In Vitro and In Vivo Inhibition of Primary Transcription of Vesicular Stomatitis Virus by a Defective Interfering Particle

PAULINE H. S. BAY AND M. E. REICHMANN*

Department of Microbiology, University of Illinois, Urbana, Illinois 61801

Received 9 March 1981/Accepted 28 August 1981

A unique defective interfering (DI) particle, generated by a heat-resistant (HR) mutant of Indiana serotype vesicular stomatitis virus, was capable of inhibiting primary transcription by heterologous New Jersey serotype virions. The correlation between this phenomenon and the lowering of viral yields from doubly infected cells was investigated by the construction of chimeric DI particles containing the HR DI particle genome with a thermolabile polymerase. At the nonpermissive temperature, these DI particles were unable to self-transcribe, inhibit virion primary transcription, or reduce virion yield, but were able to be replicated. These results suggested that self-transcription of the HR DI particle genome was a prerequisite for heterotypic interference, but not for its own replication. Inhibition of virion primary transcription by HR DI ribonucleocapsids was also observed in vitro. At low HR DI to virion ribonucleocapsid ratios, the extent of inhibition was concentration dependent, whereas at high ratios, the amount of inhibition was concentration independent, approaching a limiting maximum value. A speculative mathematical model, which quantitatively accounts for these data, is presented. According to this model, the higher affinity for polymerase molecules by the HR DI ribonucleocapsids is explained in terms of dissociation events during transcription, which are more frequent in the longer virion ribonucleocapsids.

The genomes of most defective interfering (DI) particles of vesicular stomatitis virus (VSV) map at the 5' terminus of the virion RNA (34, 40). These particles interfere with infections by their respective homotypic virions at the level of RNA replication (12, 22). Since their genomes do not contain a complete cistron, they do not produce active mRNA's in vivo (12, 22) or in vitro (31). Instead, a short oligonucleotide of unknown function, approximately 50 nucleotides in length, is generated in vivo (27) and in vitro (31, 38, 39) by transcription of the DI particle templates. Interference with virion primary transcription could not be detected, even at high DI particle to virion ratios in doubly infected cells (12, 22).

However, one exceptional DI particle, generated by a heat-resistant (HR) mutant of VSV, has been described (24, 25). The genome of this particle was shown to map at the 3' end of the VSV RNA (34, 40), although more recent partial nucleotide sequence analyses indicated that it also contained a sequence of approximately 350 nucleotides derived from the 5' end of the virion RNA (5, 8, 23). It appears that the RNA of this particle was generated by an internal deletion, rather than by the read-back mechanism postulated for the other VSV DI particles (10, 18). This DI particle, VSI HR Toronto DI 0.56 (3',

8%) (28), also referred to in the literature as LT or HR DI particle, was capable of interfering heterotypically (25, 34) with infections by the Concan subtype of the distantly related New Jersey (NJ) serotype virus (30). The four structural proteins whose complete cistrons are contained in the RNA of this particle were shown to be expressed in vivo (4), and their corresponding mRNA species were transcribed in vitro (7). Recently, we have shown that in contrast to the other DI particles of VSV, this particle interfered in vivo with primary transcription by homotypic and heterotypic virions and exhibited unexpected UV inactivation kinetics (2). We have now examined the interference with virion primary transcription in vitro, and the results presented here demonstrate the competitive nature of this phenomenon by showing its dependence on the concentration ratios of DI particles to virions. The relevance of this interference to the lowering of virion yields from doubly infected cells was also investigated. For this purpose, we constructed protein-nucleic acid chimeras containing HR DI particle genomes and well-defined Glasgow (G) temperature-sensitive (*ts*) mutant proteins by methods previously described (4, 36). We show that chimeras with proteins of the *ts*G11 and *ts*G13 mutants were unable to transcribe at the nonpermissive tem-

perature as a result of the thermosensitive L proteins (13). We also demonstrate that under restrictive conditions these chimeric DI particles did not interfere with primary transcription by the virion. Moreover, interference with virion replication was also abolished under these conditions. Despite the lack of interference, these HR DI particles were replicated at the nonpermissive temperature, indicating that the doubly infected cells contained a biologically active HR DI particle template. On the other hand, HR DI particle chimeras, produced in the presence of the *ts*G31 mutant and containing a Glasgow wild-type L protein (16, 20, 41), behaved essentially like the wild type under nonpermissive conditions. These results indicate that self-transcription of the HR DI particle genome is a necessary step for interference with primary transcription by the virion and that this partial inhibition is in turn required for interference with virion replication. However, HR DI particle replication can take place independently of either primary transcription of the HR genome or the interference phenomenon.

MATERIALS AND METHODS

Cells and viruses. Baby hamster kidney cells (BHK-21 clone 13) were grown in monolayers as described previously (2, 17).

The following VSV isolates were used in this study: Indiana serotype, Glasgow wild type, *ts*G11, *ts*G13, *ts*G31 (26, 29), Hu (San Juan subtype) (11), HR (Toronto subtype) (25), and the NJ serotype (Concan subtype) (25, 30).

Purification of virions, DI particles, particle-bound RNAs, DI particle chimeras, and viral RNCs. Virions, DI particles, and particle-bound RNAs were prepared as reported previously (2) except for virus used in the in vitro transcription experiments. In these experiments, 3E buffer was replaced by transcription (T) buffer (0.1 M NaCl, 0.005 M MgCl₂, 0.05 M Tris-hydrochloride, pH 8.0).

HR DI particles with altered proteins (chimeras) were obtained from cells coinfecting with the appropriate virion at a multiplicity of infection of 5 and a purified HR DI particle solution at a concentration of 0.05 absorbance units at 260 nm (*A*₂₆₀). The procedure used was that described by Chow et al. (4) except that the incubation was at 31°C for 11 h. Resultant chimeric DI particles were purified in the same manner as normal DI particles (2). Chimeric DI particles from the third passage were utilized in these experiments.

