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Defective-Interfering Particles of Murine Coronavirus: Mechanism of Synthesis of Defective Viral RNAs

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The mechanism of synthesis of the defective viral RNAs in cells infected with defective-interfering (DI) particles of mouse hepatitis virus was studied. Two DI-specific RNA species, DIssA of genomic size and DIssE of subgenomic size, were detected in DI-infected cells. Purified DI particles, however, were found to contain predominantly DIssA and only a trace amount of DIssE RNA. Despite its negligible amount, the DIssE RNA in virions appears to serve as the template for the synthesis of DIssE RNA in infected cells. This conclusion was supported by two studies. First, the uv target size for DIssE RNA synthesis is significantly smaller than that for DIssA. Second, when purified DIssE RNA was transfected into cells which had been infected with a helper virus, DIssE RNA could replicate itself and became a predominant RNA species in the infected cells. Thus, DIssE RNA was not synthesized from the genomic RNA of DI particles. By studying the relationship between virus dilution and the amount of intracellular viral RNA synthesis, we have further shown that DIssE RNA synthesis requires a helper function, but it does not utilize the leader sequence of the helper virus. In contrast, DIssA synthesis appears to be helper-independent and can replicate itself. Thus DIssA codes for a functional RNA polymerase.

INTRODUCTION

Mouse hepatitis virus (MHV), a member of the Coronaviridae, contains a single-stranded and positive-sense RNA of approximately 6×10^6 Da (Lai and Stohlman, 1978; Wege *et al.*, 1978). In infected cells, the genomic RNA of MHV encodes an RNA-dependent RNA polymerase (Brayton *et al.*, 1982, 1984; Mahy *et al.*, 1983) which is utilized for the synthesis of a genomic-sized negative-stranded RNA (Lai *et al.*, 1982b). The negative-stranded RNA then serves as the template for the synthesis of six subgenomic and one genome-sized mRNA (Brayton *et al.*, 1984; Lai *et al.*, 1982b). These mRNAs are arranged in the form of a 3′ coterminal "nested" set, i.e., the sequences of each mRNA are contained entirely within the next larger mRNA (Lai *et al.*, 1981; Leibowitz *et al.*, 1981). In addition, each mRNA has a common leader sequence, which is derived from the 5′ end of the genome (Lai *et al.*, 1982a, 1983, 1984; Spaan *et al.*, 1983). Several pieces of evidence demonstrate that MHV utilizes a novel mechanism of leader RNA-primed transcription for the synthesis of mRNAs (Baric *et al.*, 1983, 1985; Makino *et al.*, 1986).

We have previously reported the generation of defective-interfacing (DI) particles during high multiplicity passages of the JHM strain of MHV (MHV-JHM) (Makino *et al.*, 1984a). In purified virus particles, only one RNA species, which is slightly smaller than the genomic RNA of the standard virus, is detected. In DI-infected cells, the synthesis of most of the standard viral mRNAs is inhibited, but the cells contain three distinct RNA species (Makino *et al.*, 1985). The first species, DIssA, is equivalent to DI virion RNA, and is eventually incorporated into the virus particles. This RNA differs from the standard virus genomic RNA in that it contains multiple sequence changes distributed throughout the genome, except for regions encoding RNA polymerases and nucleocapsid (N) protein. The second species is mRNA 7, which is indistinguishable from the mRNA 7 made by the standard virus. The synthesis of this mRNA is not inhibited in DI-infected cells. Thus, a normal amount of N protein, which is encoded from this mRNA, is synthesized in DI-infected cells. The third RNA species is a novel subgenomic RNA species, which is single-stranded and polyadenylated. Oligonucleotide fingerprinting studies suggest that it represents sequences derived from various noncontiguous parts of the genome. Interestingly, this RNA differs in size and sequence at different DI passage levels. This RNA was not detected in purified virus particles (Makino *et al.*, 1984a).

