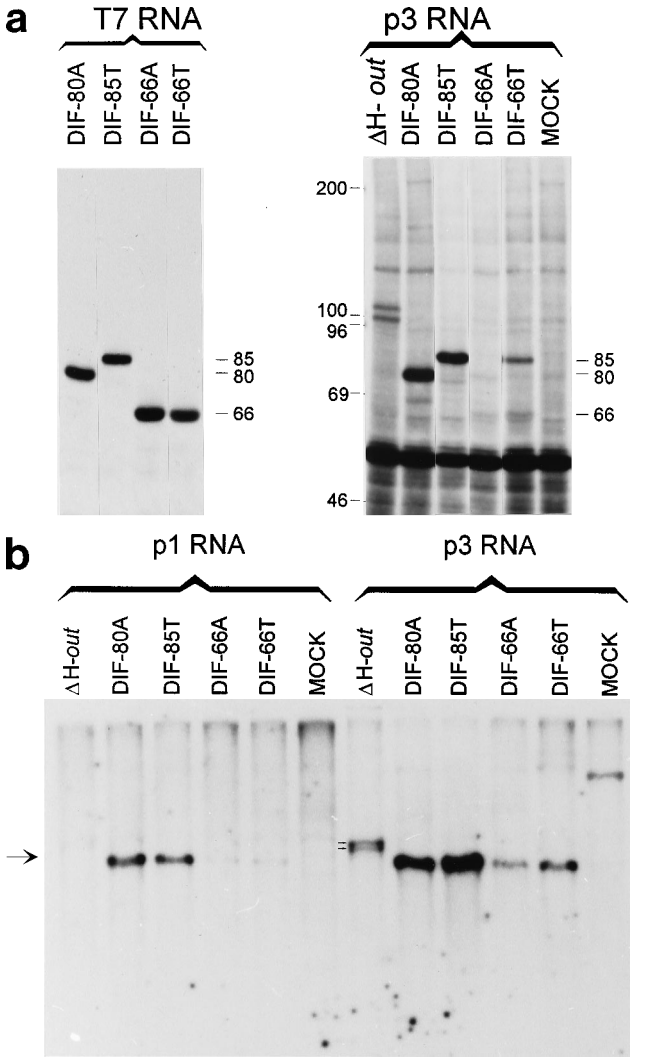

FIG. 2. (a) In vitro translation of synthetic and p3 intracellular RNAs in rabbit reticulocyte lysates. pDIF-80A, pDIF-85T, pDIF-66A, and pDIF-66T were transcribed with T7 RNA polymerase. The resulting RNAs were translated in vitro (T7 RNA lanes). MHV-A59-infected L cells were transfected with in vitro-transcribed DI RNAs. Progeny virus stocks were harvested and passaged. p3 RNAs were extracted and translated (p3 RNA lanes). Labelled proteins were directly analyzed by SDS-polyacrylamide gel electrophoresis. The positions of molecular weight markers (in thousands) are indicated to the sides of the gels. (b) Hybridization analysis of p1 and p3 intracellular RNAs. p1 and p3 RNAs were isolated and separated on formaldehyde-agarose gels and hybridized to 5′-end-labelled oligonucleotide 116. The DIF DI RNAs are indicated by the large arrow, and the two small arrows point to the two RNA species present in ΔH-*out* p3.

shown). Accordingly, specific 0.8-kb amplification products were detected for DISP3 and DISP6 but not for DISP3eco and DISP6eco when p3 RNA samples were subjected to RT-PCR (Fig. 3 and not shown). Thus, these results clearly show that for the DISP derivatives, translation of the inserted spike sequence is essential for propagation. Interestingly, DISP6 which contains the UAA stop codon between the spike and ORF1b sequences, accumulated invariably to levels similar to those of DISP3 which carries a full-length ORF (Fig. 5). This result indicates that translation of the ORF1b and N sequences is not required for these DI RNAs. However, translation of these sequences could still prove to be important for propagation if escape mutants had arisen rapidly during passage of DISP6.