Virion and DI ribonucleocapsids (RNCs) were prepared by a modification of the procedure of Carroll and Wagner (3). Virus in T buffer at a concentration of 5 *A*₂₆₀ units was pelleted in a Beckman R40 rotor at 30,000 rpm and 4°C for 55 min. Pellets were then suspended in 1 ml of nucleocapsid suspension buffer (T buffer containing 0.025% Triton N101 [Sigma Chemical Co., St. Louis, Mo.], 4 mM dithiothreitol, and 0.15 M NaCl) by gentle pipetting, layered on 11-ml linear 10 to 50% glycerol gradients in T buffer, and centrifuged in a Beckman SW40 rotor for 3 h at 30,000 rpm and 10°C. Viral RNCs were removed by side

puncture with a 20-gauge needle on a 1-ml syringe and stored at 4°C no longer than 3 days before use.

In vitro transcription reaction conditions and analysis of product. Conditions for in vitro transcription reactions were based on the procedure of Moyer et al. (21). The final reaction contained 100 mM NaCl, 5 mM MgCl₂, 4 mM dithiothreitol (Sigma), 50 mM Tris-hydrochloride (pH 8.0), 1 mM ATP, CTP, and GTP, 25 μM UTP (Sigma), and 250 μCi of prelyophilized [³H]UTP (New England Nuclear, Boston, Mass.) per ml (final concentration of [³H]UTP ranged from 10 to 20 μM). Reactions were initiated by the addition of viral RNCs, and the mixtures were incubated at 29°C for the indicated times. The typical reaction volume was 0.1 ml, and the total concentration of viral RNCs varied with each experiment. After incubation, samples were diluted 10- to 15-fold with T buffer and centrifuged in a Beckman R40 rotor at 30,000 rpm for 35 min to pellet viral cores. Supernatants were then transferred to sterile test tubes (13 by 100 mm) and made 0.3 M in sodium acetate; the RNA was precipitated overnight at -20°C with 3 volumes of absolute ethanol. After centrifugation in a Sorvall HB-4 rotor at 7,000 rpm for 30 min at 4°C, the RNA was dried under nitrogen, dissolved in water, and stored at -70°C until analyzed or assayed (see below).

In vitro transcription products were electrophoresed in 2.4% polyacrylamide gels (0.6 by 10 cm) for 3.0 h at 6 mA per gel under constant-current conditions. The conditions for electrophoresis were described previously (42). The gels were analyzed for radioactivity after slicing and solubilizing the matrix in 6.5 ml of 3% Protosol in Econofluor (New England Nuclear) as described previously (37).

Assays for in vivo and in vitro interference by DI particles. DI particle interference was measured at three levels: infectious viral particle production, in vivo virion primary transcription, and in vitro viral transcription. Interference with virion production was determined by a plaque assay of infectious progeny obtained 18 h after viral infection in the presence and absence of DI particles (2). Interference with virion transcription was determined by comparing the quantities of viral transcripts synthesized in the presence and absence of DI particles or RNCs. Virion products were distinguished from those of DI particles or RNCs by annealing excess radioactively labeled or unlabeled viral RNA with transcripts synthesized in vivo or in vitro, respectively (2). This distinction was possible because of the lack of homology between the genomes of NJ virions and Indiana DI particles (33, 35).

RESULTS

In vitro interference with virion RNA transcription by HR DI particles. Recent reports demonstrated that the M protein of VSV inhibited virion transcription (3, 6). This inhibition was encountered when purified virions or DI particles were disrupted with detergent in the transcription mixture. To avoid the masking of the HR DI particle's interfering ability by the inhibitory effect of the M protein, we purified the RNCs of virion and DI particles after treatment with detergent and prior to their addition to the in vitro transcription system. Optimal transcrib-

ing RNCs were obtained after Triton N101 treatment followed by purification of the disrupted particles on linear glycerol gradients. DI RNCs were readily separated from contaminating virion RNCs, as demonstrated in Fig. 1, which illustrates the separation obtained between the largest DI RNC (HR) used in this study and the virion RNC. The isolated nucleocapsids always contained approximately 5% of the original M protein content of the complete particle when analyzed by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). However, this level of M protein did not interfere significantly with virion RNA transcription. This was demonstrated by an analysis of the effect of a 20-fold excess of purified disrupted nucleocapsids of VSI San Juan DI 0.3 (5', 5%) (Hu DI) on virion transcription. No difference in virion transcription was found in the presence or absence of this DI nucleocapsid, which was also contaminated to the same degree with M protein (see Fig. 3A).

FIG. 1. Glycerol gradient profile of HR DI and HR virion RNCs. Purified HR DI particles and HR virions labeled with [^3H]uridine and prepared in T buffer were pelleted in a Beckman R40 rotor at 30,000 rpm and 4°C for 55 min. Pellets were then resuspended in 1 ml of nucleocapsid suspension buffer and centrifuged on a linear 10 to 50% glycerol gradient in T buffer as described in Materials and Methods. After centrifugation, the tube was bottom punctured and fractionated into 10-drop (0.2 ml) samples. A 10- μl portion of each sample was spotted on a glass-fiber filter and analyzed for radioactivity (4).

FIG. 2. In vitro transcription products of NJ virion RNCs. NJ virion RNCs (A_{260} , 0.225/ml of reaction mix) purified off a glycerol gradient were added to an in vitro transcription system (final volume, 0.16 ml) as described in the text. After incubation at 29°C for 5 h, the sample was prepared for electrophoresis on 2.4% polyacrylamide gels as described in Materials and Methods. rRNA was isolated from [^{14}C]uridine-labeled BHK cells (42) and coelectrophoresed with the sample as an internal gel marker. The gels were electrophoresed for 3.0 h at 6 mA per gel, cut into 1-mm slices, solubilized, and counted as described in Materials and Methods.

