

## Detection of Vesicular Stomatitis Virus RNA and Its Defective-Interfering Particles in Individual Mouse Brains

DAVID R. CAVE,<sup>†</sup> FRED S. HAGEN,<sup>‡</sup> EDUARDO L. PALMA,<sup>§</sup> AND ALICE S. HUANG\*

*Division of Infectious Diseases, Children's Hospital Medical Center, and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115*

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To develop a highly sensitive and direct assay for defective interfering (DI) particles of vesicular stomatitis virus (VSV), we reverse transcribed RNA from DI particles and cloned the DNA in pBR322 and used it as hybridization probes. At the lower limit, cDNA of about 850 nucleotides detected 150 pg of VSV RNA. For differentiation of hybridizable sequences found in the RNA of DI particles from complementary or identical sequences in the L mRNA or standard genomic RNA of VSV, RNA obtained from mouse brains was first separated by size, blotted onto nitrocellulose, and then hybridized to in vitro-labeled cDNA probe. Genomic VSV, DI, or L mRNA sequences from one-half of the brain of an infected mouse were detectable, whereas uninfected mice failed to react with this specific probe. When mice were infected intranasally with  $10^8$  PFU of standard VSV, most of them died between days 6 and 7, and the detection of standard genomic RNA correlated with paralysis and death. DI RNA was not detected in these mice. When mice were infected with  $10^8$  PFU of standard VSV together with an equivalent amount of DI particles, similar results were obtained. When fewer DI particles were inoculated together with standard virus, significant protection of mice occurred together with the detection of DI RNA. These results indicate that DI particles are protective in vivo and that the details of the virus-host interaction may resemble the cyclic growth patterns in cell cultures for standard VSV and its DI particles.

The study of viral pathogenesis necessitates the detection of virus and its products in animal tissues. Infectious virus and viral antigens are readily assayed, but defective products often go undetected. Recent advances in the preparation of nucleic acid probes has made it possible to detect noninfectious virus even when it is involved in abortive infections that fail to produce viral proteins. Defective interfering (DI) particles of vesicular stomatitis virus (VSV) have been postulated to play a role in both acute and chronic infections (13). To study the replication of these particles, a sensitive and direct detection system for DI particles would be required. Hybridization after size fraction of VSV RNA species would provide such an assay.

This VSV system is a good one for developing and testing such hybridization assays because a great deal is already known about VSV RNA synthesis and the competition between VSV DI particles and standard infectious virus. Moreover, beginning with the descriptions by Sabin and Olitsky (21-23), infection of mice by VSV preparations and the sequelae are well documented (7, 8, 10, 12, 16, 18, 28). Resistance to infection can be manipulated so that overall host immunity no longer plays a role (15, 16).

In this report the presence of small amounts of DI particles correlated with some protection from the lethal effects of VSV. These preliminary results are in contrast to those of previous studies in which DI particle RNA was not directly detected and protection was afforded by DI particles only when the ratio of DI particles to infectious standard VSV was  $10^2:1$  or greater (8, 10, 11, 15, 18). Similar findings have been reported in hamsters (9). An interpretation of these divergent observations is offered in the Discussion.

\* Corresponding author.

<sup>†</sup> Present address: Department of Medicine, University Hospital, Boston, MA 02118.

<sup>‡</sup> Present address: Zymos, Seattle, WA 98103.

<sup>§</sup> Present address: Department of Virology, Centro de Investigaciones en Ciencias Veterinarias, Instituto Nacional de Tecnologia Agropecuaria, Castelar, Buenos Aires, Argentina.

### MATERIALS AND METHODS

**Virus system.** The growth, purification, and assay of standard VSV (Indiana serotype, San Juan strain) and its DI particles (DI-T) have been described in detail previously (14, 19, 25).