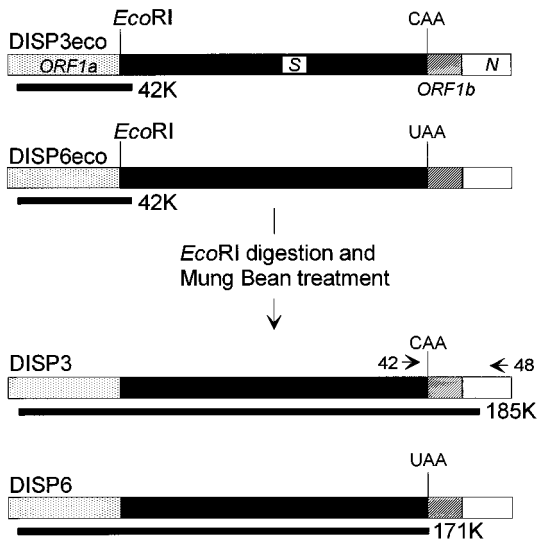

FIG. 3. Schematic representation of the DISP DI RNAs. DISP3 and DISP6 were obtained after *Eco*RI and mung bean digestion of DISP3eco and DISP6eco, respectively. Sequences derived from the 5′ terminus (*ORF1a*), the S gene (*S*), ORF1b (*ORF1b*), and the 3′ terminus (*N*) of the MHV genome are indicated by different shadings. The DI ORFs are depicted as black bars, and the molecular weights (in thousands [K]) of the DI polypeptides are shown. The CAA and UAA codons at the S-ORF1b junctions are shown. The arrows in DISP3 represent the positions of oligonucleotides 48 and 42.

Such escape mutants would be expected to have lost the UAA stop codon at the spike-ORF1b fusion, thereby restoring the full-length ORF. In vitro translation of p3 RNA samples from DISP3 and DISP6 showed that these DI RNAs encoded the predicted 171- and 185-kDa polypeptides, although the translation products are visible only as faint bands (Fig. 4b). These data demonstrate that escape mutants had not been selected

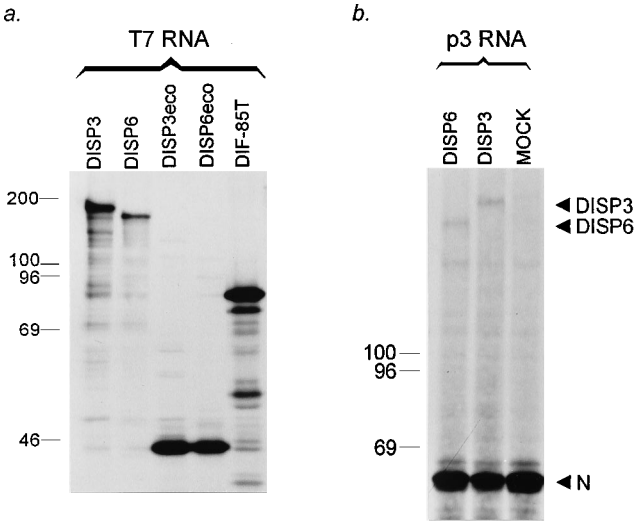

FIG. 4. In vitro translation of synthetic and p3 intracellular RNAs. Labelled proteins were directly analyzed by SDS-polyacrylamide gel electrophoresis. (a) Translation of in vitro-transcribed RNAs from DISP3, DISP6, DISP3eco, DISP6eco, and DIF-85T. (b) MHV-A59-infected L cells were transfected with the in vitro-transcribed DI RNAs. Progeny virus stocks were harvested and passaged. p3 RNAs obtained after initial mock transfection or transfection with DISP3 or DISP6 were isolated and translated in vitro. The positions of molecular weight markers (in thousands) are indicated to the sides of the gels.