The in vitro transcriptional products of NJ virion RNCs prepared as outlined in Materials and Methods were analyzed by electrophoresis in 2.4% polyacrylamide gels (Fig. 2). This profile indicated that the major size classes of VSV mRNA were synthesized under the in vitro transcription conditions used. Similar profiles were also obtained by Moyer et al. (21) under reducing and nonreducing conditions.

In contrast to Hu DI RNCs, the addition of increasing amounts of purified HR DI nucleocapsids inhibited virion transcription. A characteristic time course of the NJ virion RNC transcription in the presence and absence of a 20-fold excess of HR DI RNCs is shown in Fig. 3A (squares and triangles, respectively). The transcription of virion RNA was monitored by annealing with excess unlabeled NJ virion RNA and is expressed in terms of RNase-resistant

FIG. 3. Time course of NJ RNC in vitro transcriptions in the presence and absence of DI particles. NJ virion RNCs (A_{260} , 0.03/ml of reaction mix) in the absence and presence of Hu or HR DI RNC (A_{260} , 0.2/ml of reaction mix and 0.3/ml of reaction mix, respectively) were added to an in vitro transcription system as described in the text. At hourly intervals up to 5 h of incubation at 29°C, samples were centrifuged to pellet viral cores, and RNAs in the supernatant were precipitated with 3 volumes of absolute ethanol (see Materials and Methods). Portions of sample from each time point were then annealed to excess nonradioactive NJ virion RNA (A) or Indiana virion RNA (B) probes. Results are expressed in terms of RNase-resistant counts. Symbols: Δ , NJ RNCs; $\bullet$, NJ RNCs and Hu DI RNCs in a 1:20 ratio; $\blacksquare$, NJ RNCs and HR DI RNCs in a 1:20 ratio.

counts. The RNA of the NJ serotype of VSV has less than 5% sequence homology with the RNA of the Indiana serotype as measured by annealing (33, 35). Therefore, by this method, virion transcription could be distinguished from the simultaneous transcription of the HR DI RNC. The data show that the transcription by the virion nucleocapsid was considerably inhibited in the presence of a 20-fold excess of HR DI nucleocapsids. On the other hand, as already mentioned, a 20-fold excess of Hu DI RNCs did not affect virion transcription (Fig. 3A, circles). In addition, transcription by HR DI RNCs in the presence of NJ virion nucleocapsids was followed by annealing with an excess of unlabeled Indiana serotype virion RNA (Fig. 3B, squares).

The results of a reciprocal experiment, in which HR DI nucleocapsid transcription in the presence of a 20-fold excess of virion RNCs was

measured, indicated that no inhibition of this transcription took place. In fact, transcription by HR DI nucleocapsids was increased by 29 to 45% under these conditions (Table 1). These data also indicated that the HR DI RNCs were not saturated with RNA polymerases.

The concentration effect of HR DI nucleocapsids on virion RNC transcription in vitro is shown in Fig. 4, where the inhibition of virion transcription after 3 h of incubation is plotted as a function of DI to virion nucleocapsid ratios, R , in the range of 1 to 20. The solid line, which fits the data, is a theoretical curve given by the following relationship, where I is equal to the percent inhibition of virion transcription:

$$I = \left[1 - \frac{1 + 0.62R}{1 + 0.83R} \right] \times 100 \quad (1)$$

The derivation of this equation is discussed in detail in the Discussion.

Construction of HR DI particles, *ts* in transcription. Although HR DI particles contain L protein, the cistron for this protein is absent from their genome (34, 40). The L protein must therefore be contributed by the helper virus. When cells were coinfecting with purified HR DI particles and *ts*G13 or *ts*G11 virions, the progeny HR DI particles were assumed to contain a defective L protein. To eliminate any contaminating HR virion, the HR DI particles were prepared by three consecutive passages with the mutant virion followed by purification after each passage. After the first passage, the purified HR DI particles were assayed for contaminating virions at the permissive (31°C) and nonpermissive (39°C) temperatures. The difference in the plaque assay at the two temperatures was already four orders of magnitude, indicating that very little of the original HR contaminating virion remained in the preparation. Upon five or more passages nontranscribing variants of the HR DI particle were generated in high proportion, in agreement with reports from other laboratories (8, 23). As a compromise, HR DI particles from the third passage were used in subsequent experiments. Since the Glasgow and HR virion M proteins exhibit different electrophoretic mobilities in polyacrylamide gels (4), the formation of chimeras was monitored by examining protein profiles of purified progeny particles (data not shown). The purity of these various HR DI particles was further tested for their specific ability to interfere heterotypically and to transcribe their RNA as compared with the other DI particles (data not shown). A similar procedure was used during the formation of HR DI chimeras containing *ts*G31 proteins.

The temperature sensitivity of transcription

TABLE 1. In vitro transcription of HR DI RNCs in the presence of a 20-fold excess of NJ virion RNCs^a

Expt ^b	RNase resistance after annealing with excess unlabeled Indiana virion RNA (cpm)		Increase in HR DI RNC transcription ^c (%)
	+ NJ virion RNCs	- NJ virion RNCs	
1	3,136	2,156	145
2	3,441	2,563	134
3	5,918	4,591	129

^a HR DI RNCs (A_{260} , 0.0225/ml of reaction mixture) in the absence and presence of a 20-fold excess of NJ virion RNCs (A_{260} , 0.225/ml of reaction mixture) were added to an in vitro transcription system as described in Materials and Methods. After 3 h of incubation at 29°C, samples were diluted 15-fold with T buffer and centrifuged to pellet viral RNCs. RNAs in the supernatant were precipitated with 3 volumes of absolute ethanol prior to annealing to excess nonradioactive Glasgow Indiana virion RNA. Results have been corrected for nonspecific annealing attributable to self-annealing of the transcription products.

