Utilization of Homotypic and Heterotypic Proteins of Vesicular Stomatitis Virus by Defective Interfering Particle Genomes for RNA Replication and Virion Assembly: Implications for the Mechanism of Homologous Viral Interference

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Defective interfering (DI) particles of Indiana serotype of vesicular stomatitis virus (VSVInd) are capable of interfering with the replication of both homotypic VSVInd and heterotypic New Jersey serotype (VSVNJ) standard virus. In contrast, DI particles from VSVNJ do not interfere with the replication of VSVInd standard virus but do interfere with VSVNJ replication. The differences in the interfering activities of VSVInd DI particles and VSVNJ DI particles against heterotypic standard virus were investigated. We examined the utilization of homotypic and heterotypic VSV proteins by DI particle genomic RNAs for replication and maturation into infectious DI particles. Here we show that the RNA-nucleocapsid protein (N) complex of one serotype does not utilize the polymerase complex (P and L) of the other serotype for RNA synthesis, while DI particle genomic RNAs of both serotypes can utilize the N, P, and L proteins of either serotype without serotypic restriction but with differing efficiencies as long as all three proteins are derived from the same serotype. The genomic RNAs of VSVInd DI particles assembled and matured into DI particles by using either homotypic or heterotypic viral proteins. In contrast, VSVNJ DI particles could assemble only with homotypic VSVNJ viral proteins, although the genomic RNAs of VSVNJ DI particles could be replicated by using heterotypic VSVInd N, P, and L proteins. Thus, we concluded that both efficient RNA replication and assembly of DI particles are required for the heterotypic interference by VSV DI particles.

The mechanism of homologous viral interference mediated by defective interfering (DI) particles has been the subject of investigation for several decades, with the expectation that an understanding of homologous viral interference at the molecular level may lead us to the development of virus-specific, antiviral therapeutic agents. DI particles are subgenomic virus particles, which are generated from the standard virus during undiluted, high-multiplicity passages. DI particles depend on the viral proteins provided by the standard virus for their replication and maturation, yet they interfere with the replication of standard virus in vitro and in vivo. Vesicular stomatitis virus (VSV) has been frequently utilized to study the mechanism of the homologous viral interference, since VSV DI particles are physically separable from the standard virus. We isolated two panhandle-type DI particles from the two different serotypes of VSV, VSVInd DI particle (IΔDI) from Indiana serotype (26) and VSVNJ DI particle (NIΔDI) from New Jersey serotype (4), and characterized their genomic structures and interfering activities in our laboratory (4). Among several types of DI particles of VSV, the panhandle-type DI particles are the most commonly isolated. Panhandle-type DI particles contain various lengths of inverse complementary sequences at the 3′ and 5′ termini of their genome (13, 21). The 3′ genomic terminus of the panhandle-type DI particle is exactly the same as the 3′ terminus of the plus-strand antigenome of the standard virus. The essential role of the genomic termini for replication and maturation of the panhandle-type DI particles was demonstrated by in vitro reconstitution assay (2, 5, 11). However, it has not been determined whether or not the genomic RNA of one serotype can be utilized as a template for RNA replication by the heterotypic polymerase complex. To determine how genomic RNAs and RNP complexes of DI particles interfere with the replication of the standard virus rather than primary transcription of mRNAs (9, 10, 14). The transcription of VSV mRNA from the ribonucleoprotein (RNP) complex of one serotype is carried out only by the homotypic polymerase complex, which has been demonstrated by in vitro reconstitution assay (2, 5, 11). However, it has not been determined whether or not the RNA-N protein complex of one serotype can be utilized as a template for RNA replication by the heterotypic polymerase complex.
particles from one serotype can utilize the proteins from the other serotype of VSV, we constructed four different cDNA plasmids encoding chimeric DI RNA genomes representing VSVInd [pIDI(50-50) and pIDI(255-268)] and VSVNJ [pNJDI (50-50) and pNJDI(227-188)]. To understand the molecular mechanism that leads to the different outcomes of the heterotypic interference by VSVInd and VSVNJ DI particles, we investigated the replication and maturation of DI particles in the presence of homotypic and heterotypic VSV proteins using the VSV reverse genetics system. Here we show the difference between VSVInd DI particles and VSVNJ DI particles in terms of the ability to utilize heterotypic proteins for genome replication as well as particle assembly and maturation.