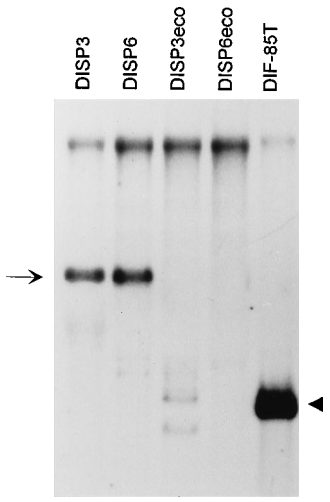

FIG. 5. Hybridization analysis of intracellular RNAs. MHV-A59-infected L cells were transfected with synthetic DI RNAs, and progeny virus was passaged. p3 intracellular RNAs were isolated, separated in formaldehyde-agarose gels, and hybridized to 5′-end-labelled oligonucleotide 116. The DISP RNAs are indicated by the arrow, and DIF-85T RNA is indicated by the black triangle.

for. To confirm this, the 0.8-kb RT-PCR products obtained for DISP3 and DISP6 were sequenced with oligonucleotide 42. Direct sequence analysis showed that the UAA stop codon is still present in DISP6 RNA (Fig. 6). Thus, translation of ORF1b and N sequences is not required for propagation of the DISP DI RNAs. From our combined data, we conclude that translation per se, rather than the information contained in the translated sequence, is essential for DI RNA propagation.

Translation of a large ORF is required for accumulation of DI RNAs in DI RNA-transfected and MHV-infected cells. Having established that the requirement for translation is not sequence specific, we next sought to determine whether translation exerts its role in DI RNA-transfected and MHV-infected cells or after passage of the virus-DI mixture. Therefore, we constructed two new DI RNAs that differ only in a single nucleotide. The structures and the ORFs of these 4.1-kb DI RNAs, ΔP-*130* and ΔP-*30*, are shown in Fig. 7. ΔP-*130* contains a full-length ORF encoding a 130-kDa polypeptide, whereas ΔP-*30* contains a UGA termination codon at 1.1 kb from the 5′ end and encodes a 30-kDa polypeptide. ΔP-*130* and ΔP-*30* were designed such that recombination of these DI RNAs with the viral genome (42) yields a 5.5-kb DI RNA containing a full-length ORF coding for a 184-kDa polypeptide (3). Because ΔP-*30* contains a truncated ORF, the 5.5-kb recombinant DI RNA represents a potential escape mutant (3). Therefore, selection of escape mutants after initial transfection of ΔP-*30* RNA can be monitored by comparing the accumulation of recombinant DI RNAs during passage of ΔP-*130* and ΔP-*30*.

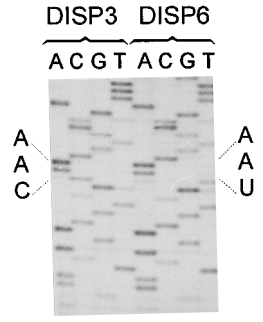

FIG. 6. Sequence analysis of PCR DNA. p3 intracellular RNAs obtained after initial mock transfection or transfection with DISP3 or DISP6 were subjected to RT-PCR with oligonucleotides 42 and 48. PCR DNA samples were directly sequenced with oligonucleotide 42. The results obtained for DISP3 and DISP6 are shown. The CAA and UAA codons from DISP3 and DISP6 are indicated.

In vitro translation of RNA transcripts confirmed that ΔP-*130* and ΔP-*30* encoded the expected polypeptides (not shown). ΔP-*130* and ΔP-*30* transcripts were transfected into MHV-A59-infected cells, and the progeny virus was passaged once. To preclude detection of input DI RNAs, viral RNAs were metabolically labelled with [^{3}H]uridine. Labelled RNAs were isolated from transfected cells (p0 RNA) and from cells infected with p0 virus (p1 RNA). As shown in Fig. 8, replication of ΔP-*130* RNA was readily detected in transfected cells. Accordingly, ΔP-*130* RNA accumulated to high levels in cells infected with p0 virus and only small quantities of the 5.5-kb recombinant DI RNA were detected (Fig. 8). In contrast, hardly any label was incorporated into ΔP-*30* RNA in transfected cells (Fig. 8). The poor replication of ΔP-*30* RNA was also evident from the analysis of p1 RNA: only small quantities of ΔP-*30* RNA were detected, and the 5.5-kb recombinant RNA prevailed. Thus, the recombinant DI RNA, containing a full-length ORF, had a strong selective advantage over ΔP-*30*. ΔP-*30* accumulated much less efficiently than ΔP-*130* did, both in transfected cells and during the first passage. Identical results were obtained by DNA transfection and in vivo transcription of ΔP-*130* and ΔP-*30* by a vaccinia virus-encoded T7 RNA polymerase (5), followed by superinfection with MHV-A59 (not shown) (16).