^b Each experiment was performed independently from the others and used different preparations of nucleocapsids.

^c Percent increase in HR DI RNC transcription was calculated by comparing results obtained in the presence of NJ RNCs with those in the absence of NJ RNCs.

FIG. 4. Concentration effect of HR DI RNCs on in vitro transcription by NJ virion RNCs. NJ virion RNCs (A_{260} , 0.03/ml of reaction mix) in the absence or presence of various concentrations of HR DI RNC were added to an in vitro transcription system as described in Materials and Methods. After 3 h of incubation at 29°C, samples were processed as described in the text prior to annealing with an excess nonradioactive NJ virion RNA probe. Percent inhibition of NJ transcription was determined by comparison of RNase-resistant counts in the presence of DI particles with those in a sample without DI particles. In the absence of DI particles, 10,364 cpm were rendered RNase resistant. The solid line represents the theoretical curve, and the broken line represents maximum percent inhibition as discussed in the text.

by the chimeric HR DI particles was examined in vivo. Intracellular primary transcription of the HR DI particle genome in the presence of cycloheximide and actinomycin D was measured 4.5 h after infection by annealing, as described previously (2). The results obtained at the permissive and nonpermissive temperatures, shown in Table 2, demonstrate that the *tsG13* and *tsG11* chimeric particles were thermosensitive in primary transcription. In contrast, the wild-type HR DI particle and the *tsG31* chimeric HR DI particle transcribed at both temperatures (Table 2).

Inhibition of virion transcription by chimeric HR DI particles. The thermolabile nature of primary transcription by certain chimeric HR DI particles was utilized to determine whether this event is mandatory for inhibition of virion primary transcription. The latter was monitored in the presence of *tsG11* and *tsG13* chimeric HR DI particles at the permissive and nonpermissive temperatures in vivo. The results shown in Table 3 indicate that at the nonpermissive temperature, in the absence of primary transcription by the *tsG13* or *tsG11* chimeric HR DI particles, no inhibition of virion primary transcription took place. However, at the permissive temperature, in the presence of primary transcription by these HR DI particles, inhibition of virion primary transcription was observed. The wild-type and *tsG31* chimeric HR DI particles inhibited virion primary transcription at both temperatures. The inhibition at the higher temperatures was always greater than at the lower temperatures. The reason for this is unknown.

Heterotypic interference with virion replication by chimeric HR DI particles. It was not clear whether the inhibition of virion primary transcription by the HR DI particle was relevant to its ability to interfere with virion replication. The correlation between these two activities was

TABLE 2. Primary transcription of the HR DI and HR DI chimera particle genomes^a

DI particle	RNase resistance after annealing with labeled HR DI RNA (cpm) ^b		cpm at 39°C/ cpm at 31°C
	31°C	39°C	
HR DI	8,051	9,760	1.00
HR DI with <i>ts</i> G11 proteins	2,452	254	0.10
HR DI with <i>ts</i> G13 proteins	2,657	66	0.02
HR DI with <i>ts</i> G31 proteins	2,853	2,740	0.96

^a BHK cells in 60-mm tissue culture plates (2×10^6 cells) were pretreated with 5 μ g of actinomycin D per ml for 1 h. The cells were then infected with the various HR DI particles (A_{260} , 0.06). After adsorption at room temperature for 30 min, the cells were washed once with sterile saline and covered with medium containing actinomycin D and 50 μ g of cycloheximide per ml and were incubated at the appropriate temperature. At 4.5 h postinfection, the cells were washed once with sterile saline, and intracellular RNAs were extracted (34). To determine the amount of HR DI particle transcriptional products, a sample of cytoplasmic RNA was annealed (17) to an excess amount of ³H-labeled HR DI particle RNA probe labeled in all four bases (2) with a specific activity of approximately 0.1 μ Ci/ μ g of RNA.

^b The relative concentration of accumulated transcriptional products is expressed as the total ³H counts per minute of probe which could be made RNase resistant after annealing. The results were corrected for nonspecific annealing by subtracting ³H counts per minute of HR DI particle RNA rendered RNase resistant after annealing with intracellular RNA from mock-infected cells.

examined by determining the extent of heterotypic interference with NJ virion replication by chimeric HR DI particles. Characteristic interference data are shown in Table 4. These data indicated that, when no inhibition of virion primary transcription occurred, virion yields were minimally affected by the HR DI particles. The *ts*G11 and *ts*G13 chimeric HR DI particles reduced virion yields by almost two logs at the permissive temperature, whereas at the nonpermissive temperature interference was essentially nil. On the other hand, wild-type and *ts*G31 chimeric HR DI particles which inhibited virion primary transcription at the two different temperatures (Table 3) also reduced virion yields at both temperatures (Table 4).