We are interested in the mechanism of synthesis of DI RNA species in DI-infected cells. Specifically, we asked the following two questions: (1) How are the novel intracellular subgenomic DI RNA species synthesized? Since subgenomic DI RNAs detected in DI-infected cells are not detected in DI particles (Makino *et al.*, 1984a), it seems plausible that all of the sub-

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genomic DI RNAs are transcribed *de novo* from the negative-strand RNA template of DI virion RNA. This mechanism would require multiple jumping during transcription. However, it is also possible that DI particles incorporate a trace amount of subgenomic DI RNA, which can replicate itself in infected cells. The latter mechanism would be more similar to the DI RNA of other virus systems. (2) Does DI genomic RNA code for a functional RNA polymerase which can replicate DI genomic RNA without helper viruses? This question arises from the oligonucleotide fingerprinting analysis of DI genomic RNA. This RNA contains multiple deletions as compared to the standard virus genome; however, the 5′ end of the genome, which presumably encodes viral RNA polymerases, is essentially intact (Makino *et al.*, 1985). The studies presented in this report revealed that coronavirus DI RNA synthesis involves several unique features.

MATERIALS AND METHODS

Viruses and cell culture

The MHV-JHM and MHV-A59 were used as nondefective standard viruses. The serially passaged MHV-JHM stock at passage level 17 was used as the source of DI particles (Makino *et al.*, 1985). This virus stock inhibits the growth of standard MHV-JHM to about 50–60% (Makino *et al.*, 1984a). All viruses were propagated in DBT cells as described previously (Makino *et al.*, 1984a). Mouse L-2 cells (Lai *et al.*, 1981) were used for RNA transfection studies.

Purification of viruses

^{32}P-labeled viruses were purified according to published procedures (Makino *et al.*, 1983) with slight modifications. Virus was collected at 14 hr postinfection (p.i.) and clarified by low-speed centrifugation. The supernatants were then placed on discontinuous sucrose gradients consisting of 10 and 50% (w/w) sucrose in NTE buffer (0.1 *M* NaCl, 0.01 *M* Tris–HCl, pH 7.2, and 0.001 *M* EDTA). After centrifiugation at 26,000 rpm for 3 hr at 4° in a Beckman SW 28.1 rotor, the virus band at the interphase between 10 and 50% sucrose was collected, diluted with NTE buffer, and centrifuged in a 10 to 60% continuous sucrose gradient at 26,000 rpm for 19 hr at 4°. After centrifugation, fractions were collected and the position of virus was determined by locating the radioactive virus peak.

Preparation of virion RNA and virus-specific intracellular RNA

Viral RNA was extracted from purified viruses by procedures described previously (Makino *et al.*, 1984a). MHV-specific intracellular RNA was labeled with [^{32}P]orthophosphate for 1 hr, from 7 hr to 8 hr p.i. in the presence of 2.5 μg/ml actinomycin D and extracted with phenol/chloroform as previously described (Makino *et al.*, 1984b).

Agarose gel electrophoresis

RNA was separated by electrophoresis on 1% agarose gels after denaturation with 1 *M* glyoxal (McMaster and Carmichael, 1977). For nondenaturing gels, RNA was dissolved in 10 m*M* sodium phosphate (pH 7.0) containing 1 m*M* EDTA, 0.1% SDS, 0.1% bromphenol blue, and 0.1% xylene cyanol FF, and mixed with an equal volume of glycerol and then applied to 1% agarose gel in electrophoresis buffer (10 m*M* sodium phosphate, pH 7.0). Preparative gel electrophoresis in 1 or 1.3% urea–agarose gels was performed as previously described (Makino *et al.*, 1984a). The RNA was eluted from gel slices by the method of Langridge *et al.* (1980).

uv irradiation of DI particles

Four hundred microliters of DI virus samples in growth medium was spread onto 60-mm Falcon plastic dishes. At a distance of 50 cm under a 30-W germicidal lamp, virus samples were irradiated for various periods of time. After uv irradiation, DI virus was mixed with nonirradiated MHV-JHM, and the mixture was immediately inoculated onto DBT cells. Virus-specific RNA synthesis was determined as described above.

RNA transfection

Confluent L-2 cells on 60-mm plastic dishes were first infected with MHV-A59 at a multiplicity of infection (m.o.i.) of 5. After 1 hr of adsorption, viruses were removed and the cells were washed once with prewarmed Eagle's minimum essential medium (MEM). Gel-purified RNA (l μg) in 200 μl of transfection buffer (600 μg/ml DEAE-dextran, 0.14 *M* LiCl, 10 m*M* HEPES, pH 7.5, and 1 m*M* $MgCl_2$) was added to the cells at room temperature. After 30 min of incubation, the inoculum was removed and the cells were washed once with prewarmed MEM. The infected cells were then incubated with 4 ml of MEM containing 5% fetal calf serum at 37° for 13 hr. Culture fluid was harvested, clarified, and stored at −70°. Four hundred microliters of the virus samples was used to infect new monolayers of DBT cells for subsequent virus passages.