DISCUSSION

Role of the DI RNA ORF. We have previously demonstrated that the DI RNAs of MHV-A59 require translation of a large ORF for efficient propagation (3). The data presented in this report confirm and extend the previous observations. We now show that translation of a full-length ORF is not required: DISP6 accumulates to levels similar to those of DISP3, indicating that translation of ORF1b and N sequences is not essential for the DISP DI RNAs. Similarly, translation of N sequences does not confer a selective advantage to DIF-85T compared with DIF-80A. Thus, the relative length of the ORF or the distance of the termination codon to the 3′ end may determine the effect of translation. In this view, DI RNAs with very short ORFs relative to the length of the genome (e.g., DISP3eco and DISP6eco) cannot be propagated, whereas DI RNAs with larger though not full-length ORFs (e.g., DIF-80A and DISP6) do not have a strong selective disadvantage when compared with their counterparts carrying a full-length ORF. Next, we provide evidence that the requirement for translation is not sequence specific. This nonspecificity is most clearly illustrated by the fact that translation of the MHV-A59 spike ORF, which is not present in any DI RNA identified so far (18, 22, 39), is necessary for survival of the DISP DI RNAs. Although all DI RNAs contain (partial) ORF1a sequences, it seems very unlikely that a truncated ORF1a polypeptide has a specific *cis*-acting function, since DISP3eco and DISP6eco encode such products yet do not accumulate. Thus, the observation that unrelated sequences must be translated upon insertion into a DI virus RNA genome strongly argues against a role of the DI virus polypeptide. Finally, the results obtained by the metabolic labelling of ΔP-*130* and ΔP-*30* show that the role of