MATERIALS AND METHODS

Plasmids. Plasmids containing the VSVInd DI particle genome with λ sequences [pIDI(50-50) and pIDI(255-268)] and plasmids containing the VSVNJ DI particle genome (Hazelhurst strain) with λ sequences [pNJDI(50-50) and pNJDI(227-188)] were generated by replacing internal sequences of the VSV DI particle genomes with 2,322 bp of bacteriophage λ DNA. The internal region of the 2,322-bp HindIII fragment sequence as shown in Fig. 1. The cDNA clones of VSV genes from VSVInd and the Hazelhurst strain of VSVNJ were constructed in the pluScriptor II KS vector (Stratagene, La Jolla, CA). The clones are nucleocapsid protein N gene clones (pBKS-NI and pBKS-NNJ), phosphoprotein P gene clones (pBKS-PI and pBKS-PNJ), matrix protein M gene clones (pBKS-MI and pBKS-MNJ), and glycoprotein G gene clones (pBKS-GI and pBKS-GNJ). The expression of the proteins from the cDNA clones has been confirmed previously in our laboratory (4). We have described construction, expression, and functional analyses of the plasmids encoding L proteins of VSVNJ (pBKS-LNJ) recently (15). A plasmid encoding VSVInd L (pGem4-LI) was kindly provided by M. Schubert (National Institutes of Health [NIH], Bethesda, MD). Expression of the VSV proteins from these plasmids was under the control of the T7 transcriptional promoter.

Recovery of chimeric DI particles from cDNA transfection. Chimeric DI particles were recovered from cDNAs by transfection using the calcium phosphate precipitation method and reverse genetics of VSV (16, 24).

Virus, chimeric DI particle preparation, and antibody. A heat-resistant strain of VSVInd (originally obtained from L. Prevec, McMaster University, Hamilton, Ontario, Canada) and the Hazelhurst strain of VSVNJ (a gift from S. Emerson, NIH) were used for the study. Recombinant vaccinia virus, vTF7-3 (6), was a kind gift from Bernard Moss (NIH) and was used as the source of T7 DNA-dependent RNA polymerase in the VSV reverse genetics system. Stock of vTF7-3 was prepared in TK-143 cells as described by Mackett et al. (20).

The chimeric DI particles of VSV recovered from cDNA transfection were amplified by a gradient centrifugation using 5% to 30% linear sucrose gradients prepared in TNE buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA). The collected chimeric DI particles were stored at −80°C.

To estimate DI particle concentration, the total protein of the DI particle was quantified with a protein assay kit (Bio-Rad, Hercules, CA).

Polyclonal rabbit antibodies against total proteins of VSVInd and VSVNJ were prepared previously in our laboratory using sucrose gradient-purified and lysed whole viruses (4).

Analysis of RNA synthesis. For the analysis of chimeric DI RNA replication in the presence of the N, P, and L proteins of VSV, one of the chimeric plds encoding the positive-strand antigenomic RNA of chimeric DI and plasmids encoding N, P, and L of VSVInd or VSVNJ were transfected into BHK-21 cells, which were preinfected with recombinant vaccinia virus vTF7-3, at an MOI of 5. Transfection was carried out using Lipofectin (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. The concentrations of the plasmids used for the transfection were 1.4 pmol (5 to 5.5 μg) of chimeric pDHs and 6 μg, 3 μg, and 1 μg of pN, pP, and pL, respectively. Chimeric DI RNA-N protein complexes were isolated from cell lysates prepared at 30 h posttransfection by immunoprecipitation. Immunoprecipitation of RNA-N protein complexes and RNA extraction were done as described previously (15).

The level of chimeric DI RNA synthesis was determined by Northern blot analysis using λ sequence-specific riboprobes λ-E/E1 and λ-E/E2 (15). The RNA bands were detected by autoradiography, and the intensity of the bands was measured using a densitometer (ImageMaster; Amersham-Pharmacia, Piscataway, NJ).