**Preparation of cloned probes.** Purified DI-T were phenol extracted, and the RNA was further purified through a sucrose gradient. The reextracted RNA, about 10  $\mu$ g in 50  $\mu$ l, was reverse transcribed with avian myeloblastosis virus (2.1 U/ $\mu$ l; Life Sciences, St. Petersburg, Fla.) with fragments of calf thymus DNA as primer (60 ng/ $\mu$ l) in the presence actinomycin D (4). After incubation at 42°C for 1 h, 130 ng of DNA was made. After phenol-chloroform and ether extraction, the DNA was passed through a G-75 Sephadex column. Klenow reagent (New England Biolabs, Cambridge, Mass.) was added to complete double-stranded DNA synthesis by the procedures described by Bothwell et al. (4). After treatment with S1 nuclease, the DNA was ligated into the vector  $\lambda$  charon 16A with *Eco*RI linkers d(GGAATTCC) (3). The DNA was packaged into  $\lambda$  heads, and phage stocks were grown and screened by DNA hybridization with <sup>32</sup>P-labeled cDNA reverse transcribed from the RNA of DI-T (2). Approximately 500 plaques were obtained. Of 12 plaques picked for purification and further cloning, 7 were positive for VSV sequences and were subcloned into pBR322 at the *Eco*RI site and transfected into *Escherichia coli* C600. Those with inserts were selected by growth in M9 medium with ampicillin but without tetracycline. Plasmid DNA for analysis was prepared by the cleared-lysate method (4). To obtain large amounts for nick translation, chloramphenicol-amplified plasmid DNA was prepared by the standard procedures outlined by Schleif and Wnesink (24), except for a modification of the lysis buffer by substituting Triton X-100 (0.1%) for the detergent. Six individual plasmid DNA preparations that hybridized to DI-T RNA sequences were found to contain inserts of about 200 to 850 base pairs. Clones 5 and 6 represented the smallest and largest, respec-

tively, of the DNA inserts and were used for the experiments described here.

**Nick translation.** Plasmids containing clones 5 and 6 were labeled with [ $^{32}$ P]dCTP (specific activity,  $\geq 3,000$  Ci/mmol; New England Nuclear Corp., Boston, Mass.) by nick translation (20). The final specific activity of the  $^{32}$ P-labeled plasmid DNA was  $\geq 10^8$  cpm/ $\mu$ g of DNA.

**Infection of mice.** Male and female CD1 mice (Charles River Breeding Laboratories, Wilmington, Mass.) aged 9 to 10 months were inoculated upon recovery from ether anesthesia per nasam with 20  $\mu$ l of phosphate-buffered saline (pH 7.4) containing either VSV at  $10^8$  PFU or the same amount of VSV and different concentrations of DI-T. Control animals were inoculated with phosphate-buffered saline alone. The animals were then observed daily, and those that developed obvious hind limb paralysis were sacrificed immediately. A few animals died before autopsy and were discarded. At 14 days, all the remaining animals were sacrificed. Autopsies were performed and the brain of each animal was removed, cut in half sagittally, and stored at  $-70^\circ\text{C}$  for infectivity and RNA assays.

**RNA extraction.** For gels and hybridizations, one-half of each mouse brain was weighed and homogenized in 4 M guanidine thiocyanate (Tridom, Inc., Hauppauge, N.Y.) with 40 strokes in a Dounce homogenizer (6). The RNA was separated through a step gradient of 95.5% CsCl and 40% CsCl in 25 mM sodium acetate–1 mM EDTA (pH 5.0) by centrifugation in a Beckman SW50.1 rotor at 34,000 rpm for 16 h at  $15^\circ\text{C}$ . The RNA pellet was resuspended in 8 M guanidine hydrochloride–30% ethanol and precipitated in the presence of sodium acetate (pH 5.0). The pellet was dissolved in water, precipitated twice with 70% ethanol, and then quantitated spectrophotometrically. RNA at a concentration of 5  $\mu$ g/ml of water was stored at  $-70^\circ\text{C}$ . Marker RNAs from purified virions or from VSV-infected cells were obtained by the same method.

