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Interference Among Defective Interfering Particles of Vesicular Stomatitis Virus

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Three defective interfering (DI) particles of vesicular stomatitis virus (VSV), all derived from the same parental standard San Juan strain (Indiana serotype), were used in various combinations to infect cells together with the parental virus. The replication of their RNA genomes in the presence of other competing genomes was described by the hierarchical sequence: DI 0.52 particles > DI 0.45 particles ≥ DI-T particles > standard VSV. The advantage of one DI particle over another was not due simply to multiplicity effects nor to the irreversible occupation of limited cellular sites. Interference, however, did correlate with a change in the ratio of plus and minus RNA templates that accumulated intracellularly and with the presence of new sequences at the 3′ end of the DI genomes. DI 0.52 particles contained significantly more nucleotides at the 3′ end that were complementary to those at the 5′ end of its RNA than did DI-T or DI 0.45 particles. The first 45 nucleotides at the 3′ ends of all of the DI RNAs were identical. VSV and its DI particles can be separated into three classes, depending on their terminal RNA sequences. These sequences suggest two mechanisms, one based on the affinity of polymerase binding and the other on the affinity of N-protein binding, that may account for interference by DI particles against standard VSV and among DI particles themselves.

When viruses grow to high titer, deletion mutants arise. They become enriched in the population, especially if they have the capacity to interfere with the growth of the standard wild-type parent (12, 14, 45). To study the molecular basis of this interference, we have used vesicular stomatitis virus (VSV) because of its asymmetric bullet shape, which permits the separation of shorter deletion mutants from the parental wild type (15). This separation has led to the characterization of individual size classes of defective interfering (DI) particles and the reconstruction of experiments with known amounts of standard and DI particles.

The primary competition between DI particles and standard VSV occurs intracellularly during the step of RNA replication or genome amplification (17, 29, 30). Very little is known about this mechanism, because RNA replication is initiated early during infection, and all of the proteins that are involved have not been identified. It is known, however, that input multiplicities affect this interference, that the small size of the interfering RNA alone cannot account for its ability to compete during replication, and that the derivation of a DI particle in relation to the strain of standard helper virus determines in part the degree of competition and the subsequent ability of the DI RNA to be replicated (14).

Continuous undiluted passages of the same VSV strain may result in the appearance of several different size classes of DI particles, with some or one-size particles eventually dominating the viral population (11, 21, 31, 37). These results show that interference occurs not only between DI particles and standard virus, but also among different DI particles. By examining DI particles, all derived from the same parental standard virus, it is suggested here that, besides RNA replication, there is another rate-limiting step during virus maturation when interference occurs. We found that the ability to interfere increased with the extent of complementary sequences found at the termini of the RNA.

MATERIALS AND METHODS

Materials. The source of all materials has been recently reported (35) except for the following. Cytidine 3′,5′-bisphosphate and [γ-^{32}P]ATP were purchased from New England Nuclear Corp., Boston, Mass. Actinomycin D was a kind gift from Merck Sharp & Dohme, Rahway, N.J. Practical-grade acrylamide and electrophoretic-grade *N,N′*-methylene-bisacrylamide were purchased from Eastman Kodak Chemical Co., Rochester, N.Y. Aminobenzyloxymethyl (ABM)-cellulose paper was purchased from

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Schleicher & Schuell Co., Keene, N.H. Polynucleotide kinase and RNA ligase from T4-infected *Escherichia coli* were purchased from P-L Biochemicals, Inc., Milwaukee, Wisc. Calf intestine alkaline phosphatase was purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. RNases T_1, T_2, A, and U_2 were purchased from Calbiochem-Boehring Corp., San Diego, Calif. RNase physarum M was a kind gift from Helen Donis-Keller.

Cells and viruses. BHK, CHO, and mouse L cells were grown as suspension cultures in Joklik modified Eagle minimal essential medium supplemented with 5% bovine serum and nonessential amino acids. Cloned and sucrose gradient-purified standard VSV of the Indiana serotype (San Juan strain) and three size classes of DI particles derived from this same standard strain were used. According to a recent agreement (36), the three DI particles are designated: VSI ts^+ SJ DI-T/DI 0.33 (5') (15, 19); VSI ts^+ SJ DI 0.52 (5') (19, 34); and VSI ts^+ SJ DI LT/DI 0.45 (5') (5). DI 0.45 was kindly supplied by Sue Emerson (University of Virginia, Charlottesville). All DI particles were purified by two rounds of sucrose gradient centrifugation. Their effective multiplicity was determined as described by Huang and Manders (17).

Infection of cells and extraction of cytoplasmic RNA. Infection of 4×10^6 cells at 34°C with the indicated multiplicities for each experiment was done essentially as described (9, 35). When RNA was examined, the cells were treated with actinomycin D at 5 μg/ml at the beginning of infection and 0.1 mCi of ^{32}P per ml at the indicated times. Cytoplasmic extracts were made with the nonionic detergent Nonidet P-40 (17). Phenol-chloroform extractions of the RNA from the cytoplasm were done as described previously (9, 35).

Separation of RNA. For the separation of RNA species ranging from 30 to 12,000 nucleotides, two different gel systems (10% polyacrylamide and 1.5% agarose) were used as previously described (35). To provide quantitative comparisons of the amounts of RNA, either the autoradiogram was scanned or the bands were excised from the gels and the radioactivity was counted. Conclusions based on the incorporated radioactivity (counts per minute) were similar to those made when molar amounts of RNA were calculated.

Elution of RNA from 10% polyacrylamide gels. After electrophoresis of RNA on a polyacrylamide gel, the gel was covered with Saran Wrap and autoradiographed. Using the autoradiogram as a template, the region of the gel containing labeled RNA was excised and sliced into pieces of 2 mm^3. Small gel cubes were then transferred to a siliconized tube together with 2 volumes of elution buffer (0.4 M sodium acetate, 0.01 M Tris, pH 7.8, and 0.2% sodium dodecyl sulfate) containing 50 μg of yeast carrier RNA per ml. Elution proceeded overnight at 22°C with intermittent mixing. The small gel pieces were rinsed with 1 additional volume of elution buffer. The combined eluates were then filtered through a 0.45-μm filter to retain any remaining particulate polyacrylamide. The RNA was then precipitated by 2 volumes of ethanol.