Replication of the HR DI particle genome in the absence of interference. The inability of chimeric HR DI particles containing a thermolabile L protein to interfere with virion primary transcription or production at the restrictive temperature raised the question of whether their

RNCs were biologically stable under this condition. Previously, standard VSV DI particles have been shown to be replicated by virions of a heterologous serotype and not exhibit a strong degree of interfering ability (1, 36). This phenomenon has been attributed to a slow rate of interaction between the incoming DI RNC and the heterologous, putative virion-encoded replicase protein or factor (36). Presumably, a similar event would occur if the chimeric HR DI RNCs were biologically stable. To resolve this issue, the progeny from mixed infections of *ts*G13 chimeric HR DI particles and NJ virion particles were examined. The particles were labeled in their RNA with [³H]uridine and [³H]adenosine and separated by sucrose gradient centrifugation. Characteristic profiles are shown in Fig. 5. In cells infected with virion alone, a radioactive peak consistent with the sedimentation properties of the virion was obtained (Fig. 5A, solid circles). In mixed infections with wild-type HR DI particles at 31°C (not shown) or at 39°C, the major peak was consistent with the sedimentation properties of the HR DI particle (Fig. 5A, open circles). As a result of interference, the viral progeny was reduced. A similar profile was obtained from mixed infections with the *ts*G13 chimeric HR DI particles and NJ virions at the permissive temperature (Fig. 5B, open circles). At the nonpermissive temperature the two peaks corresponding to virion and DI particles were much larger as a result of a lack of interference (Fig. 5B, solid circles). The nature of the HR DI

TABLE 3. Inhibition of NJ VSV primary transcription by various HR DI particles^a

Infecting particles	RNase resistance after annealing with labeled NJ virion RNA (cpm)		Inhibition (%)	
	31°C	39°C	31°C	39°C
NJ virion	10,078	15,288	0	0
NJ virion + HR DI	7,106	6,598	29	57
NJ virion + HR DI with <i>ts</i> G11 proteins	7,659	15,417	24	0
NJ virion + HR DI with <i>ts</i> G13 proteins	8,745	15,512	13	0
NJ virion + HR DI with <i>ts</i> G31 proteins	7,641	10,341	24	32

^a Experimental conditions are similar to those described for Table 1 except that cells were infected with NJ virions (multiplicity of infection, 6) in the presence or absence of the various HR DI particles (A_{260} , 0.06) and that a ³H-labeled NJ RNA probe labeled in all four bases (specific activity, 0.1 μ Ci/ μ g) (2) was used instead of HR DI particle RNA.

TABLE 4. Heterotypic interfering ability of the HR DI and HR DI chimera particles^a

DI particle ^b	Log ₁₀ reduction of infectivity (PFU/ml) ^c	
	31°C	39°C
HR DI	2.42	2.55
HR DI with <i>ts</i> G11 proteins	1.82	0.10
HR DI with <i>ts</i> G13 proteins	1.85	0.08
HR DI with <i>ts</i> G31 proteins	2.02	1.78

^a BHK cell monolayers in 100-mm tissue culture plates (2.7×10^6 cells) were infected with NJ virions (multiplicity of infection, 6) in the presence and absence of various HR DI particles for 30 min at room temperature, rinsed twice with sterile saline, and then covered with fresh medium. At 18 h after infection, samples were removed and assayed at the respective temperatures for plaque-forming ability (34).

^b Concentration of DI particle solution = $0.06 A_{260}$ units.

^c Results are expressed as the log₁₀ difference of infectious particles produced from BHK cells coinfecting with virions and DI particles as compared with cells infected with virions only. In the absence of DI particles, the yield of virions was 8×10^8 plaque-forming units (PFU)/ml at 39°C and 5.5×10^8 PFU/ml at 31°C.

particle peak was further verified by in vitro transcription assays. Activity resulting in Indiana serotype RNA transcripts was confined to this position in the gradient and coincided with the HR DI particle peak (data not shown). These results indicated that the *ts*G13 chimeric HR DI particle served as a template for its replication even in the absence of interference with virion production.

DISCUSSION

The purpose of constructing HR DI particle chimeras which were *ts* in self-transcription was to determine whether a correlation existed between this activity and the ability of the particle to interfere with virion primary transcription and replication. As shown in Tables 2, 3, and 4, such a correlation has been demonstrated. At 39°C when self-transcription by *ts*G11 and *ts*G13 chimeric HR DI particles was negligible (see Table 2), inhibition of virion transcription (Table 3) and interference with virion replication (Table 4) were also absent. However, under identical conditions, wild-type and *ts*G31 chimeric HR DI particles self-transcribed and interfered (Tables 2, 3, and 4). Although the in vitro stabilities of chimeric and wild-type HR DI nucleocapsids at 39°C were observed to be similar (data not

shown), the chimeric DI particles may be unstable at this temperature. The disintegration of a biologically active nucleocapsid would lead to a loss of all its activities, and the conclusions about the correlation between DI particle self-transcription and interference would be invalid. The results in Fig. 5B indicated that this was not the case. The replication of the *ts*G13 chimeric HR DI particle at the nonpermissive temperature clearly demonstrated the presence of a biologically active nucleocapsid in the cell and showed that the absence of primary transcription was due to the lesion in its L protein rather than to its physical instability. It should also be noted that DI particles normally generated by the *ts*G11 virion were reported to interfere with a higher efficiency at the restrictive (39°C) than at the permissive (31°C) temperature (29). Therefore, it is unlikely that the mutated L proteins could have destabilized the DI nucleocapsids.

A similar reduction of virion transcription in the presence of HR DI RNCs was also observed in vitro (Fig. 3A). The extent of inhibition was concentration dependent at low ratios of HR DI to virion nucleocapsids and reached a saturation level at high ratios (Fig. 4). Although these interactions occurred between heterologous templates and enzymes, such active combinations have been previously reported (4, 32). These in vitro data indicated that this phenomenon was not host cell mediated but involved direct interactions between the two types of nucleocapsids. The concept of a competitive inhibition of virion primary transcription by HR DI particles is difficult to interpret. Recently reported nucleotide sequences of HR DI particle and virion RNA indicate that they are identical in regions which are likely to be pertinent to the binding of active polymerases (15). This presents a problem in explaining how this DI particle could have a greater affinity for the enzyme than the virion. Moreover, the concentration independence of the inhibition at higher HR DI particle to virion ratios cannot be accounted for by this interpretation. As a result of these difficulties, a model, at present highly speculative, which can account for both phenomena is proposed below.