Oligonucleotide fingerprinting

Purified ^{32}P-labeled RNA were digested with RNase T1 and the resistant oligonucleotides were separated

by two-dimensional polyacrylamide gel electrophoresis as previously described (Makino *et al.*, 1984a).

RESULTS

RNA species in DI virus particles

Our previous studies (Makino *et al.*, 1984a, 1985) suggest that, among different intracellular DI-specific RNA species, only the genome-sized DIssA RNA can be incorporated into virus particles. However, we could not rule out the possible presence of a small amount of subgenomic DI RNA species in DI virus particles. To reexamine this issue, intracellular RNA and RNA isolated from the purified virus released from the same DI-infected cells were compared directly. In the present study, the DI-containing MHV-JHM stock at passage level 17 was used as the source of DI particles. At this passage level, two DI RNA species, DIssA and DIssE, and mRNA 7 were detected in infected cells (Makino *et al.*, 1985) (Fig. 1, lane b). The amount of DIssE was different from preparation to preparation of virus stocks (data not shown) (see below). To characterize the RNA species in purified DI particles, a modified procedure was used for virus purification to include virus particles of lower density. A single virus band with a buoyant density of 1.19 g/cm^3 was detected (data not shown). This virus preparation contains both standard MHV-JHM genomic RNA and DI genomic RNA (Fig. 1, lane a). As shown previously, the DI genomic RNA in the purified virus particles represents the packaged DIssA RNA detected in infected cells (Makino *et al.*, 1985). No other RNA species are detectable. However, a prolonged exposure of the autoradiogram in this study revealed that the DI virus population contained a trace amount of mRNA 7 and DIssE (Fig. 1, lane c), suggesting that a minute amount of these subgenomic RNAs could be packaged. The presence of DIssE RNA, though in small quantity, in DI particles necessitated the distinction between DIssA and DIssE itself as the RNA template for the synthesis of DIssE RNA in DI-infected cells.

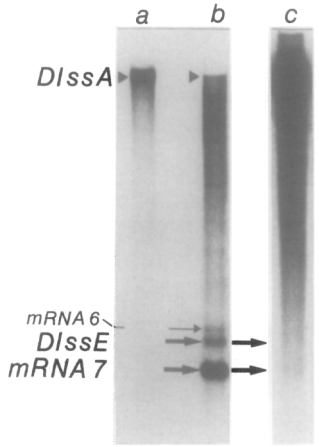

FIG. 1. Agarose gel electrophoresis of glyoxal-denatured ^{32}P-labeled virion RNA (a,c) and intracellular RNAs (b) of DI at the same passage level. Lane c represents a longer exposure of the autoradiogram of lane a.

uv inactivation kinetics of DI RNAs

To determine whether DIssE RNA is transcribed directly from DI genomic RNA or is replicated from the small amount of DIssE present in DI particles, we studied the effects of uv irradiation of DI particles on DI RNA synthesis. This study is based upon the chain-terminating effects of uv photoproducts in the RNA template. Potential sites for these uv lesions are distributed randomly along the RNA, and one uv lesion is sufficient to terminate transcription. Thus, inactivation of RNA synthesis by uv light occurs with pseudo one-hit kinetics. In the present study, lesions are induced on the DI genomic RNA by uv irradiation of virus particles. Therefore, the synthesis of negative-stranded RNA is terminated at the uv lesions on input virion RNA. If DIssE RNA is transcribed from the negative template of DI genomic RNA, the inactivation kinetics of the synthesis of both DIssA and DIssE should be the same. On the contrary, if DIssE RNA is replicated directly from the template of its own size, the DIssE RNA synthesis should be more resistant to uv irradiation than DIssA RNA, since DIssA has a much larger target size.