**Northern hybridization.** A total of 28  $\mu$ g of total RNA from individual mice was denatured in 50% deionized formamide–20 mM sodium acetate–20 mM EDTA–20 mM morpholinepropanesulfonic acid buffer (pH 7.3; Sigma Chemical Co., St. Louis, Mo.)–6% formaldehyde for 10 min at  $60^\circ\text{C}$  and then rapidly cooled. The RNA was then electrophoresed in 1% agarose gels containing 6% formaldehyde at  $4^\circ\text{C}$  for 20 h at 4 V/cm. The gel was run in 20 mM sodium acetate–20 mM EDTA dissolved in 20 mM morpholinepropanesulfonic acid buffer.

The RNA was then transferred by blotting as described previously (1), except nitrocellulose paper (BA 85; Schleicher & Schuell Co., Keene, N.H.) was used (27). Transfer of RNA from the gel was performed at  $4^\circ\text{C}$  overnight with  $20\times$  SSC ( $1\times$  SSC = 0.15 M NaCl plus 0.015 M sodium citrate) buffer. The nitrocellulose sheets were then air dried and baked in vacuo at  $80^\circ\text{C}$  for at least 2 h. Hybridization was then performed as described below for dot hybridizations.

**Dot hybridization.** The sensitivity and specificity of the cDNA probes were evaluated by dot blotting (5, 26) on nitrocellulose paper (BA 85; Schleicher & Schuell). One microliter containing serially diluted, phenol-extracted (19) standard VSV RNA was dotted onto dry nitrocellulose paper previously equilibrated in  $4\times$  SSC, air dried, and baked in vacuo at  $80^\circ\text{C}$  for 2 h.

Prehybridization of blots with 30  $\mu$ g of sheared calf thymus DNA per ml was performed at  $42^\circ\text{C}$  for 2 to 4 h with 100  $\mu$ l of RNA buffer (0.775 M NaCl–0.15 M Tris base [pH 7.5]–40 mM monobasic sodium phosphate–60 mM dibasic sodium phosphate), containing 10 mM vanadyl adenosine,

50% deionized formamide, 0.1% sodium dodecyl sulfate, and Denhardt's reagent (26), per  $\text{cm}^2$ .

Just before hybridization, the probe was denatured by heating for 3 min at  $100^\circ\text{C}$  in the presence of 20  $\mu$ g of calf thymus DNA and then added to the buffer together with 10% dextran sulfate. Approximately  $3 \times 10^4$  cpm of probe per  $\text{cm}^2$  was used in 50  $\mu$ l of buffer per  $\text{cm}^2$ . Hybridization was performed at  $42^\circ\text{C}$  for 24 h with occasional agitation.

After hybridization, the nitrocellulose sheets were washed twice for 10 min each with  $2\times$  SSC containing 0.1% sodium dodecyl sulfate and then twice again with  $0.1\times$  SSC containing 0.1% sodium dodecyl sulfate. The sheets were then air dried and autoradiographed on Cronex film with an intensifier screen at  $-70^\circ\text{C}$ . Development of the films took place within days.

## RESULTS

**Detection of VSV RNA by dot hybridization.** To determine the sensitivity of the hybridization assay on nitrocellulose for VSV RNA, we hybridized  $^{32}$ P-labeled clones 5 and 6 to different concentrations of standard VSV RNA. Figure 1 shows the results obtained with clone 6, which detected 5 pg of RNA. The smaller clone 5 was 10-fold less sensitive (data not shown). Although the gradation of radioactive DNA bound to the nitrocellulose was not readily discernible by eye, cutting out these dots and counting the radioactivity gave a linear relationship between the amount of unlabeled VSV RNA and the amount of hybridized [ $^{32}$ P]DNA. When these probes were hybridized to similar concentrations of cellular rRNA, positive signals were not detected (data not shown).