Genomic RNA and mRNA synthesis of the standard virus and genomic RNA synthesis of the chimeric DI particles in cells coinfected with the standard virus were examined by labeling infected cells with [3H]uridine (30 Ci/ml) (NEN, Boston, MA) in the presence of 10 μg/ml actinomycin D. The cells were radio-labeled for 2 h at 4 h postinfection. RNAs were isolated from the cells and analyzed by electrophoresis in a 1% agarose formaldehyde gel (3). After the completion of gel electrophoresis, the gel was fixed with a solution containing 30% methanol and 10% acetic acid for 60 min. The gel was washed twice with methanol for 30 min each and then treated with a 3% solution of 2,5-diphenyloxazole (Sigma-Aldrich, St. Louis, MO) for 3 h. The gel was rehydrated in distilled H2O for 60 min and dried using a gel drier (Bio-Rad). The RNA bands were detected by autoradiography.

FIG. 1. Schematic representation of chimeric pDHs containing bacteriophage λ sequences. The internal region of the 2,322-bp HindIII fragment of λ DNA is shown as a shaded bar. Numbers in the names of the plasmids indicate the length (in nucleotides) of wild-type sequences at the 3′ and 5′ genomic termini of chimeric DI RNAs. The sizes of chimeric DI RNAs encoded from the plasmids are shown at the right side of the plasmids. Eighty-eight-nucleotide deletions (88 nts) in the λ DNA of pNJDI(50-50) are shown under the shaded bar. T7P, HDV, and T7T indicate the T7 transcriptional promoter, hepatitis delta virus ribozyme sequences, and T7 transcriptional terminator, respectively. Numbers in the bracket indicate conserved 3′ and 5′ VSV-specific nucleotide sequences of DI particle genomes.
The interfering activities of purified DI particles were examined by the standard virus yield reduction assay as described previously (26, 27, 31).

RESULTS

Usage of P and L proteins by RNA-N protein complex of DI particles. The functional template for the replication of the DI particle is the genomic RNA encapsidated with N protein. It has been demonstrated that the polymerase complex (P and L proteins) of isolated RNP can be interchanged with the added P and L proteins (8). It may be possible that the capability of RNPs of DI particles from one serotype to utilize P and L proteins provided by the standard VSV of the other serotype determines the heterotypic interfering activities of DI particles. Therefore, we examined the utilization of P and L proteins of both VSV\textsubscript{Ind} and VSV\textsubscript{NJ} by DI genomic RNA-N protein complex from both serotypes of VSV. We analyzed the synthesis of two of the DI RNAs [IDI(50-50) and NJDI(50-50)] (Fig. 1) in various combinations of N, P, and L proteins from the two different serotypes. The detection of RNA synthesis by Northern blot analysis is depicted in Fig. 2. Figure 2B shows the replication of IDI(50-50) in the presence of different combinations of N, P, and L proteins from VSV\textsubscript{Ind} and VSV\textsubscript{NJ}. T7 RNA polymerase-mediated positive-strand RNA synthesis and encapsidation were detected in all combinations (Fig. 2B, open block arrow); however, VSV RNA polymerase-derived negative-strand RNA synthesis was detected with only three combinations of N, P, and L proteins; (i) N\textsubscript{Ind}, P\textsubscript{Ind}, and L\textsubscript{Ind}; (ii) N\textsubscript{NJ}, P\textsubscript{NJ}, and L\textsubscript{NJ}; and (iii) N\textsubscript{NJ}, P\textsubscript{Ind}, and L\textsubscript{NJ} (Fig. 2B, lanes 2, 4, and 10). NJDI(50-50) replicated in the presence of the same combinations of N, P, and L proteins as IDI(50-50) (Fig. 2C), although the efficiency of replication in the presence of N\textsubscript{Ind}, P\textsubscript{Ind}, and L\textsubscript{Ind} was lower than that in the presence of N\textsubscript{NJ}, P\textsubscript{NJ}, and L\textsubscript{NJ}. Synthesis of NJDI(50-50) RNA in the presence of N\textsubscript{NJ}, P\textsubscript{Ind}, and L\textsubscript{NJ} was also detected, indicating that not only IDI(50-50) but also NJDI(50-50) can be replicated by the com-
bination of NNJ, P Ind, and LNJ proteins. These results demonstrate that N, P, and L proteins of one serotype do not exclude the genomic RNAs of VSV DI particles as long as all three proteins are from the same serotype or three proteins are in the combination of NNJ, P Ind, and LNJ. The results also demonstrate that DI RNP complex from one serotype does not utilize P and/or L proteins from the other serotype. It was interesting to see that replication of chimeric DIs was carried out by the combination of NNJ, P Ind, and LNJ but not with the combination of NInd, P NJ, and LInd. These results clearly demonstrate that PInd can interact with N and L proteins from both serotypes of VSV. In contrast, PNJ can interact only with N and L proteins of VSVNJ.