The DI virus stock was irradiated with uv light for various periods of time and was then used to coinfect DBT cells with standard MHV-JHM. The ^{32}P-labeled intracellular virus-specific RNA was analyzed by agarose gel electrophoresis. The resolution of DIssA and mRNA 1 of MHV-JHM was poor in agarose gel electrophoresis under denaturing conditions (Fig. 2, lanes a–f) (Makino *et al.*, 1985). However, under nondenaturing conditions, both RNAs were clearly separated (Fig. 2, lanes g–j) probably due to differences in the secondary structure between both RNAs. The latter electropherograms were used for determining the uv sensitivity of DIssA. It is evident that the synthesis of DIssA RNA was inhibited by uv light at a much faster rate than that of DIssE RNA, indicating that the sizes of template RNA of DIssE and DIssA are different. The target sizes of the template RNA for both RNAs were calculated to be equivalent to the physical sizes of these two RNAs, respectively (data not shown). Thus, DIssE RNA is most likely replicated from the small amount of DIssE RNA packaged in the DI particles, but not from the DI genomic RNA, which is the predominant RNA species in virus particles.

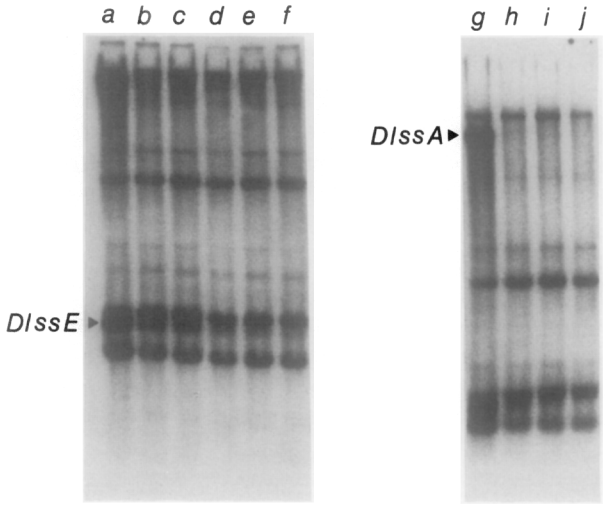

FIG. 2. Effects of uv irradiation of DI particles on DI RNA synthesis. DI-containing virus stocks were uv-irradiated for 0 sec (a,g), 20 sec (b,h), 40 sec (c,i) 60 sec (d,j), 90 sec (e), and 120 sec (f), respectively. The uv-irradiated virus samples were used to coinfect DBT cells with MHV-JHM at an m.o.i. of 2. The ^{32}P-labeled intracellular viral RNA was analyzed by electrophoresis on agarose gels after glyoxal denaturation (a to f) or without denaturation (g to j).

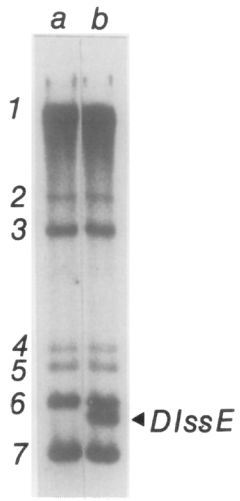

FIG. 3. The appearance of DIssE RNA in DBT cells after transfection with DIssE RNA and infection with MHV-A59. L-2 cells were infected with MHV-A59 and then transfected with purified DIssE RNA in the presence of DEAE-dextran. The culture fluid of infected cells was harvested at 14.5 hr p.i. and then passaged once in DBT cells. The virus harvested after the second passage was then used to infect DBT cells. The ^{32}P-labeled intracellular viral RNA was extracted, denatured with glyoxal, and analyzed by agarose gel electrophoresis. Lane a, mock-transfected. Lane b, DIssE-transfected.

Transfection of DIssE RNA

The uv inactivation study suggested that DIssE RNA replicates independently of DIssA RNA. To support this conclusion, we examined whether DIssE can be synthesized in the absence of DIssA RNA. For this study, DIssE RNA was purified from the gel and used to transfect L-2 cells which had been infected with MHV-A59 to serve as a helper virus. The virus harvested from the transfected L-2 cells was passaged an additional time on DBT cells before virus-specific RNA in infected cells was analyzed by agarose gel electrophoresis (Fig. 3). Clearly, the virus obtained from DIssE RNA-transfected culture synthesized DIssE RNA, while the virus from cells infected with helper virus alone did not. The absence of DIssA in both intracellular RNA samples was confirmed by nondenaturing agarose gel electrophoresis (data not shown). This study conclusively demonstrated that DIssE is replicated from its own template which is, in turn, transcribed from the small amount of DIssE RNA packaged in DI particles.