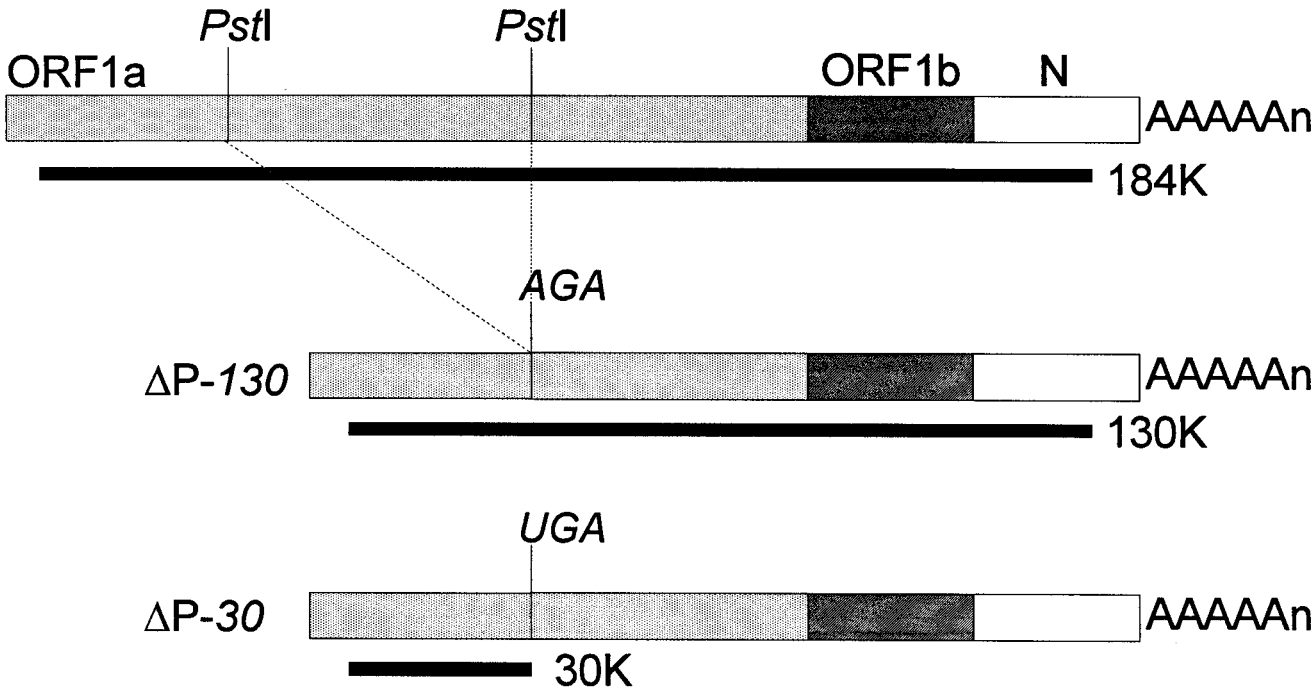

FIG. 7. Structures of ΔP-*130* and ΔP-*30*. The ORFs are depicted as black bars. Regions derived from different parts of the MHV genome are indicated as described in the legend to Fig. 1. The introduced UGA and AGA codons are shown.

translation is already evident in DI RNA-transfected and MHV-infected cells.

Taken together, our data indicate that translation affects either the production or degradation of DI RNAs, since these are the processes that determine RNA accumulation in transfected cells. We cannot rule out that there are additional roles of translation, for instance, in RNA packaging or uncoating (see below). One interpretation of our present data is that translation has a direct and sequence-independent effect on RNA synthesis, e.g., by unfolding RNA secondary structure or by delivering an essential replication factor that is associated with the translational machinery. Alternatively, translation may increase the stability or cytoplasmic half-life of the DI RNAs (3). If translation somehow protects RNA from degradation, one would indeed expect that not the sequence of the DI RNA ORF but its length is relevant and that this effect manifests itself already in transfected cells. Puzzlingly, however, the viral genomic and subgenomic RNAs do not contain full-length ORFs and would thus not be efficiently protected from degradation. It will therefore be necessary to compare the cytoplasmic half-lives of both the DI RNAs and the genomic and subgenomic RNAs.

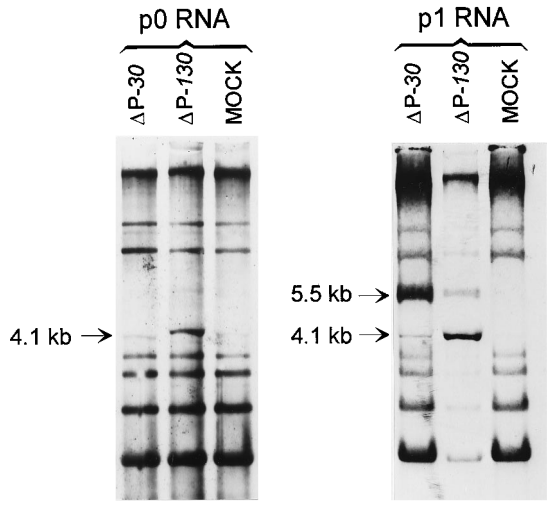

FIG. 8. Replication of ΔP-*130* and ΔP-*30*. In vitro-transcribed RNAs were transfected into MHV-A59-infected cells. Viral RNAs were labelled with [^{3}H] uridine (200 μCi/ml) and separated in formaldehyde-agarose gels. ^{3}H-labelled RNAs were visualized by enhancing the gels in methanol–20% 2,5-diphenyloxazole.

Experimental evidence for a correlation between translation and RNA stability has been reported for several cellular mRNAs (1, 10, 41). Pulak and Anderson (30) recently postulated the existence of a specific "mRNA surveillance" system which degrades mRNAs containing premature termination codons. Such aberrant termination codons could result from errors in transcription, splicing, or processing. The steady-state levels of different mRNAs were found to depend on the relative positions of the aberrant termination codons; this result is reminiscent of our finding that the relative length of the DI RNA ORF seems to be important. Experimental evidence for this system of nonsense-mediated mRNA decay was obtained in *Caenorhabditis elegans*, but Pulak and Anderson speculated that mRNA surveillance systems could be a common property among eukaryotes. It is, however, not clear how these aberrant transcripts are recognized. In yeasts, premature translational termination was recently shown to trigger mRNA decapping followed by 5′→3′ or 3′→5′ degradation (26).