Transfer of RNA from agarose gels to DBM paper and hybridization to ^{32}P-labeled RNA. The procedure of Alwine et al. (1) was used with the following modifications. Potassium phosphate buffer, pH 6.5, was used instead of sodium borate buffer, pH 8.0. Unlabeled VSV-specific RNA extracted from virions and from the cytoplasm was first separated on a 1.5% agarose gel. After urea was diffused out of the gel, the gel was placed in 200 ml of 50 mM NaOH and rocked gently for 40 min at room temperature. The gel was then treated twice with 200 ml of 200 mM potassium phosphate buffer, pH 6.5, for 5 min at 22°C. Conversion of the ABM paper to diazobenzyloxymethyl (DBM) paper, subsequent transfer of RNA from the gel to DBM paper, and hybridization conditions all followed the procedures described by Alwine et al. (1), except that ^{32}P-labeled small RNA from cells coinfected with DI-T and standard VSV was the hybridization probe rather than ^{32}P-labeled DNA. After the unhybridized labeled RNA was thoroughly washed off, the DBM paper was placed in Seal-N-Save boilable bags (Sears Roebuck and Co.) and exposed to X-ray film with an intensifying screen at −70°C.

Extraction and purification of DI particle RNA. Purified DI particles were diluted two- to three-fold with Earle saline and then pelleted by centrifugation at 89,300 × *g* for 90 min. The pelleted particles were suspended in ETS buffer (0.01 M EDTA, 0.01 M Tris, pH 7.4, and 0.2% sodium dodecyl sulfate) and repeatedly extracted with phenol-chloroform. Virion RNA was precipitated with ethanol. The RNA resuspended in ETS buffer was loaded onto a 15 to 30% (wt/wt) sucrose gradient made in NETS buffer (0.15 M NaCl in ETS buffer). Centrifugation was for 17 h at 89,300 × *g*. The gradient fractions were collected and monitored by UV absorbance. Full-length DI-particle genomic RNA was then pooled and ethanol precipitated.

Radioactive labeling of the termini of the RNA of DI particles. Purified RNAs from DI particles were labeled at the 3' end by ligation of [^{32}P]cytidine 3',5'bisphosphate according to England and Uhlenbeck (6) as modified by Schubert et al. (40). After labeling, the RNA was again purified through sucrose gradients. For labeling at the 5' end, purified RNAs were first suspended in 10 mM Tris, pH 8.4, boiled for 1.5 min, and then rapidly chilled to 4°C. Calf intestine alkaline phosphatase was added to a ratio of 10^{-3} U per μg of RNA. After 30 min at 34°C, the RNA was extracted with phenol-chloroform repeatedly and then ethanol precipitated. The RNA was suspended in 20 μl of buffer consisting of 50 mM Tris, pH 8.4, 10 mM $MgCl_2$, and 5 mM dithiothreitol. [γ-^{32}P]ATP at 100 μCi was added, and the whole sample was lyophilized. Then 20 μl of sterile double-distilled water containing 10 U of polynucleotide kinase was added for 30 min at 37°C. RNA was again extracted with phenol-chloroform repeatedly and then ethanol precipitated.

Isolation of self-complementary terminal sequences. End-labeled RNAs at 1 to 4 μg from DI particles were mixed with 50 μg of yeast carrier RNA in 25 μl of distilled water. Equal volumes of 2× hybridization buffer (1× = 0.4 M NaCl, 0.01 M Tris, pH 7.4, and 0.01 M EDTA) were added, and then the RNA sample was sealed in a capillary tube and incubated at 70°C for 2 h. Samples were pipetted into 200 μl of hybridization buffer containing, per milliliter, RNase T_1 at 50 U, RNase T_2 at 5 U, and RNase A at 100 μg. Digestion proceeded at 37°C for 30 min. After digestion, samples were extracted repeatedly with phenol-chloroform in the presence of added carrier RNA (50 to 100 μg) and precipitated with ethanol. To denature double-strand-

ed RNA, the RNA was suspended in 10 mM Tris, pH 7.4, boiled for 1.5 min, and rapidly chilled on ice.

RESULTS

Interference among different DI particles. Three different DI particles, all isolated from the San Juan strain of VSV, were used in various combinations together with standard VSV to infect cells in order to test whether any one of the DI particles was particularly more effective than the others during interference. This was measured by comparing the amount of intracellular RNA specific to each DI particle.

As shown by the absence of standard virus-specific 40S RNA, each of the DI particles (DI 0.45, DI 0.52, and DI-T) was capable of completely inhibiting the replication of standard VSV without affecting primary transcription of the L, G, N, NS, and M mRNA's (Fig. 1, slots a, f, and g). These results measuring RNA correlated well with inhibition of infectivity. When identical combinations were used and plaque-forming ability of standard VSV was assayed, all three DI particles, singly or in combination, completely inhibited the growth of VSV (D. D. Rao and A. S. Huang, unpublished data). Radioactive material between the origin and 40S RNA probably represents contaminating DNA and some trapped RNA. Agarose gel patterns of RNA obtained from cells infected with only standard VSV have been published (19, 34, 35).