Usage of homotypic and heterotypic N, P, and L proteins by DI particle genomic RNA for RNA replication. The genomic RNAs of DI particles from both serotypes containing only 50 nucleotides of terminal sequences can utilize N, P, and L proteins as long as all three proteins are from the same serotypes (Fig. 2). The panhandle-type DI particles contain cis-acting promoter sequences at the 3’ termini of both positive- and negative-strand genomes. It has been previously demonstrated that the VSVInd DI particle genome requires only 45 nucleotides of 3’ and 5’ genomic terminal sequences to replicate efficiently (18). The minimal number of terminal nucleotides required for efficient replication of VSVNJ DI particles has not been determined previously. The NInd genome contains 71-nucleotide-long inverse complementary sequences (15), which are longer than that of IInd (54 nucleotides). We compared the genomic RNA replication between DI particles containing 50 nucleotides of genomic end sequences, IInd(50-50) and IInd(50-50), and DI particles containing more than 50 nucleotides of genomic end sequences, IInd(255-268) and NJInd(227-188). To determine the replication efficiency of DI RNAs using homotypic and heterotypic N, P, and L proteins, DI RNA synthesis was analyzed in the presence of N, P, and L proteins from either VSVInd or VSVNJ as depicted in Fig. 2. The amounts of encapsidated plus-strand RNA synthesized from the chimeric pDI by T7 RNA polymerase were similar among the chimeric DI particles (Fig. 3, open block arrow), indicating that approximately equal numbers of templates were available for negative-strand RNA synthesis by the VSV RNA polymerase. All chimeric DI RNAs were synthesized by the VSVInd N, P, and L proteins with differing efficiencies. Although chimeric VSVNJ DI RNAs were synthesized by the heterotypic VSVInd N, P, and L proteins, the amount of chimeric VSVNJ DI RNA synthesis was significantly lower than that of chimeric VSVInd DIs (Fig. 3A). In contrast, the N, P, and L proteins of VSVNJ supported replication of chimeric IInd(50-50) and IInd(255-268) as efficiently as its homotypic NJInd(227-188) particle (Fig. 3B). Our results demonstrate that genomic RNAs of DI particles can utilize N, P, and L proteins from either serotype, but only IInd RNA can replicate with high efficiency using N, P, and L proteins from both serotypes.

In the presence of VSVInd N, P, and L proteins, similar amounts of negative-strand RNAs were synthesized from IInd(50-50) and IInd(255-268), indicating that 50 nucleotides of IInd at both the 3’ and 5’ genomic termini are sufficient for replication and encapsidation (Fig. 3A, lanes 2 and 3). In contrast, NJInd(50-50) particles were only 30% as efficient in the synthesis of negative-strand RNA as NJInd(227-188) (Fig. 3B, lanes 3 and 4). These results suggest that the VSVNJ DI particles require more than 50 nucleotides of terminal genomic sequences for the optimum level of genomic RNA replication.
Functional RNP complex (Fig. 3A). Therefore, we decided to use VSV Ind standard virus (26, 28). On the other hand, VSV NJ DI particles recovered by using VSV Ind N, P, M, G, and L proteins. VSV NJ was used as a helper virus for the DI particles recovered by using VSV Ind N, P, M, G, and L proteins. The presence of chimeric DI particles in the culture fluid was determined by infecting fresh BHK-21 cells with the culture fluid from the first amplification and Northern blot analysis using the λ sequence-specific riboprobe λ-E2.