Kim et al. (12) have shown that translation of a large ORF is required for the propagation of the 2.2-kb MHV-JHM DI DIssE (23). DIssE consists of three noncontiguous fragments of the viral genome that have been joined in frame, creating a

full-length ORF for a 63-kDa polypeptide (23). The DI RNA-specific polypeptide migrates as a 88-kDa molecule (23). Kim and coworkers studied a DIssE derivative, NE-1, that contains a short ORF coding for a 7.5-kDa polypeptide as the result of a 1-nt deletion at position 376 from the 5′ end of the DI virus RNA genome. After transfection of NE-1 into MHV-A59-infected cells followed by one passage, only escape mutants that had restored the full-length ORF were detected. In contrast, these escape mutants were not detected in DI RNA-transfected and MHV-infected cells or in the resulting p0 virus stock. The researchers therefore concluded that, unlike our DI system, translation is not required for RNA replication or protection against RNA degradation and hypothesized that translation might play a role during the uncoating process (12). At present, we cannot explain why these data differ from ours with respect to the role of translation in transfected cells. One possibility is that translation has two roles, i.e., protection of RNA from decay and uncoating. To explain the data presented by Kim et al., one would then have to assume that DIssE is protected from degradation by some other factor, e.g., a specific RNA structure. Alternatively, in-frame escape mutants could have accumulated slowly in DIssE-transfected cells and might have escaped detection somehow. In this respect, it is of interest that a very faint 88-kDa protein band, which might be diagnostic for the appearance of in-frame escape mutants, can be detected in NE-1-transfected cells and is absent in mock-transfected cells (12).

The phenomenon that DI RNAs carry large ORFs resulting from in-frame fusion of the genomic fragments is not unique for coronaviruses. For the defective RNAs of potexvirus clover yellow mosaic virus, translation of a full-length fusion ORF is essential for propagation in planta (45). The DI RNAs of the bromovirus broad bean mottle virus contain single in-frame deletions (32). Also, the DI RNAs of the negative-strand RNA viruses Bunyamwera virus (28) and tomato spotted wilt virus (31) carry large fusion ORFs on their antigenome strands. Experimental evidence that translation of these ORFs is important for DI virus survival has not yet been presented. However, in all these cases it will be of interest to study whether translation protects the RNAs from degradation. A DI RNA-specific polypeptide is also produced by a naturally occurring bovine diarrhea virus DI RNA, but in this case expression of the DI virus polypeptide itself is most probably related to a cytopathogenic phenotype of the DI-containing virus stock (38). For poliovirus RNA genomes, it has been demonstrated that translation of an internal sequence of the genome is required in *cis* (27). Although it could be excluded that translation led to an increase in RNA stability, it is as yet unclear why translation in *cis* is required for poliovirus replication (27).

DI RNAs can be used as cloning vectors for DI virus-directed mutagenesis. We recently reported (42) that MHV DI RNAs can be used to introduce site-specific mutations into the genome of MHV-A59 by exploiting the high-frequency RNA recombination that occurs in MHV-infected cells (20). As a necessary prelude for future applications of DI virus-directed mutagenesis (42), we have explored whether MHV sequences that do not occur in natural DI RNAs can be cloned into a synthetic DI RNA without impairing its capacity to be replicated and packaged. The fact that insertion of a 3.6-kb fragment of the MHV-A59 spike gene into DIF-85T gives rise to a viable DI RNA indicates that the MIDI derivatives can be used as cloning vectors, provided that a large ORF is maintained. In principle, the inserted sequences could be expressed via a subgenomic RNA by inserting short intergenic promoter sequences—without interrupting the ORF—directly upstream of the insert (40). We are currently studying whether DISP3 and DISP6 can be used as tools to mutagenize the MHV spike gene by DI virus-directed mutagenesis.