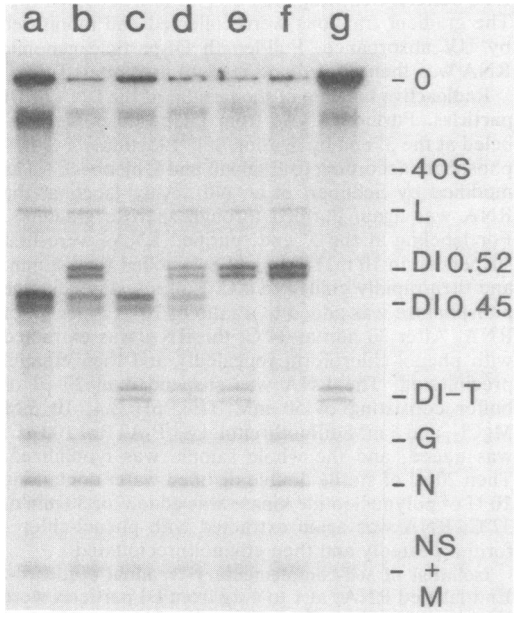

FIG. 1. Interference among DI particles of VSV. BHK cells in five cultures were infected with the indicated viruses at multiplicities of 20 each. ^{32}P was added to the cultures at 1 to 6 h postinfection. Cytoplasmic RNA was then extracted, and equal portions were separated on a 1.5% agarose gel. (a) Standard VSV plus DI 0.45 particles; (b) standard VSV plus DI 0.45 plus DI 0.52 particles; (c) standard VSV plus DI 0.45 plus DI-T particles; (d) standard VSV plus DI 0.45 plus DI 0.52 plus DI-T particles; (e) standard VSV plus DI 0.52 plus DI-T particles; (f) standard VSV plus DI 0.52 particles; (g) standard VSV plus DI-T particles.

When cells were triply infected with any two of the DI particles and standard VSV, 40S RNA synthesis was still inhibited, but there were varying degrees of replication of the genomes of DI particles. DI 0.45 and DI-T particles replicated about equally well in each other's presence (Fig. 1, slot c), whereas DI 0.52 particles had an advantage over the two smaller DI particles. When the individual bands were excised and the radioactivity was compared by Cerenkov counting, DI 0.52 particles synthesized between 1.2 and 2.5 times the molar amount of plus and minus strands synthesized by the smaller DI particles. The advantage increased with time (Fig. 1, slots b and e). When all three DI particles were added to cells in the presence of standard virus, there was an overall reduction in the total amount of RNA replication for each of the genomes, but DI 0.52 still appeared to replicate its RNA somewhat better than the other two DI particles (Fig. 1, slot d).

These results indicate that all three DI particles interfered effectively against the parental standard VSV. Among the DI particles, the largest one appeared to have a replicative advantage over the two smaller ones. In only one cycle of growth, interference against standard virus was complete, whereas interference among DI particles was only partial.

Symmetry of replication. During standard VSV genome replication, plus and minus RNA templates accumulate asymmetrically, with the bulk of the RNA being of the minus polarity (27, 44). It has been postulated that differences between plus and minus strands in their binding affinities for polymerases may account for this asymmetry and that if binding affinities of DI templates are altered, the differential accumulation of the plus and minus strands may be changed as well (16, 33). Because the plus and minus strands of RNA specific to the replication of VSV DI particles have been recently separated on gels (19), the two templates were measured directly.

To test the above hypothesis, the doublet bands of DI RNAs from coinfected cells were quantitated, and the ratio of plus strands (bottom band) to minus strands (top band) was determined (Table 1). DI 0.52 particles synthesized virtually equivalent amounts of plus and minus strands, whereas the two smaller DI particles made plus and minus RNA in the ratio of 40:60.

TABLE 1. Ratio of intracellular plus and minus RNA specific to DI particles[a]

DI particles	%	
	Plus strand	Minus strand
DI 0.52	49 ± 3	51 ± 2
DI 0.45	40 ± 1	60 ± 1
DI-T	40 ± 1	60 ± 1

[a] BHK cells were coinfected with standard VSV and one of the three DI particles, each at a multiplicity of 20, and exposed to ^{32}P from 0 to 6 h. Then the RNA was extracted, separated on an agarose gel, and exposed to X-ray film. The bands representing intracellular RNA of the size of the genome of each DI particle were quantitated by scanning the film. The assumption was made that the faster-migrating band is plus stranded and the slower-migrating band is minus stranded (19).

These direct measurements confirm previous results for DI-T particles obtained by RNA hybridization studies (18). This is in contrast to the ratio of 20:80 for the intracellular plus and minus RNA strands of standard VSV (27). These results indicate that as the interfering capability of the DI particles increased, there appeared to be a more equal accumulation of plus and minus RNA strands.

Interference by DI 0.52 particles against DI-T particles and standard VSV in mouse L, CHO, and BHK cells. Because DI-T and DI 0.45 particles appeared to be nearly equivalent in their interfering potential and because DI 0.45 particles have been studied elsewhere (40, 41), we chose to focus on DI 0.52 and DI-T particles for detailed comparative studies. Initial attempts were to select a cell line which showed the greatest degree of interference. Three different cell lines were each triply infected with standard VSV and both DI particles, all at equal multiplicities. When equal portions of the RNA from each cell type were separated on 1.5% agarose gels, RNA specific to DI 0.52 particles was always the predominant species (Fig. 2). RNA of the size of standard virus genomes, migrating at 40S, was undetectable, but some RNA specific to DI-T particles was seen (Fig. 2). These results indicate that all three cell lines supported interference by DI 0.52 particles against standard VSV and DI-T particles. In addition, as was seen in Fig. 1, interference among the DI particles was quantitatively different from interference with standard virus by DI particles.

Parenthetically, the total amount of ^{32}P incorporated into the RNA from equivalent numbers of cells was highest for BHK cells. This reflects the different abilities of these cell types to incorporate ^{32}P into total virus-specific RNA, even though the production of infectious progeny was equivalent for each of these three cell lines (G. J. Freeman, Ph.D. thesis, Harvard University, Cambridge, Mass., A79). Because of the ability of suspended BHK cells to incorporate ^{32}P into VSV RNA, these cells were chosen for all subsequent experiments.