The competitive nature of the inhibition of virion transcription requires that the available enzyme be released after or during transcription into a pool from which it is redistributed among the available HR DI and virion templates. The redistribution of the enzyme is determined by the relative binding constants and relative concentrations of the two templates. The relative contribution of polymerase molecules into the pool by the virion and HR DI particle templates is determined by the relative quantities of the

FIG. 5. Sucrose gradient profiles of particles produced by BHK cells infected with VSV (NJ) in the presence or absence of wild-type and *tsG13* chimeric HR DI particles at 31°C and 39°C. BHK cell monolayers in 75-cm² tissue culture flasks (5×10^6 cells) were infected with NJ virions (multiplicity of infection, 3) in the presence and absence of various HR DI particles for 30 min at room temperature, rinsed twice with sterile saline, and then covered with fresh medium containing [³H]uridine and [³H]adenosine, each at 5 μ Ci/ml. At 18 h after infection at the indicated temperature, the virion progeny was pelleted by ultracentrifugation at 25,000 rpm for 90 min at 4°C in a Beckman R40 rotor. The pellets were then suspended overnight in T buffer, layered on linear 10 to 35% sucrose in T buffer gradients, and centrifuged at 30,000 rpm and 4°C for 60 min in a Beckman SW40 rotor. Tubes were fractionated after bottom puncture into 10-drop (0.2 ml) samples. A 50- μ l portion of each sample was spotted on a glass-fiber filter and analyzed for radioactivity (4). The figure shows profiles based on the counts per minute in the portions and obtained from cells infected with the following inocula and incubation temperatures: (A) ●, NJ virions at 39°C; ○, NJ virions and 0.06 A_{260} units of HR DI particles at 39°C; (B) ●, NJ virions and 0.06 A_{260} units of *tsG13* chimeric HR DI particles at 39°C; ○, NJ virions and 0.06 A_{260} units of *tsG13* chimeric HR DI particles at 31°C.

two RNCs and the amount of transcribing enzymes on each.

If the number of polymerase molecules per virion template and per HR DI particle template is α and β , respectively, and the concentration of the two templates is $[V]$ and $[HR]$, respectively, then the total size of the pool will be proportionate to $\alpha[V] + \beta[HR]$. Assuming that there are n_1 and n_2 equivalent binding sites per virion and per HR DI particle templates, respectively, then the polymerase molecules in the pool will be redistributed at equilibrium between these binding sites as follows: the fraction $(\alpha[V] + \beta[HR])n_1[V]/n_1[V] + n_2[HR]$ will bind to the virion templates, and the fraction $(\alpha[V] + \beta[HR])n_2[HR]/n_1[V] + n_2[HR]$ will bind to the HR DI particle templates. The rate of virion transcription in the mixture will be proportionate to the number of the redistributed polymer-

ase molecules bound to the virion as compared with $\alpha[V]$, the number of these molecules transcribing virion templates in the absence of interference. Percent inhibition by HR DI particles is therefore given by the following equation:

$$I = \left[1 - \left(\frac{\alpha[V] + \beta[HR]}{n_1[V] + n_2[HR]} \right) \left(\frac{n_1[V]}{\alpha[V]} \right) \right] \times 100 \quad (2)$$

After rearranging, the following simplified form is obtained:

$$I = \left[1 - \frac{1 + (\beta/\alpha)R}{1 + (n_2/n_1)R} \right] \times 100 \quad (3)$$

where R is the concentration ratio of the HR DI RNCs to virion RNCs. When R is very much

larger than one, equation 3 reduces to:

$$I = \left[1 - \left(\frac{\beta}{\alpha} \right) / \left(\frac{n_2}{n_1} \right) \right] \times 100 \quad (4)$$

Under these conditions inhibition is no longer a function of R . Equation 4 represents the maximum attainable inhibition which is only a function of the relative "affinity" parameters.

The affinity parameters in equation 4 are made up of two parts: (i) the relative contributions by HR DI particles and virions of polymerase molecules into the free "pool" and (ii) the relative number of binding sites on the two particles. A numerical estimate of these values can be obtained as follows. Since the protein compositions of DI particles and virions are approximately the same (14, 43), the ratio of the sizes of the two particles will be equivalent to the relative amount of transcribing polymerase molecules contributed by each into the pool. Using 100 nm and 180 nm for the length of the HR DI particle and VSV NJ virion, respectively (9, 24), the value of 0.56 for β/α is obtained. However, the best fit of the experimental data in Fig. 4 was obtained by changing the value of this parameter to 0.62. In addition to the 3' binding site in the leader sequence, it was assumed that polymerase molecules could also be bound during transcription at the beginning of each cistron, as suggested by the model of independent initiation followed by sequential elongation (41). If so, the virion particle would have a total of six binding sites whereas the HR DI particle, in which the L cistron is not transcribed, would have a total of five binding sites. Thus, the value of $n_2/n_1 = 5/6 = 0.83$. Equation 1, which contains these parameters, yields the solid line shown in Fig. 4, which fits the experimental data reasonably well. With other sets of experimental data, best fits were obtained when the numerical parameters in equation 1 were slightly changed, the alterations never amounting to more than a few percent. When R in equation 1 becomes very large, I approaches the asymptotic value of 25.3%. This maximum inhibition is shown by the broken line in Fig. 4.

The maximum interference theoretically predicted at high HR DI particle to virion ratios was not observed in vivo. It should be noted, however, that the in vivo conditions could not be strictly controlled. Under these experimental conditions, cycloheximide is only 95 to 97% effective (19), which would permit some amplification of primary transcription products. Moreover, some cellular events may have been implicated.