Assembly of DI particles using homotypic and heterotypic proteins. Assembly and maturation of VSV is mediated by the interaction of RNP complexes with matrix proteins (M) and glycoproteins (G) (19, 22, 34). VSV Ind DI particles are able to replicate and mature in cells coinfected with either VSV N, P, M, G, and L proteins. VSV Ind was used as a helper virus for the DI particles recovered by using VSV Ind N, P, M, G, and L proteins. The presence of chimeric DI particles in the culture fluid was determined by infecting fresh BHK-21 cells with the culture fluid from the first amplification and Northern blot analysis using the λ sequence-specific riboprobe λ-E2.

Genomic terminal sequences of DI particles confer interfering activity. We examined the interfering activities of purified IDI(50-50) and NJDI(227-188) against the homotypic and heterotypic standard viruses to determine the role of genomic termini in homologous viral interference. BHK-21 cells were infected at an MOI of 3 with standard VSV (VSV Ind or VSV NJ) and superinfected with various concentrations (16 ng, 64 ng, 256 ng, and 1,024 ng) of DI particles. At 18 h after infection, culture medium was harvested and the titer of the standard virus was measured by plaque assay. IDI(50-50) interfered with the replication of both VSV Ind and VSV NJ. The yield reduction of the standard virus was correlated with the increased amount of input DI particles in both homotypic and heterotypic viral interference assays (Fig. 5). Although IDI particles could interfere heterotypically with the replication of VSV NJ, 6- to 20-fold more IDI(50-50) particles were required to achieve the same level of interference as with the homotypic standard virus (Fig. 5A). NJDI(227-188) particles also showed the same level of interfering activity as their homotypic wild-type VSV NJ DI particles (data not shown). These VSV NJ DI particles interfered with the replication of the homotypic VSV Ind standard virus, resulting in a standard virus yield from 300- to 1,000-fold less at the highest concentrations of input DI particles. However, these VSV NJ DI particles did not interfere with the replication of the heterotypic VSV Ind standard virus, even with the highest concentration (Fig. 5B). These results demonstrate that the interfering activity of the panhandle-type DI particles of VSV was conferred by the 3′ and 5′ genomic termini, which contain promoter sequences, but not by the internal region of the DI particle genome.