Relative accumulation of RNA species during interference between DI 0.52 and DI-T particles. To measure the rate of inhibition of synthesis of RNA at hourly intervals after infection, ^{32}P-labeled RNA from cells coinfected with standard VSV and each or both of the DI particles was separated and quantitated. Figure 3 shows the accumulation of the intracellular RNAs from each of the three sets of infected cells. The accumulation of total plus and minus strands of DI RNAs was compared with that of the mRNA for the N protein from the same cells. When cells were coinfected with either one of the DI particles and standard VSV, DI RNA replication occurred to about the same extent (Fig. 3a,b). When cells were triply infected, total RNA synthesis was reduced to approximately 70% of that in cells coinfected with only one type of DI particle and standard VSV (Fig. 3c). In these

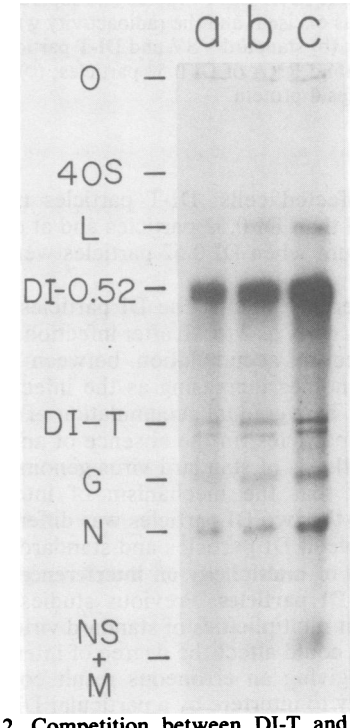

FIG. 2. Competition between DI-T and DI 0.52 particles in different cell lines. Mouse L (a), CHO (b), and BHK (c) cells were each triply infected with standard VSV, DI 0.52 particles, and DI-T particles, each at a multiplicity of 20. ^{32}P was added from 1 to 6 h after infection. Equal amounts of the cytoplasmic RNA obtained from these infected cells were separated on 1.5% agarose gels.

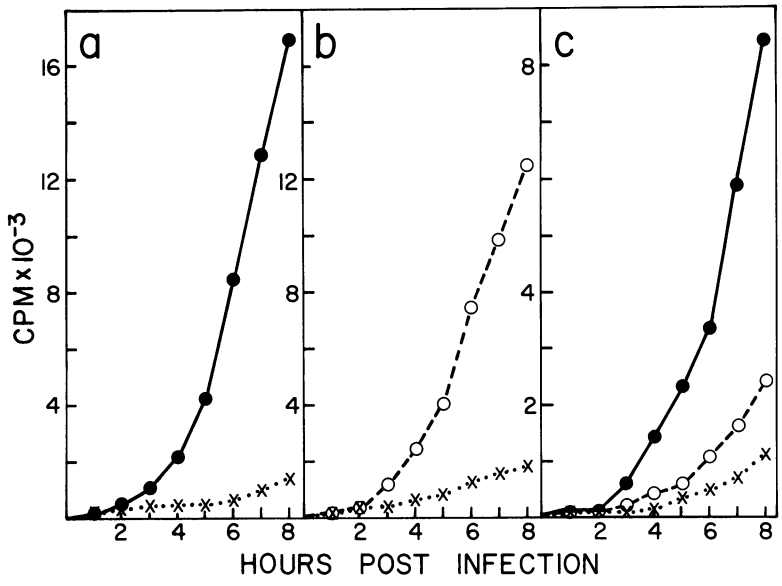

FIG. 3. Accumulation of intracellular RNA species in cells coinfected with standard VSV and DI particles. BHK cells were coinfected with standard VSV and either DI 0.52 or DI-T particles. Another culture of cells was triply infected with standard VSV and both DI particles. All multiplicities were at 20 each. ^{32}P was added at the time of infection together with actinomycin D. At hourly intervals, equal portions were removed, and cytoplasmic RNA was extracted and separated on 1.5% agarose gels. Each of the VSV-specific RNA-containing bands was excised, and the radioactivity was quantitated by Cerenkov counting. (a) Standard VSV and DI 0.52 particles; (b) standard VSV and DI-T particles; (c) standard VSV and both DI particles. Symbols: (●) plus- and minus-strand RNA of DI 0.52 particles; (○) plus- and minus-strand RNA of DI-T particles; (×) mRNA for the nucleocapsid protein.

triply infected cells, DI-T particles replicated less well than DI 0.52 particles and at only 25% the amount when DI 0.52 particles were absent (Fig. 3c).

Interference among the DI particles was detected as early as 2 to 3 h after infection, with the difference in accumulation between the two RNA genomes increasing as the infection progressed. This gradual accumulation of genomes of DI-T particles, in the absence of any detectable synthesis of standard virus genomes, again suggests that the mechanism of interference between the two DI particles was different from that between DI particles and standard VSV.

Effects of multiplicity on interference between the two DI particles. Previous studies showed that input multiplicities of standard virus and DI particles could affect the degree of interference, thereby giving an erroneous result concerning the ability to interfere by a particular DI particle (20, 43). To ensure that the partial inhibition by DI 0.52 particles against DI-T particles was not due simply to multiplicity, cells were triply infected with DI 0.52 particles together with standard VSV, each at a constant multiplicity of 20, and then with DI-T particles over a fivefold range. As a measure of interference, the total radioactivity in plus and minus RNA of the two DI particles was compared.

In these triply infected cells, RNA of DI-T particles accumulated to the same reduced amount despite increased multiplicities of DI-T particles (Fig. 4). The lack of enrichment of RNA of DI-T particles at higher multiplicities indicated that the inhibitory effect of DI 0.52 particles on DI-T particles was multiplicity independent.

On the other hand, at the higher total multiplicities, RNA of DI 0.52 particles was decreased (Fig. 4). This reverse effect of increased multiplicity of DI-T particles on the interfering DI 0.52 particles relates to an expected multiplicity-dependent event where the competition for proteins involved in RNA replication is equal between the two incoming DI genomes.