The theoretical considerations discussed above suggested that the affinity for the poly-

merase enzymes was not solely determined by the dissociation constant at the binding site (which may be identical for both particles), but also by subsequent dissociation events after the polymerase moved away from the binding site. Since the virion lost a considerably larger number of polymerase molecules during transcription than the DI particle, whereas the number of polymerase binding sites on the two particles was almost the same, the net effect resulted in a lower polymerase affinity for the virion. This highly speculative model is consistent with the in vitro kinetic data. It also suggests the HR DI RNC transcriptional ability was not fully saturated. The data in Table 1 demonstrated this lack of saturation since transcription of the HR DI particle genome was greater in the presence of virions than in their absence.

According to this model, self-transcription of the HR DI particle was required for inhibition of virion transcription so that binding sites for polymerase molecules were opened, thereby enabling a competition to occur for a limited supply of these enzymes. In the case of the chimeras containing thermosensitive L proteins, at 39°C the binding sites were probably permanently occupied during the early events of infection. Thus, a competition for polymerase molecules could not take place. Even though the inhibition of virion primary transcription was small relative to the observed overall interference, amplification of this effect throughout the infectious cycle would greatly reduce virion yields. Such amplification would take place as a result of the competition between nascent virion and DI particle RNCs for newly synthesized L proteins which can only be encoded for by the virion templates. However, it is clear that more experiments are required to either verify or reject this model. The most important of these are in vitro polymerase binding experiments using virion and HR DI RNCs stripped of polymerases as binding templates. Furthermore, the model predicts that stripped HR DI RNCs should be more efficient in inhibiting virion transcription than untreated HR DI RNCs. An attempt is being made to perform these experiments.

ACKNOWLEDGMENTS

We thank W. M. Schnitzlein for critical reading of the manuscript, Delphine Pillote for excellent technical assistance, and Marj Gillett for typing this manuscript.

This work was supported in part by Public Health Service research grant AI 12070 from the National Institute for Allergy and Infectious Diseases. P.B. was recipient of a U.S. Public Health Service Predoctorate Traineeship from a Cellular and Molecular Biology Training Grant (GM 7283).

LITERATURE CITED

1. Adachi, I., and R. A. Lazzarini. 1978. Elementary aspects of autointerference and the replication of defective interfering virus particles. *Virology* 87:152-163.