Structure of DIssE RNA in cells coinfected with MHV-A59

We have previously demonstrated that, during mixed infection with two different MHVs, the leader sequence from one virus could be detected on the mRNAs of the coinfecting virus at a very high frequency (Makino *et al.,* 1986). To determine whether DIssE is synthesized by the same mechanism as standard subgenomic mRNAs, i.e., utilizing a free leader RNA, we determined whether the A59 leader RNA could be utilized for the replication of DIssE RNA. Cells were coinfected with MHV-A59 and the DI particles of MHV-JHM at an m.o.i. of 1 each, and virus-specific intracellular RNAs were examined by agarose gel electrophoresis (Fig. 4). The data showed that the synthesis of MHV-A59 mRNAs was inhibited to the same extent as MHV-JHM by DI particles. However, the synthesis of mRNA 7 was not inhibited. The synthesis of DIssE was stimulated by coinfection with MHV-A59 (Fig. 4, lanes 5 and 8). This stimulation was not observed when MHV-JHM was used as a coinfecting virus (data not shown). The structure of DIssE obtained from cells coinfected with MHV-A59 and that from cells infected with DI only were compared. As shown in Fig. 5, the oligonucleotide fingerprints of DIssE from these two sources are indistinguishable. Both contain JHM-specific leader T_1-oligonucleotide No. 8 and other DIssE-specific T_1 spots (Makino *et al.,* 1985). The leader-specific oligonucleotide of MHV-A59, which would have been electrophoretically distinguishable (Makino *et al.,* 1986), was not detected in DIssE isolated from coinfected cells. This result was not due to the absence of MHV-A59 leader RNA synthesis since the synthesis of MHV-A59 mRNA 7 was not completely inhibited (Fig. 4, and data not shown). Oligonucleotide fingerprinting analysis of this RNA in-

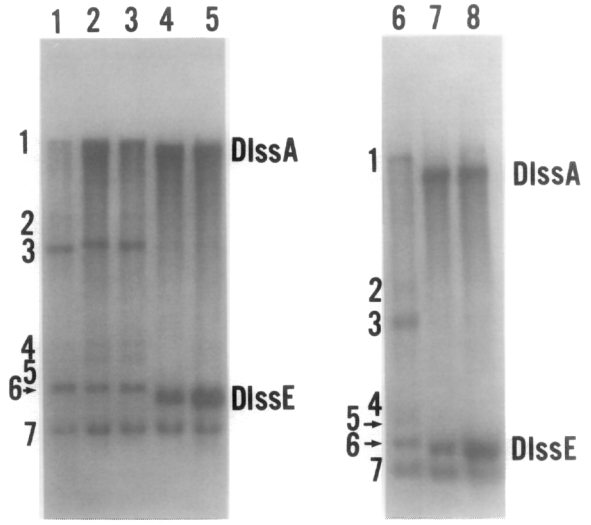

FIG. 4. Electrophoretic analysis of intracellular RNAs from cells mix-infected with different viruses. The ^{32}P-labeled intracellular RNAs were separated by agarose gel electrophoresis after glyoxal treatment (1–5) or without denaturation (6–8). Lane 1, single infection with MHV-JHM; lane 2, single infection with MHV-A59; lanes 3, 6, mixed infection with MHV-A59 and MHV-JHM; lanes 4, 7, single infection with DI-containing MHV-JHM; lanes 5, 8, mixed infection with MHV-A59 and DI-containing MHV-JHM.

dicated that the MHV-A59-specific leader RNA was present in the same molar quantity as other MHV-A59 sequences (data not shown). Therefore, we conclude that the synthesis of DIssE is the result of replication of its own template, and does not involve a free leader RNA species, which is required for the transcription of standard subgenomic mRNA.