Effect of cycloheximide on the initial competition between DI-T and DI 0.52 particles. Previously, it was found that an advantage was recovered by DI-T particles over DI 0.52 particles if cells were infected with DI-T particles and standard VSV for a period before superinfection with DI 0.52 particles (19). To determine whether this superinfection exclusion was due to the ability of input DI-T particles to bind irrevers-

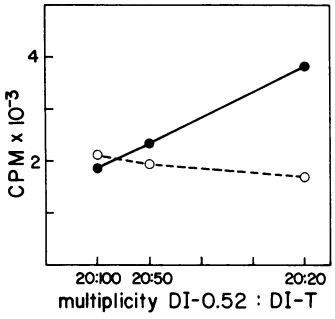

FIG. 4. Effects of relative multiplicities on the competition between DI-T and DI 0.52 particles. Three cultures of BHK cells were infected with standard VSV and DI 0.52 particles, each at a multiplicity of 20, and with multiplicities of DI-T particles at 20, 50, and 100. ^{32}P was present from 1 to 6 h post-infection. Cytoplasmic RNA was extracted and separated on 1.5% agarose gels. Bands containing subgenomic-sized DI RNA, both plus and minus strands, were excised, and the radioactivity was quantitated by Cerenkov counting. Symbols: (●) RNA of DI 0.52 particles; (○) RNA of DI-T particles.

ibly to limited resources or sites during the period before superinfection, cycloheximide was added during the period of superinfection. This prevents genome amplification without inhibiting attachment and uncoating (17, 24). Cells coinfected with standard VSV and DI-T particles were treated or not with cycloheximide for 0.5 or 1 h and then superinfected with DI 0.52 particles, followed by the removal of cycloheximide 0.5 h later.

Figure 5a shows the usual predominance of the larger DI RNA when cells were triply infected at the same time. When DI-T particles had an advantage of 0.5 or 1 h, there was a relative decrease in the ability of DI 0.52 to replicate its RNA (Fig. 5b,d). However, if cycloheximide were present to prevent the amplification of input DI-T genomes, then the RNA of DI 0.52 was replicated to advantage over DI-T RNA (Fig. 5c,e).

These results indicate that input DI-T particles did not irreversibly tie up limited resources of the cell, but that superinfection exclusion of DI 0.52 particles required the initial increase in DI-T templates. The end result of prior infection by one DI particle was an increase in their effective multiplicity. Therefore, prior infection, without RNA replication, did not irreversibly tie up limited resources or occupy sites which control RNA replication.

Hybridization of small RNA to RNA from standard VSV and DI particles and from infected cells. Sequence differences at the 3′ end of RNA genomes are postulated to account for the ability of DI particles to compete against standard VSV (13, 23). It therefore becomes important to examine the sequences at the RNA termini of both DI genomes in order to determine whether any differences could account for the ability of the larger DI particle to inhibit the smaller DI particle. Hybridization of the RNA genomes in solution gave 9% self-annealing for both DI 0.52 and DI-T particles (19). This suggests that the larger genome may have more self-complementary sequences than the smaller genome. Oligonucleotide analysis indicates that both are derived from the *L* gene and contain sequences specific to the 5′ end of the VSV RNA (10, 19).

To test for sequence differences at the 3′ end of the two DI RNAs, we took advantage of the presence of a small RNA, 46 nucleotides in length, which is synthesized in large amounts in cells coinfected with standard VSV and DI parti-

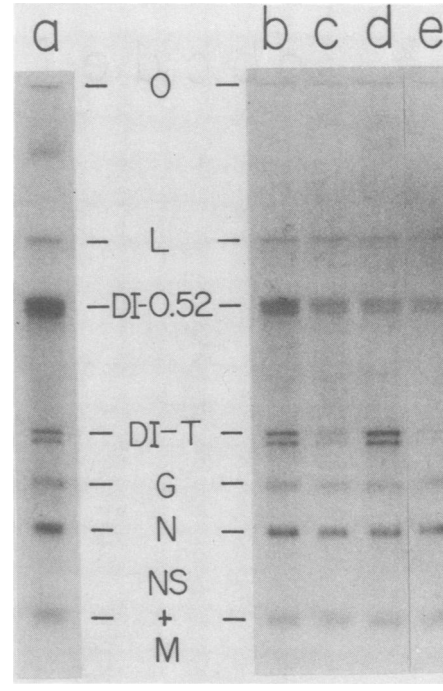

FIG. 5. Reversal by cycloheximide of the competitive advantage of DI particles due to prior infection. BHK cells at 20 × 10^6 cells/ml were infected with standard VSV and DI-T particles at a multiplicity of 20 each and then divided into five equal portions. Addition of cycloheximide at 100 μg/ml, superinfection with DI 0.52 particles at a multiplicity of 20, and exposure to ^{32}P were as follows. RNA was harvested at 6 h after infection and separated on a 1.5% agarose gel. (a) DI 0.52 particles at 0 h, ^{32}P at 1 to 6 h; (b) DI 0.52 particles at 0.5 h, ^{32}P at 1.5 to 6 h; (c) cycloheximide at 0 to 1 h, DI 0.52 particles at 0.5 h, ^{32}P at 1.5 to 6 h; (d) DI 0.52 particles at 1 h, ^{32}P at 2 to 6 h; (e) cycloheximide at 0 to 1.5 h, DI 0.52 particles at 1 h, ^{32}P at 2 to 6 h.

cles (34). Because small RNA is complementary to the 3′ end of DI RNAs (23), it can be used to determine the identity or divergence of sequences at the 3′ end by cross-hybridization.

To do so, ^{32}P-labeled small RNA was prepared from cells coinfected with DI-T particles and standard VSV (34, 35). Small RNA was hybridized against unlabeled virion RNA prepared from standard VSV or DI particles, as well as against total unlabeled intracellular RNA from singly or coinfected cells. The unlabeled RNA was first separated on agarose gels and then transferred to paper before hybridization. Small RNA failed to anneal to the negative-strand RNA from standard VSV, whereas it bound to the negative-strand RNA from either DI 0.52 or DI-T particles (Fig. 6c,e). RNA from coinfected cells was recognized by small RNA if they were DI genomes or their complementary plus strand (Fig. 6d,f).