In cells coinfected with VSV DI particles and the standard virus, DI particles inhibit the replication of full-length standard virus genomic RNA and secondary transcription of the mRNAs, while genomic RNA of DI particles is synthesized preferentially to a certain saturation point (4). Therefore, we examined the synthesis of standard virus genomic RNA and genomic RNA of DI particles in cells infected with both standard virus and chimeric DI particles. BHK-21 cells were infected with an MOI of 3 of VSV Ind or VSV NJ and superinfected with various concentrations of IDI(50-50) or NJDI(227-188) (1 ng, 4 ng, and 16 ng for the homotypic standard virus and 64 ng, 240 ng, and 960 ng for the heterotypic standard virus). Cells were infected with the homotypic VSV Ind proteins (Fig. 4, lanes 3 and 4), indicating that VSV NJ DI particles cannot assemble using VSV Ind proteins although their genomic RNAs can be replicated in the presence of N, P, and L proteins of VSV Ind. In contrast, all chimeric DI particles [NJDI(50-50), NJDI(227-188), IDI(50-50), and IDI(255-268)] were recovered from cDNAs when all five VSV Ind proteins were coexpressed (Fig. 4, lanes 6, 7, and 8). The amount of IDI(50-50) particle recovered was extremely low, necessitating a longer exposure to see a band on the Northern blot. These findings demonstrate that the IDI genome replicates and assembles using proteins from VSV NJ, as expected. However, genomes of NJDI particles cannot be assembled using VSV Ind proteins, despite the fact that the genomes of these DI particles can be replicated at a low level in the presence of N, P, and L proteins of VSV Ind. We also found that IDI(50-50) and NJDI(50-50) matured poorly with VSV NJ proteins. These results suggest that the VSV Ind proteins can assemble only with VSV Ind genomes and that an appropriate length of genomic terminal sequences is required in the assembly of VSV NJ and VSV Ind virions.
labeled with \[^{3}H\]uridine (30 μCi/ml) for 2 h. Total cellular RNAs were isolated and analyzed in a 1% agarose formaldehyde gel. The RNA bands were visualized by autoradiography. I\(_{DI}(50-50)\) showed a dramatic inhibition of VSV\(_{Ind}\) genomic RNA and mRNA synthesis with increasing amounts of DI particles (Fig. 6). The synthesis of I\(_{DI}(50-50)\) genomic RNA increased gradually with larger amounts of input DI particles (Fig. 6A). I\(_{DI}(50-50)\) inhibited genomic RNA and mRNA synthesis of VSV\(_{NJ}\) as well, but much greater amounts of DI particles were required to obtain the same level of interfering activity compared to the VSV\(_{Ind}\) RNA synthesis (Fig. 6A). The amount of I\(_{DI}(50-50)\) genomic RNA synthesized with the help of VSV\(_{NJ}\) was not as large as that of I\(_{DI}(50-50)\) genomic RNA synthesized in the presence of homotypic VSV\(_{Ind}\). Similar levels of I\(_{DI}(50-50)\) genomic RNA were synthesized in cells coinfected with 240 ng and 960 ng of the DI particles and the VSV\(_{NJ}\) standard virus (Fig. 6A, lanes 7 and 8). NJ\(_{DI}(227-188)\) inhibited genomic RNA and mRNA synthesis of VSV\(_{NJ}\) in a gradual manner with increasing concentration of the DI particles (Fig. 6B). In contrast, NJ\(_{DI}(227-188)\) did not affect the genomic RNA and mRNA synthesis of VSV\(_{Ind}\), although we used as much as 960 ng per culture dish (Fig. 6B).

To our surprise, a small amount of NJ\(_{DI}(227-188)\) RNA was replicated with the help of the VSV\(_{Ind}\) standard virus (Fig. 6B). We have confirmed that the low level of NJ\(_{DI}(227-188)\) RNA synthesis was not the result of copurified VSV\(_{NJ}\) standard virus.

FIG. 5. Interfering activity of chimeric DI particles. The yields of the standard viruses represent the mean value of duplicate plaque assays. (A) Yield reduction of VSV standard viruses by I\(_{DI}(50-50)\). (B) Yield reduction of VSV standard viruses by NJ\(_{DI}(227-188)\). Error bars represent the standard deviation of the mean of three separate assays.

FIG. 6. Analysis of VSV RNA synthesized in cells coinfected with chimeric DI particles and the standard viruses. (A) Synthesis of I\(_{DI}(50-50)\) genomic RNA and genomic RNA and mRNA of VSV\(_{Ind}\) and VSV\(_{NJ}\) standard virus in the presence of various concentrations of input I\(_{DI}(50-50)\). (B) Synthesis of genomic RNA of NJ\(_{DI}(227-188)\) and genomic RNA and mRNA of VSV\(_{Ind}\) and VSV\(_{NJ}\) with various concentrations of NJ\(_{DI}(227-188)\). Arrows indicate the genomic RNAs of the chimeric DI particles, Std, standard virus; L, G, N, and M/P, mRNA of each gene.
in the DI particle preparation (data not shown). The result was confirmed by Northern blot analysis using the λ sequence-specific riboprobe, which is much more sensitive and detects internal λ sequences in the N1DI(227-188) RNA directly (data not shown). These results show that VSVNJ DI particles can replicate at a very low efficiency with the help of the VSVInd standard virus, although N1NJ failed to interfere heterotypically.