2. Bay, P. H. S., and M. E. Reichmann. 1979. UV inactivation of the biological activity of defective interfering particles generated by vesicular stomatitis virus. *J. Virol.* 32:876-884.
3. Carroll, A. R., and R. R. Wagner. 1979. Role of the membrane (M) protein in endogenous inhibition of *in vitro* transcription by vesicular stomatitis virus. *J. Virol.* 29:134-142.
4. Chow, J. M., W. M. Schnitzlein, and M. E. Reichmann. 1977. Expression of genetic information contained in the RNA of a defective interfering particle of vesicular stomatitis virus. *Virology* 77:579-588.
5. Clerx-van Haaster, C., J. P. Clewley, and D. H. L. Bishop. 1980. Oligonucleotide sequence analyses indicate that vesicular stomatitis virus large defective interfering virus particle RNA is made by internal deletion: evidence for similar transcription polyadenylation signals for the synthesis of all vesicular stomatitis virus mRNA species. *J. Virol.* 33:807-817.
6. Clinton, G. M., S. P. Little, F. S. Hagen, and A. S. Huang. 1978. The matrix (M) protein of vesicular stomatitis virus regulates transcription. *Cell* 15:1455-1462.
7. Colonna, R. J., R. A. Lazzarini, J. D. Keene, and A. K. Banerjee. 1977. *In vitro* synthesis of messenger RNA by a defective interfering particle of vesicular stomatitis virus. *Proc. Natl. Acad. Sci. U.S.A.* 74:1884-1888.
8. Epstein, D. A., R. C. Herman, I. Chien, and R. A. Lazzarini. 1980. Defective interfering particle generated by internal deletion of the vesicular stomatitis virus genome. *J. Virol.* 33:818-829.
9. Hartford, S. L., J. A. Lesnaw, W. H. Flygare, R. MacLeod, and M. E. Reichmann. 1975. Physical properties of New Jersey serotype of vesicular stomatitis virus and its defective particles. *Proc. Natl. Acad. Sci. U.S.A.* 72:1202-1205.
10. Huang, A. S. 1977. Viral pathogenesis and molecular biology. *Bacteriol. Rev.* 41:811-821.
11. Huang, A. S., and R. R. Wagner. 1966. Defective T particles of vesicular stomatitis virus. II. Biological role in homologous interference. *Virology* 30:173-181.
12. Huang, A. S., and E. K. Manders. 1972. Ribonucleic acid synthesis of vesicular stomatitis virus. IV. Transcription by standard virus in the presence of defective interfering particles. *J. Virol.* 9:909-916.
13. Hunt, D. M., S. U. Emerson, and R. R. Wagner. 1976. RNA⁻ temperature-sensitive mutants of vesicular stomatitis virus: L-protein thermosensitivity accounts for transcriptase restriction of group I mutants. *J. Virol.* 18:596-603.
14. Kang, C. Y., and L. Prevec. 1969. Proteins of vesicular stomatitis virus. I. Polyacrylamide gel analysis of viral antigens. *J. Virol.* 3:404-413.
15. Keene, J. D., M. Schubert, and R. A. Lazzarini. 1979. Terminal sequences of vesicular stomatitis RNA are both complementary and conserved. *J. Virol.* 32:167-174.
16. Lafay, F. 1974. Envelope proteins of vesicular stomatitis virus: effect of temperature-sensitive mutations in complementation groups III and V. *J. Virol.* 14:1220-1228.
17. Leamnson, R. N., and M. E. Reichmann. 1974. The RNA of defective vesicular stomatitis virus particles in relation to viral cistrons. *J. Mol. Biol.* 85:551-568.
18. Leppert, M., L. Kort, and D. Kolokofsky. 1977. Further characterization of Sendai virus DI-RNAs: a model for their generation. *Cell* 12:539-552.
19. Lewis, J. B., H. Esche, J. E. Smart, B. W. Stillman, M. L. Harter, and M. B. Mathews. 1979. Organization and expression of the left third of the genome of adenovirus. *Cold Spring Harbor Symp. Quant. Biol.* 44:493-508.
20. Metzel, P. S., and M. E. Reichmann. 1981. Characterization of vesicular stomatitis virus mutants by partial proteolysis. *J. Virol.* 37:248-255.
21. Moyer, S. A., M. J. Graubman, E. Ehrenfeld, and A. K. Banerjee. 1975. Studies on the *in vivo* and *in vitro* messenger RNA species of vesicular stomatitis virus. *Virology* 42:958-968.
22. Perrault, J., and J. J. Holland. 1972. Absence of transcriptase activity and transcription-inhibiting ability in defective interfering particles of vesicular stomatitis virus. *Virology* 50:159-170.
23. Perrault, J., and B. L. Semler. 1979. Internal genome deletions in two distinct classes of defective interfering particles of vesicular stomatitis virus. *Proc. Natl. Acad. Sci. U.S.A.* 76:6191-6195.
24. Petric, M., and L. Prevec. 1970. Vesicular stomatitis virus—a new interfering particle, intracellular structures, and virus-specific RNA. *Virology* 41:615-630.
25. Prevec, L., and C. Y. Kang. 1970. Homotypic and heterotypic interference by defective particles of vesicular stomatitis virus. *Nature (London)* 228:25-27.
26. Pringle, C. R. 1970. Genetic characteristics of conditional lethal mutants of vesicular stomatitis virus induced by 5-fluorouracil, 5-azacytidine, and ethyl methane sulfonate. *J. Virol.* 5:559-567.
27. Rao, D. D., and A. S. Huang. 1979. Synthesis of a small RNA in cells coinfecting with standard and defective interfering particles of vesicular stomatitis. *Proc. Natl. Acad. Sci. U.S.A.* 76:3742-3745.
28. Reichmann, M. E., D. H. L. Bishop, F. Brown, J. Crick, J. J. Holland, C.-Y. Kang, R. Lazzarini, S. Moyer, J. Perrault, L. Prevec, C. R. Pringle, R. R. Wagner, J. S. Younger, and A. S. Huang. 1980. Proposal for a uniform nomenclature for defective interfering viruses of vesicular stomatitis virus. *J. Virol.* 34:792-794.
29. Reichmann, M. E., C. R. Pringle, and E. A. C. Follett. 1971. Defective particles in BHK cells infected with temperature-sensitive mutants of vesicular stomatitis virus. *J. Virol.* 8:154-160.
30. Reichmann, M. E., W. M. Schnitzlein, D. H. L. Bishop, R. A. Lazzarini, S. T. Beatrice, and R. R. Wagner. 1978. Classification of the New Jersey serotype of vesicular stomatitis virus into two subtypes. *J. Virol.* 25:446-449.
31. Reichmann, M. E., L. P. Villarreal, D. Kohne, J. Lesnaw, and J. J. Holland. 1974. RNA polymerase activity and poly (A) synthesizing activity in defective T particles of vesicular stomatitis virus. *Virology* 58:240-249.
32. Repik, P., A. Flamand, and D. H. L. Bishop. 1976. Synthesis of RNA by mutants of vesicular stomatitis virus (Indiana serotype) and the ability of wild-type VSV New Jersey to complement the VSV Indiana *ts* G 1-114 transcription defect. *J. Virol.* 20:157-169.
33. Repik, P., A. Flamand, H. F. Clark, J. F. Obijeski, P. Roy, and D. H. L. Bishop. 1974. Detection of homologous RNA sequences among six rhabdovirus genomes. *J. Virol.* 13:250-252.
34. Schnitzlein, W. M., and M. E. Reichmann. 1976. The size and the cistronic origin of defective vesicular stomatitis virus particle RNAs in relation to homotypic and heterotypic interference. *J. Mol. Biol.* 101:307-325.
35. Schnitzlein, W. M., and M. E. Reichmann. 1977. Interference and RNA homologies of New Jersey serotype isolates of vesicular stomatitis virus and their defective particles. *Virology* 77:490-500.
36. Schnitzlein, W. M., and M. E. Reichmann. 1977. A possible effect of viral proteins on the specificity of interference by defective vesicular stomatitis virus particles. *Virology* 80:275-288.
37. Schnitzlein, W. M., and M. E. Reichmann. 1980. Inhibition of vesicular stomatitis virus replication by adenosine. *Virology* 103:123-137.
38. Schubert, M., J. D. Keene, R. A. Lazzarini, and S. U. Emerson. 1978. The complete sequence of a unique RNA species synthesized by a DI particle of VSV. *Cell* 15:103-112.
39. Semler, B. L., J. Perrault, J. Abelson, and J. J. Holland. 1978. Sequence of a RNA templated by the 3'-OH RNA terminus of defective interfering particles of vesicular stomatitis virus. *Proc. Natl. Acad. Sci. U.S.A.* 75:4704-4708.
40. Stamminger, G., and R. A. Lazzarini. 1974. Analysis of

- the RNA of defective VSV particles. *Cell* 3:85-93.
41. Testa, D., P. K. Chanda, and A. K. Banerjee. 1980. Unique mode of transcription *in vitro* by vesicular stomatitis virus. *Cell* 21:267-275.
42. Unger, J. T., and M. E. Reichmann. 1973. RNA synthesis in temperature-sensitive mutants of vesicular stomatitis virus. *J. Virol.* 12:570-578.
43. Wagner, R. R., T. A. Schnaftman, and R. M. Snyder. 1969. Structural proteins of vesicular stomatitis virus. *J. Virol.* 3:395-403.