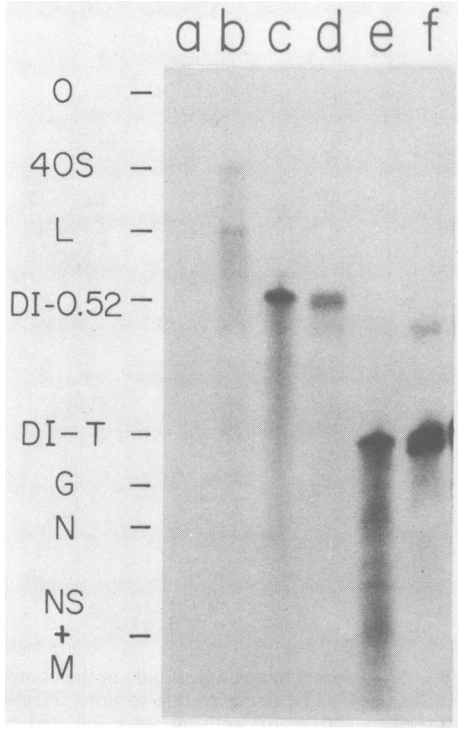

FIG. 6. Northern blot hybridization of small RNA to VSV-specific RNA species. Virion RNAs at 0.5 to 1 μg from standard VSV or its DI particles and intracellular RNA at 3 μg from singly or coinfected cells were separated on a 1.5% agarose gel, transferred to DBM paper, and hybridized to ^{32}P-labeled small RNA (34). (a) RNA from standard VSV; (b) RNA from standard VSV-infected cells; (c) RNA from DI 0.52 particles; (d) RNA from cells coinfected with standard VSV and DI 0.52 particles; (e) RNA from DI-T particles; (f) RNA from cells coinfected with standard VSV and DI-T particles.

Small amounts of hydridization occurred between small RNA and intracellular 40S RNA, presumably due to the complementary sequences on the large plus strand which comigrated with virion 40S RNA (Fig. 6b). Annealing of small RNA to the L mRNA from standard VSV-infected cells suggests that some L mRNA was not terminated, but contained run-through sequences with 3′ ends identical to those of the plus strand of 40S RNA (42). In Fig. 6f, the minor species which bound small RNA was due to a contaminating DI particle in our DI-T preparation.

These hybridization results show that there were sequences in common between the genomes of DI 0.52 and DI-T particles which were not found on the negative-strand RNA of standard VSV. These sequences were presumably at the 3′ end. Therefore, if the two DI genomes differed in their RNA sequence at the 3′ terminus, it would be due to less than 20% divergence within the first 46 nucleotides or internal to these nucleotides. Moreover, hybridization of small RNA to both plus and minus strands of DI RNAs indicated sequence homology at the 3′ ends of these RNAs and suggested the presence of at least 46 nucleotides that were terminal and complementary at the 3′ and 5′ ends of both strands.

Isolation of self-hybridized duplex molecules from the RNA of DI 0.52 and DI-T particles. Because the previous hybridization results were indirect, a direct approach was taken to isolate these terminal sequences by annealing. To do so, RNAs from purified DI particles were labeled at either the 5′ or 3′ end, rapidly self-annealed, and digested with RNase. The resulting duplex molecules (stems) were electrophoresed on a 10% polyacrylamide gel to determine their relative sizes.

Discrete self-hybridizable pieces of 45, 48, and 55 nucleotides were formed from the RNA of DI-T, DI 0.45, and DI 0.52 particles, respectively (Fig. 7). These lengths were determined by comparison with tRNA markers and the small RNA as well as from the sequencing results to follow. Because radioactive RNA, labeled in vitro at either terminus before hybridization and RNase digestion, appeared in the isolated stems from all three DI particles, the particles must be formed by self-annealing of RNA sequences at the 3′ and 5′ termini and not by loop-back or other internal complementary sequences. These results prove that terminal complementarity existed in the RNAs of all three DI particles. Differences in the stem sizes correlated with their ability to compete against each other.

Sequencing of end-labeled RNA from DI 0.52 and DI-T particles. To ensure that the sequences

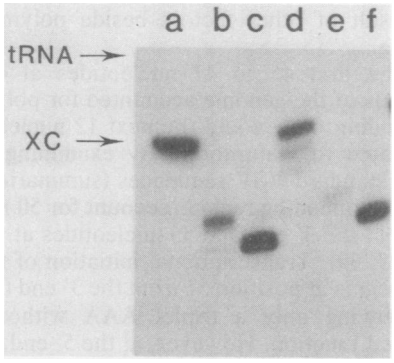

FIG. 7. Terminal complementary stems obtained from 5'- or 3'-end-labeled DI RNAs. Purified RNAs from DI 0.52, DI 0.45, and DI-T particles were terminally labeled at either the 5' or 3' terminus. The labeled RNAs were again purified on sucrose gradients, self-annealed, and digested with RNases. RNase-resistant RNAs were then separated on a 10% polyacrylamide gel. tRNA and xylene cyanol (XC) dye markers are indicated by arrows. (a) DI 0.52 RNA, 5'-end labeled; (b) DI 0.45 RNA, 5'-end labeled; (c) DI-T RNA, 5'-end labeled; (d) DI 0.52 RNA, 3'-end labeled; (e) DI 0.45 RNA, 3'-end labeled; (f) DI-T RNA, 3'-end labeled.

at the termini of the RNAs of the DI particles differed only in the extent of complementarity, sequence analysis was done on the 3'-end-labeled stems obtained from DI 0.52 and compared with DI-T particles. The 3' and 5' ends of the RNA from DI-T and DI 0.45 particles have been sequenced (40).