**DISCUSSION**

Both standard VSV and DI particles incorporate all five VSV proteins during assembly and maturation (1, 12, 32). Although panhandle-type DI particles cannot produce any VSV proteins, it is assumed that they can initiate their genomic RNA synthesis by using the polymerase complex incorporated into the DI particles (30). If DI particles are to interfere with the replication of the standard virus homotypically and/or heterotypically, the DI particle RNPs or genomic RNAs synthesized from the initial templates should be able to utilize the proteins from the standard virus efficiently. Because only the genomic RNAs encapsidated with N protein as a form of RNP complex function as templates for transcription and replication, it has been difficult to demonstrate in vitro whether or not the DI genomic RNA of one serotype can utilize the proteins of the other serotype. It has been demonstrated that VSVInd DI particles could utilize proteins from the VSVNJ standard virus for their replication in infected cells (28). However, genomic RNA synthesis of the VSVNJ DI particle has not been demonstrated in cells coinfected with the VSVInd standard virus. It has also been demonstrated that P and L proteins in the RNP are interchangeable with the newly added P and L proteins in vitro (8). Therefore, we expected that the RNP complex of VSVInd DI particles could exchange with P and L proteins of VSVNJ, but that the RNP complex of VSVNJ DI particles could not do the reverse. DI particle genomic RNAs of both VSVInd and VSVNJ replicated when N, P, and L proteins were from the same serotype or a combination of N1NJ, P1Ind, and L1NJ (Fig. 2B and C). Any other combinations of N, P, and L proteins did not support the replication of VSVInd DI particle or VSVNJ DI particle genomes. Combinations of N1Ind, P1Ind, and L1NJ or N1Ind, P1NJ, and L1NJ did not support the replication of the 1DI genome, demonstrating that the RNA-N protein template of the VSVInd DI particle or the VSVNJ DI particle cannot utilize the P and L proteins of the heterotypic standard virus directly. Overall, the data shown in Fig. 2 demonstrate that protein interactions among RNA-N protein complexes, P proteins, and L proteins play a crucial role in conferring serotypic specificity of the VSV.

It was not known previously whether or not the genomic RNAs of VSVNJ could utilize VSVInd proteins for their replication. Our results shown in Fig. 3 demonstrate that genomic RNAs of VSVNJ DI particles utilized N, P, and L proteins of VSVInd for their replication. However, the efficiency of the VSVNJ DI particle RNA replication in the presence of VSVInd N, P, and L proteins was much lower. This shows that DI particle genomic RNAs from both serotypes can utilize VSV proteins without serotypic exclusion but with differing efficiencies.

It has previously been demonstrated that panhandle-type VSVInd DI particle genomes containing 45 nucleotides of the 3′ and 5′ terminal genomic sequences replicated efficiently in the presence of homotypic proteins (23). Our data in Fig. 3 agreed with the previous results by showing that ID1DI(50-50) and ID1DI(255-268) replicated equally well with both homotypic and heterotypic N, P, and L proteins. VSVNJ DI particle genomes containing only 50 nucleotides of terminal genomic sequences [NJ1DI(50-50)] were less efficient in their replication than DI particle genomes containing more than 188 nucleotides of the VSVNJ-specific terminal genomic sequences [NJ1DI(227-188)]. Considering the long 3′ and 5′ inverse complementary sequence of the N1NJ particle genome (71 bases), it may be possible that VSVNJ DI particles containing longer inverse complementary sequences in their genomes are generated from the standard virus and are selectively amplified because of the advantage they have in utilizing the polymerase complex or other viral proteins involved in the assembly of the VSV virions.

VSV virions mature by budding through the cytoplasmic membrane as a result of the specific interaction of the RNP complex with the matrix protein (M) and glycoprotein (G) (17, 22). Since chimeric VSVNJ DI RNAs can replicate using the N, P, and L proteins of VSVInd (Fig. 3A, which suggests the assembly of functional RNP, they should be able to mature and bud out as DI particles if M and G proteins of VSVInd are provided. However, the results in Fig. 4 show that the maturation of VSVNJ DI particles using VSVInd proteins does not occur. These data can be interpreted in one of two ways. The first, a very low level of replication of NJ1DI genome using N, P, and L proteins of VSVInd results in few RNA-N protein complexes which are available for assembly by M and G proteins of VSVInd. The second is that specific genomic terminal sequences of DI particles may be involved in the maturation of VSV particles as described by others (33).