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REFERENCES

1. **Atwater, J. A., R. Wisdom, and I. M. Verma.** 1990. Regulated mRNA stability. Annu. Rev. Genet. **24:**519–541.
2. **Calain, P., and L. Roux.** 1993. The rule of six, a basic feature for efficient replication of Sendai virus defective interfering RNA. J. Virol. **67:**4822–4830.
3. **de Groot, R. J., R. G. van der Most, and W. J. M. Spaan.** 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.
4. **Fosmire, J. A., K. Hwang, and S. Makino.** 1992. Identification and characterization of a coronavirus packaging signal. J. Virol. **66:**3522–3530.
5. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83:**8122–8126.
6. **Gyllensten, U.** 1989. Direct sequencing of in vitro amplified DNA, p. 45–60. *In* H. A. Erlich (ed.), PCR technology: principles and applications for DNA amplification. Stockton Press, New York.
7. **Holland, J. J.** 1991. Defective viral genomes, p. 151–165. *In* B. N. Fields and D. M. Knipe (ed.), Fundamental virology. Raven Press, New York.
8. **Huang, A. S., and D. Baltimore.** 1970. Defective viral particles and viral disease processes. Nature (London) **226:**325–327.
9. **Huang, A. S., and D. Baltimore.** 1977. Defective interfering animal viruses, p. 73–116. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology. Plenum, New York.
10. **Kim, S.-K., C. D. Sigmund, K. W. Gross, and L. E. Maquat.** 1992. Nonsense codons in human β-globin mRNA result in the production of mRNA degradation products. Mol. Cell. Biol. **12:**1149–1161.
11. **Kim, Y.-N., Y. S. Jeong, and S. Makino.** 1993. Analysis of cis-acting sequences essential for coronavirus defective interfering RNA replication. Virology **197:**53–63.
12. **Kim, Y.-N., M. M. C. Lai, and S. Makino.** 1993. Generation and selection of coronavirus defective interfering RNA with large open reading frame by RNA recombination and possible editing. Virology **194:**244–253.
13. **Lai, M. M. C.** 1990. Coronavirus—organization, replication and expression of genome. Annu. Rev. Microbiol. **44:**303–333.
14. **Levis, R., B. G. Weiss, M. Tsiang, H. Huang, and S. Schlesinger.** 1986. Deletion mapping of sindbis virus DI RNAs derived from cDNAs defines the sequences essential for replication and packaging. Cell **44:**137–145.
15. **Lin, Y.-J., and M. M. C. Lai.** 1993. Deletion mapping of a mouse hepatitis virus defective interfering RNA reveals the requirement of an internal and discontinuous sequence for replication. J. Virol. **67:**6110–6118.
16. **Luytjes, W., H. Gerritsma, and W. J. M. Spaan.** Unpublished results.
17. **Luytjes, W., L. S. Sturman, P. J. Bredenbeek, J. Charite, B. A. M. van der Zeijst, M. C. Horzinek, and W. J. Spaan.** 1987. Primary structure of the glycoprotein E2 of coronavirus MHV-A59 and identification of the trypsin cleavage site. Virology **161:**479–487.
18. **Makino, S., N. Fujioka, and K. Fujiwara.** 1985. Structure of the intracellular defective viral RNAs of defective interfering particles of mouse hepatitis virus. J. Virol. **54:**329–336.
19. **Makino, S., M. Joo, and J. K. Makino.** 1991. A system for study of coronavirus mRNA synthesis: a regulated, expressed subgenomic defective interfering RNA results from intergenic site insertion. J. Virol. **65:**6031–6041.
20. **Makino, S., J. G. Keck, S. A. Stohlman, and M. M. Lai.** 1986. High-frequency RNA recombination of murine coronaviruses. J. Virol. **57:**729–737.
21. **Makino, S., and M. M. C. Lai.** 1989. High-frequency leader sequence switching during coronavirus defective interfering RNA replication. J. Virol. **63:**5285–5292.
22. **Makino, S., C. Shieh, J. G. Keck, and M. M. C. Lai.** 1988. Defective interfering particles of murine coronavirus: mechanism of synthesis of defective viral RNAs. Virology **163:**104–111.
23. **Makino, S., C. K. Shieh, L. H. Soe, S. C. Baker, and M. M. Lai.** 1988. Primary structure and translation of a defective interfering RNA of murine coronavirus. Virology **166:**550–560.
24. **Makino, S., K. Yokomori, and M. M. C. Lai.** 1990. Analysis of efficiently packaged defective interfering RNAs of murine coronavirus: localization of a possible RNA-packaging signal. J. Virol. **64:**6045–6053.
25. **Meinkoth, J., and G. Wahl.** 1984. Hybridization of nucleic acids immobilized on solid supports. Anal. Biochem. **138:**267–284.
26. **Muhlrad, D., and R. Parker.** 1994. Premature translational termination