Dependence or independence of helper functions for DI-specific RNA synthesis

Oligonucleotide fingerprinting analysis shows that the DIssA RNA contains multiple deletions as compared to the wild-type genome; however, the 5′-end gene A, which presumably encodes viral RNA polymerases, is essentially intact (Makino *et al.*, 1985). This raises a possibility that the DIssA RNA may encode a functional polymerase. Thus, DIssA RNA could be synthesized independently of the helper functions provided by standard viruses. To prove the DIssA encodes a functional polymerase which can synthesize its own RNA independently of helper functions, we examined the effects of serial virus dilution on the synthesis of DI-specific RNAs. Since the virus stock used in this experiment contained both DI particles and wild-type MHV-JHM, serial virus dilution would reduce the possibility of a cell being coinfected with both DI particles and standard helper viruses. Thus, if DI RNA synthesis requires helper functions from the standard virus, the DI RNA synthesis should decrease exponentially following virus dilution, according to a two-hit kinetics. In contrast, a one-hit kinetics would indicate that DI RNA can be synthesized independently of helper functions. Cells were infected with serial dilutions of either DI-containing MHV-JHM stock or standard MHV-JHM, and ^{32}P-labeled virus-specific RNA were separated by electrophoresis (Figs. 6A, 6B, 6C). The amounts of virus-specific RNAs were quantified by densitometry and the graphs were fitted by linear regression analysis (Figs. 6D, 6E, 6F). The effects of virus dilution on the synthesis of standard virus-spe-

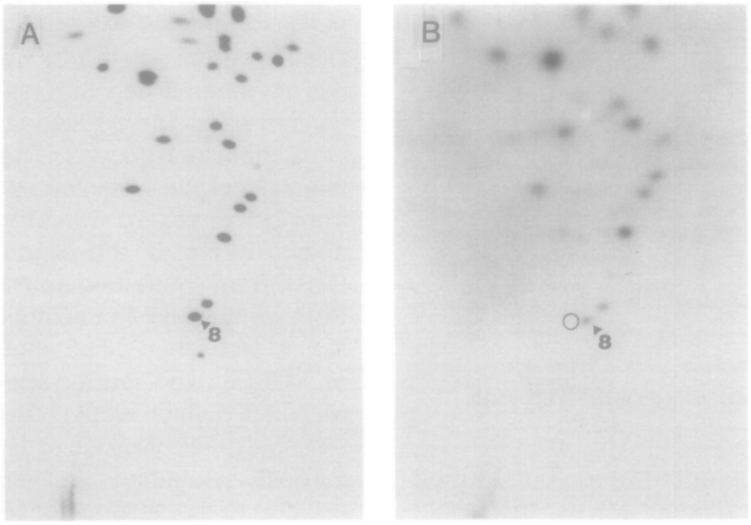

FIG. 5. Oligonucleotide fingerprints of DIssE RNA from the cells infected with DI only (A) or coinfected with DI and MHV-A59 (B). Oligonucleotide 8 is the leader-specific oligonucleotide of DIssE. The circle represents the predicted location of MHV-A59 leader-specific oligonucleotide 10 (Lai *et al.*, 1981; Makino *et al.*, 1986).