Pattnaik et al. have shown that chimeric VSVInd DI particles containing 51 nucleotides from the 3′ and 5′ genomic termini of a panhandle-type DI particle with non-VSV internal sequences could replicate and mature into infectious DI particles (23). These investigators examined the maturation of the chimeric DI particle without analyzing the interfering activity of the chimeric DI particles. We successfully recovered, amplified, and purified 1DI50(50-50), 1DI50(255-268), and NJ1DI(227-188) particles using the homotypic standard viruses (Fig. 4, lanes 1, 2, and 8). The interfering activities of the recovered chimeric DI particles were the same as those of wild-type DI particles, indicating that genomic terminal sequences encompassing the promotor sequences in panhandle-type DI particle genomes are the only elements required for interfering activity.

We propose the following model of homotypic and heterotypic viral interference mediated by VSV DI particles. In the case of homotypic viral interference, RNPs of VSV DI particles utilize their own as well as newly synthesized polymerase complexes provided by the standard virus to initiate antigenomic RNA synthesis. Once the RNAs are synthesized from the RNP of DI particles, the nascent RNA genomes are encapsidated by N proteins synthesized by the standard virus and form new RNPs. While DI particle genomic RNA replicates using N, P, and L proteins without the need for transcription, standard viruses have to transcribe mRNAs to provide the viral proteins necessary for replication. Because its 3′ and 5′ inverse complementarity of panhandle-type DI particle genome pro-
vides stronger promoter sequences to both senses of the DI particle genome (18, 23) and the DI particle is dependent on the standard virus for the N, P, and L proteins (25). DI particles have a replicative advantage over the standard virus. Consequently, DI particles interfere with the replication of the standard virus.

In the case of heterotypic interference, when cells are infected with DI particles and a heterotypic standard virus, DI particle RNA-N protein complexes from either serotype cannot utilize P and L proteins from different serotypes (Fig. 2). They require initial transcription of leader RNAs through the use of their own DI particle-associated P and L proteins. The incapability of DI RNPs to directly use VSVNJ P and L proteins may delay the replication of the DI particle genome. The delay of DI particle replication may give the VSVNJ standard virus a chance to initiate replication using the available N, P, and L proteins and subsequently results in the reduced interfering activity against the VSVNJ standard virus. This possibility is supported by an earlier observation that adding DI particles at later times of standard virus infection fails to inhibit the genomic RNA and mRNA synthesis of the standard virus (29). The efficiency of the promoter of the VSVNJ DI particle genome using the polymerase complex from VSVInd was not sufficient to compete for VSVInd P and L proteins (Fig. 3). Therefore, VSVNJ DI particles cannot interfere with the replication of VSVInd standard virus, although genomic RNAs of VSVNJ DI particles can be replicated by N, P, and L proteins of VSVInd with very low efficiency (Fig. 3).

Another difference between the VSVInd and VSVNJ DI particles in using heterotypic proteins is the inability of VSVNJ DI particles to assemble into virions using VSVInd proteins (Fig. 4). Although we cannot directly demonstrate whether or not the ability to assemble using heterotypic proteins contributes to heterotypic interference by VSVInd DI particles, we speculate that the assembly of VSVInd DI particles using VSVNJ proteins will amplify the DI particles and the amplified DI particles will spread to neighboring cells, which will result in a reduction of the standard virus yield. In contrast to the VSVInd DI particles, the VSVNJ DI particles cannot assemble using heterotypic VSVInd proteins, although their genomic RNA can be replicated by VSVInd proteins at a low level; therefore, the input DI particles are restricted to the cells initially infected. Our results suggest that both DI particle genomic RNA replication and particle assembly are prerequisites for efficient homologous viral interference. In addition, the ability of the genomic RNAs of DI particles to replicate and mature as DI particles efficiently using heterotypic VSV proteins allows DI particles to interfere heterotypically.

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REFERENCES