triggers mRNA decapping. Nature (London) **370:**578–581.
27. **Novak, J. E., and K. Kirkegaard.** 1994. Coupling between genome translation and replication in an RNA virus. Genes Dev. **8:**1726–1737.
28. **Patel, A. H., and R. M. Elliott.** 1992. Characterization of Bunyamwera virus defective interfering particles. J. Gen. Virol. **73:**389–396.
29. **Pattnaik, A. K., L. A. Ball, A. W. LeGrone, and G. W. Wertz.** 1992. Infectious defective interfering particles of VSV from transcripts of a cDNA clone. Cell **69:**1011–1020.
30. **Pulak, R., and P. Anderson.** 1993. mRNA surveillance by the Caenorhabditis elegans smg genes. Genes Dev. **7:**1885–1897.
31. **Resende, R. D. O., P. de Haan, E. van de Vossen, A. C. de Avila, R. Goldbach, and D. Peters.** 1992. Defective interfering L RNA segments of tomato spotted wilt virus retain both genome termini and have extensive internal deletions. J. Gen. Virol. **73:**2509–2516.
32. **Romero, J., Q. Huang, J. Pogany, and J. J. Bujarski.** 1993. Characterization of defective interfering RNA components that increase symptom severity of broad bean mottle virus infections. Virology **194:**576–584.
33. **Roux, L., A. E. Simon, and J. J. Holland.** 1991. Effects of defective interfering viruses on virus replication and pathogenesis in vitro and in vivo. Adv. Virus Res. **40:**181–211.
34. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
35. **Sawicki, S. G., and D. L. Sawicki.** 1986. Coronavirus minus-strand RNA synthesis and effect of cycloheximide on coronavirus RNA synthesis. J. Virol. **57:**328–334.
36. **Spaan, W., D. Cavanagh, and M. C. Horzinek.** 1988. Coronaviruses: structure and genome expression. J. Gen. Virol. **69:**2939–2952.
37. **Spaan, W. J. M., P. J. M. Rottier, M. C. Horzinek, and B. A. M. van der Zeijst.** 1981. Isolation and identification of virus-specific mRNAs in cells infected with mouse hepatitis virus (MHV-A59). Virology **108:**424–434.
38. **Tautz, N., H.-J. Thiel, E. J. Dubovi, and G. Meyers.** 1994. Pathogenesis of mucosal disease: a cytopathogenic pestivirus generated by an internal deletion. J. Virol. **68:**3289–3297.
39. **van der Most, R. G., P. J. Bredenbeek, and W. J. Spaan.** 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.
40. **van der Most, R. G., R. J. de Groot, and W. J. M. Spaan.** 1994. Subgenomic RNA synthesis directed by a synthetic defective interfering RNA of mouse hepatitis virus: a study of coronavirus transcription initiation. J. Virol. **65:** 3656–3666.
41. **Vancanneyt, G., S. Rosahl, and L. Willmitzer.** 1990. Translatability of a plant-mRNA strongly influences its accumulation in transgenic plants. Nucleic Acids Res. **18:**2917–2921.
42. **van der Most, R. G., L. Heijnen, W. J. M. Spaan, and R. J. de Groot.** 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.
43. **Vennema, H., L. Heijnen, A. Zijderveld, M. C. Horzinek, and W. J. M. Spaan.** 1990. Intracellular transport of recombinant coronavirus spike proteins: implications for virus assembly. J. Virol. **64:**339–346.
44. **Weiss, B., H. Nitschko, I. Ghattas, R. Wright, and S. Schlesinger.** 1989. Evidence for specificity in the encapsidation of Sindbis virus RNAs. J. Virol. **63:**5310–5318.
45. **White, K. A., J. B. Bancroft, and G. A. Mackie.** 1992. Coding capacity determines in vivo accumulation of a defective RNA of clover yellow mosaic virus. J. Virol. **66:**3069–3076.