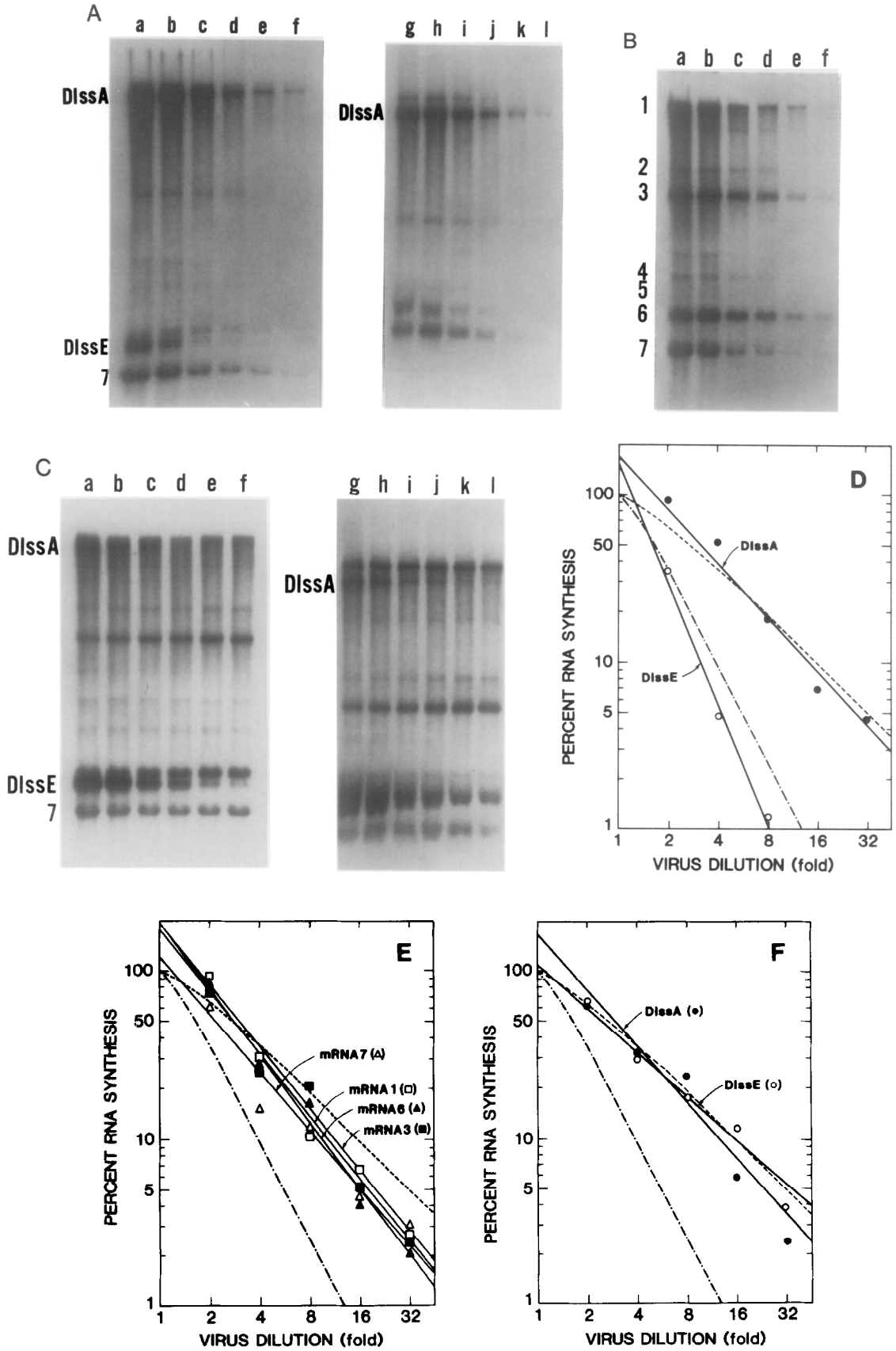

FIG. 6. Effects of virus dilution on DI RNA synthesis. (A,B,C) DBT cells were infected with twofold serially diluted DI-containing viruses (A), standard MHV-JHM (B), or a mixture of serially diluted DI viruses and an excess amount of MHV-JHM (m.o.i. = 5) (C). ^{32}P-labeled virus-specific RNAs were separated by electrophoresis following glyoxal treatment (a–f) or without glyoxal (g–l). Lanes a and g are undiluted viruses at m.o.i. of 1. The remaining lanes are twofold serially diluted viruses. (D,E,F) The autoradiographs in (A), (B), and (C) were quantified by densitometry and the graphs were fitted by linear regression analysis. Theoretical one-hit kinetics (---) and two-hit kinetics (– – –) curves are also shown. (D), (E), and (F) correspond to (A), (B), and (C), respectively.

cific mRNAs are shown in Figs. 6B and 6E. Since all MHV-specific mRNAs are transcribed from the same negative template of genomic RNA (Lai *et al.,* 1982b), the kinetics of RNA synthesis for all mRNAs vs virus dilution follows a one-hit kinetics. Similarly, the synthesis of DIssA decreased linearly following virus dilution, according to a one-hit kinetics (Figs. 6A and 6D), suggesting that DIssA was synthesized in the absence of helper functions. Thus the DI genomic RNA contains all the genetic information required for DIssA RNA synthesis. In contrast, the reduction of DIssE RNA synthesis followed a two-hit kinetics, suggesting that helper functions from standard viruses are required for DIssE synthesis (Figs. 6A and 6D). This interpretation was further tested by a similar virus dilution experiment performed in the presence of a constant amount of excess standard MHV-JHM (m.o.i. = 5). As shown in Figs. 6C and 6F, the kinetics of DIssE RNA synthesis appeared similar to that of DIssA as compared by simple regression analysis. This study demonstrated that the replication of subgenomic DI RNA requires a *trans*-acting product(s), which was supplied by the coinfecting standard virus.