Figure 8 shows the pattern of RNA fragments from DI RNAs labeled at the 3' end obtained by digestion with alkali or several different RNases. The overall pattern was identical for 45 nucleotides, indicating that the only difference between DI 0.52 and DI-T particles was in the extent of the self-complementarity at the termini. When whole RNA from DI 0.52 and DI-T particles was similarly sequenced, divergence at the 3' end was detected from the first 45 nucleotides. The exact sequences in these first 45 nucleotides agree with previously published sequences for DI-T and DI 0.45 particles except for nucleotide positions 35 and 36 (40). These results, however, were not clear enough above the first 45 nucleotides to permit further sequence determinations.

DISCUSSION

These studies relate sequences on the RNA genomes of VSV and its DI particles to possible mechanisms of interference. Interference among VSV DI particles themselves was quantitatively different from their inhibition of standard VSV growth. Interference among DI particles was incomplete when measured by RNA synthesis, whereas inhibition of standard virus growth by any one of the three DI particles resulted in complete inhibition of standard genome replication as well as infectious progeny. Moreover, inhibition of one DI particle by another could be shown to be multiplicity independent, resulting in the same partial degree of RNA replication,

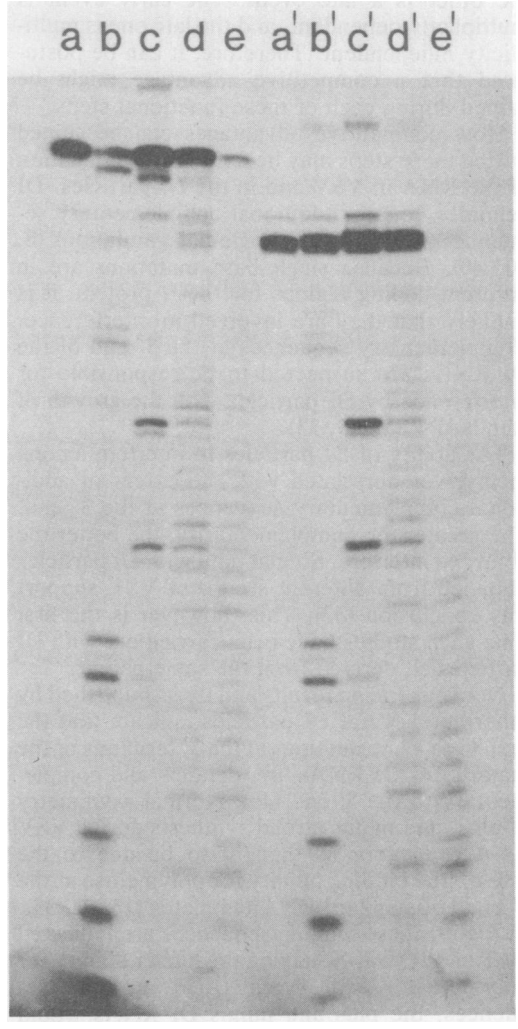

FIG. 8. Sequence comparisons of 3'-end-labeled complementary RNA from DI 0.52 and DI-T particles. RNA from the two DI particles were prepared as described in Materials and Methods. RNA stems labeled at the 3' end were then denatured by boiling and quick cooling before partial nuclease digestion by the method of Donis-Keller et al. (4). Separation was on a 20% polyacrylamide gel. (a–e) 3'-end-labeled RNA from DI 0.52 particles; (a'–e') 3'-end-labeled RNA from DI-T particles; (a, a') no treatment; (b, b') RNase T_1; (c, c') RNase U_2; (d, d') RNase physarum M; (e, e') alkaline denatured.

irrespective of the initial concentration of the suppressed DI particle.

When interference is multiplicity dependent, the mechanism is likely to be an early event involving input virions, whereas multiplicity-independent interference suggests a mechanism which occurs after the initial steps of viral multiplication. DI particles have only two essential functions: genome replication and maturation into virus particles. One is an early event; the other is a late event. The early event is multiplicity dependent, and the late one is multiplicity independent. Therefore, it can be postulated that a competitive advantage might be gained during each of these functional steps.

How competitive advantages can be gained during these steps may be found in an evaluation of the RNA in VSV and in the DI particles. DI genomes contain additional complementary sequences as well as the single-base mutations (8, 10, 40). Because single-base mutations are in different coding regions for the L protein, it is unlikely that they are involved in interference. Complementary sequences at the 3′ end of the DI RNAs are suspected to be responsible for interference by DI particles with the growth of standard VSV (13, 23).

The ability of DI particles to interfere among themselves correlated with the extent of additional complementary sequences at the 3′ end. The greater the complementarity, the better the ability to interfere. Similar data with DI particles obtained from different strains of VSV support this conclusion (32). This, however is the first time such studies have been carried out with DI particles all derived from the same parent.

Our sequencing results and those published by others on several DI particles indicate that the first 45 to 47 nucleotides at the 3′ terminus of the majority of DI RNAs are identical and complementary to the 5′ end (40). Normal asymmetry of plus- and minus-strand synthesis during VSV RNA replication is thought to be due to the differential binding affinity for polymerase at the 3′ ends of standard RNA templates (16, 22, 33). In Fig. 9, these binding sequences are indicated by T and R, with R having the higher affinity for polymerase. With symmetrical terminal sequences, the plus and minus DI RNAs would both contain R-like sequences at their 3′ ends. The results should be equal production of both plus- and minus-strand templates.

Measurement of RNA templates in this study, however, indicates approximately equal plus- and minus-strand synthesis for only DI 0.52 particles. The other two DI particles synthesize minus-strand RNA in excess over plus-strand RNA (60:40), but not at the same ratio (80:20) as standard VSV (27, 44). These results suggest that the asymmetry of RNA synthesis may be the result of other factors beside polymerase binding.