DISCUSSION

In this report, we studied the mechanism of synthesis of DI-specific RNAs in MHV DI-infected cells. Our data showed that both of the DI-specific RNA species, DIssA and DIssE, are synthesized from the negative-strand RNA template of their own sizes. In addition, DIssE requires helper viruses for its replication, while DIssA does not. Thus DIssA contains all of the functions essential for its own replication.

The template size for DIssE RNA synthesis was unexpected. Since MHV DI particles were previously shown to contain only the genome-sized RNA (Makino *et al.,* 1984a), it was suggested that DIssE RNA had to be transcribed *de novo* from the DI genomic RNA by a jumping transcription mechanism. The data in this report showed that a minute amount of DIssE RNA could be packaged along with other subgenomic mRNAs into virus particles. The studies of uv target size and transfection of DIssE RNA conclusively indicated that the DIssE RNA synthesis utilizes the negative-strand RNA template of its own size and does not rely on the DI genome. Thus, DIssE RNA is most likely replicated from the packaged DIssE RNA associated with the virus particles. This study thus indicated that DIssE RNA synthesis is different from the transcription of subgenomic mRNAs of standard MHV. This conclusion is also supported by the finding that DIssE RNA synthesis does not utilize a free leader RNA of a helper virus. Thus, DIssE RNA synthesis is more similar to the replication of MHV RNA genome.

The poor incorporation of DIssE in virus particles distinguishes the MHV DI from DIs of other viruses. The incorporation of DIssE RNA in virions may be nonspecific and random. This may account for the variation in the amount of DIssE RNA synthesized by different DI-containing virus preparations, since different DI stocks may contain different amounts of DIssE RNA in the virion. Thus, it is likely that DIssE RNA lacks packaging signals, or, alternatively, DIssE RNA may be too small to be packaged efficiently. Presently we cannot distinguish between these two possibilities.

Since DIssE RNA appears to be incorporated into virus particles nonspecifically, only a minute number of cells may be infected with DIssE-containing viruses. Despite the small number of cells infected with viruses carrying DIssE RNA, the DIssE RNA is the predominant RNA species in infected cultures. These data suggest that the replication of DIssE RNA has an enormous advantage over the synthesis of other MHV RNA species. The sequence analysis of DIssE RNA (unpublished data) shows that this RNA is composed of three discontiguous parts of the viral genome including the 5′-end leader and 3′-end of genomic RNA, and also contains some minor sequence changes within the leader sequence. It is not clear which part is responsible for this enhanced RNA synthesis. As suggested by the studies of the effects of virus dilution on DI RNA synthesis (Fig. 6), the synthesis of DIssE apparently requires *trans*-acting products provided by helper viruses. It is unlikely that free leader RNA species are the helper function, since A59 leader RNA did not reassort with DIssE RNA (Fig. 5). One candidate helper function may be viral RNA polymerase.

It is surprising that the synthesis of DIssA RNA did not require helper functions, implying that this RNA probably encodes a functional RNA polymerase. This result was consistent with our previous oligonucleotide fingerprinting studies which show that gene A, which encodes RNA polymerase, is essentially intact in DIssA RNA. Only a minor difference was detected in this region between DI and standard MHV-JHM genome (Makino *et al.,* 1985). Therefore, by definition, the DI genomic RNA present in virus particles is not defective at the level of RNA synthesis.

It has been demonstrated that mRNA 7 and its product N protein are synthesized in DI-infected cells even though most of the wild-type mRNA synthesis is inhibited (Fig.4; Makino *et al.,* 1985). We have shown that the mRNA 7 detected is most likely transcribed from the DIssA RNA genome, rather than from the helper virus genome (unpublished data). This interpretation is consistent with oligonucleotide fingerprinting studies, which show that the genomic region corresponding to mRNA 7 is intact in DIssA. It has been suggested that

N protein is important for MHV RNA synthesis (Compton *et al.*, 1987). Therefore, it is conceivable that both DIssA and mRNA 7 are synthesized from the negative template RNA of DI genomic RNA, and their gene products, RNA polymerases and N protein, are both essential for viral RNA replication.

Another consequence of the finding that DIssA is capable of autonomous RNA synthesis is that it might be possible to isolate persistently infected cell lines in which only DI genomic RNA and possibly mRNA 7 are synthesized. Such a cell line will be very useful for understanding the mechanism of persistent viral infection.

ACKNOWLEDGMENTS

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