If the first 45 to 47 nucleotides at either terminus of the genome accounted for polymerase binding, how could the next 12 nucleotides be related to maturation? By examining published standard VSV sequences (summarized in Fig. 9), noncoding regions account for 50 nucleotides at the 3′ end and 59 nucleotides at the 5′ end (25, 39). Transcriptional initiation of the *N* gene begins at position 51 from the 3′ end (7, 26, 38), leaving only a triplet AAA without an assigned function. However, at the 5′ end, after termination of the coding region for the L protein, there are 12 nucleotides in addition to the terminal 47 nucleotides. Since DI 0.52 particles contain sequences at the 3′ end of their RNA which are complementary to some of these additional nucleotides, this region between positions 48 and 59, just internal to the terminal 47, at the 5′ end may determine interactions during morphogenesis. Because the initial step of morphogenesis is encapsidation of the RNA with N protein, the interaction could be viewed as the determinant for N-protein binding.

A part of the sequence within the 12 nucleotides, 5′ AUCAAA 3′, is also found in the noncoding region of the N mRNA just before the first AUG for initiating translation (5′ m^7AmACAGUAAUCAAAAUG 3′). Such a hexamer is not found in any other noncoding region of VSV-specific RNAs. It is premature to decide whether this sequence at the 5′ ends or its complement at the 3′ ends determines the interaction between VSV RNA and N protein. Nevertheless, if the hexamer and the nucleotides surrounding it affect the affinity of the association between RNA and N protein, then the same arguments for the polymerase concerning binding affinities and symmetry of sequences would hold for the interactions of RNA and the N protein. Although the RNA of DI 0.52 particles has terminal complementarity only for a portion of the 12 nucleotides, including the hexamer, the extended symmetrical sequence in its RNA may be enough to account for its ability to compete during encapsidation as well as during replication. Blumberg et al. (B. M. Blumberg, M. Leppert, and D. Kolakofsky, Cell, in press), reasoning from a different tack, conclude similarly that the sequences responsible for initiating N-protein binding are internal to the termini. Our assignment of the internal position of these sequences differs from theirs; however, regulatory sequences and binding sequences need not be the same.

Since the general understanding of VSV RNA replication and encapsidation is scant, other hypotheses for interference mechanisms could be entertained. It is possible that the ability of

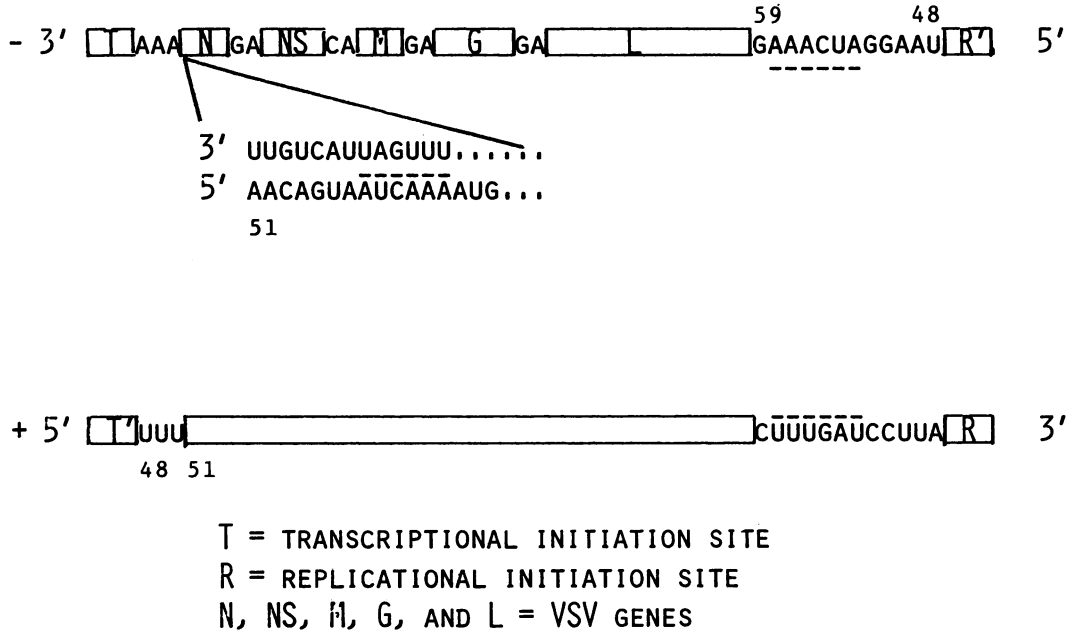

FIG. 9. Sequences at the termini of VSV RNA postulated to be responsible for RNA synthesis and encapsidation. Sequences are taken from Schubert et al. (39), McGeoch and Dolan (25), McGeoch et al. (26), and Rose (38). T, Transcriptional initiation site; R, replicational initiation site; N, NS, M, G, and L, VSV genes.

RNA templates to circularize may contribute in some way to their ability to be replicated (28). If preferential replication depends on the stability of circular template RNA, then the degree of sequence complementarity at the termini would correlate directly with such stability. On the other hand, we have made the assumption that all VSV particles, standard or defective, have about the same particle-to-infectivity ratio. If the effective multiplicities for the DI particles vary greater than threefold from our assumptions, then any hypothesis based on the degree of sequence complementarity may have to be revised.

Despite whatever mechanisms turn out to be true, there are three classes of controlling terminal sequences on the RNA genomes of VSV and its DI particles. Standard virus and the DI 0.46 particle derived from the Orsay (HR) strain contain 18 nucleotides at the 3' and 5' termini which are complementary except for a few base mismatches (32). Other DI particles contain terminal RNA sequences which are completely complementary for 45 to 48 nucleotides, as a second group, and for 55 to 60 nucleotides, as a third group (40). These three types of terminal sequences relate to a hierarchical interference pattern where the extent of complementarity results in a greater ability to compete during the virus growth cycle. These results focus the control of VSV RNA replication and encapsidation on these terminal sequences. Direct competitive binding experiments with these sequences and their regulatory proteins are needed to understand how interference occurs during nucleic acid replication and encapsidation.

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