**Migration positions of VSV RNAs on formaldehyde-agarose gels.** Because of the need to transfer large RNA from gels onto nitrocellulose paper, a more porous gel system (1% agarose-formaldehyde) was tried and the migration pattern of VSV RNA was determined. Figure 2, lane 1 shows RNA obtained from VSV-infected cells labeled with  $^{32}$ P in the presence of actinomycin D. The five VSV mRNAs, labeled L, G, N, M, and NS, were readily separated. Figure 2, lane 2 shows the migration of standard virion RNA, labeled 40S, and DI-T RNA. The position of the bands are similar to those of gels used to separate VSV RNA species in other studies (19). The density of radioactivity in this autoradiograph appeared by eye to correlate with size and not to the

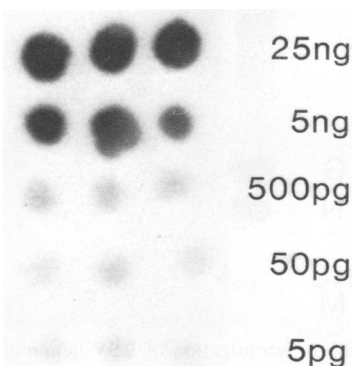


FIG. 1. Dot hybridization of VSV genomic RNA to  $^{32}$ P-labeled clone 6 DNA. Phenol-extracted RNA from purified virions was dotted in triplicate at the indicated concentrations and then hybridized to nick-translated [ $^{32}$ P]DNA.

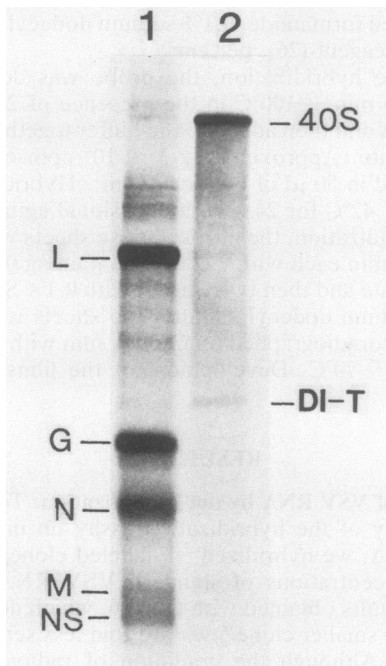


FIG. 2. Agarose gel electrophoresis of VSV RNA.  $^{32}\text{P}$ -labeled viral RNA from VSV-infected cells (lane 1) and from a mixture of standard virus and DI-T (lane 2) were extracted with the cesium chloride-thiocyanate method used for mouse brain RNA and electrophoresed on 1% agarose-formaldehyde. The gels were exposed directly to X-ray film.

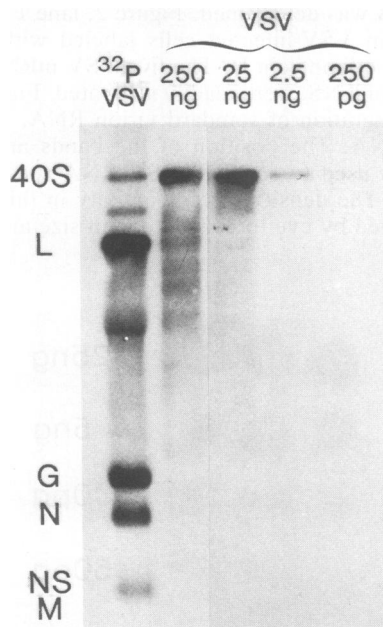


FIG. 3. Northern hybridization of VSV genomic RNA. RNA at different concentrations from purified virions was separated on gels, transferred to nitrocellulose paper, and hybridized with clone 6 [ $^{32}\text{P}$ ]DNA. The lane marked " $^{32}\text{P}$  VSV" provides marker RNAs obtained from VSV-infected cells labeled with  $^{32}\text{P}$  in the presence of actinomycin D (19).

expected molar ratios of VSV mRNA, as has been determined previously (27); however, when the major bands were excised and the radioactivity was determined, the molar amounts nevertheless gave a descending order of mRNA concentrations as follows:  $\text{N} > \text{NS} > \text{M} > \text{G} > \text{L}$ .

**Detection of VSV RNA by Northern hybridization.** By the 1% agarose-formaldehyde gel system, serial dilutions of standard VSV RNA were electrophoresed, blotted, and hybridized to clone 6 [ $^{32}\text{P}$ ]DNA. A faint signal at 40S was found with 250 pg of RNA (Fig. 3). The first lane, containing previously  $^{32}\text{P}$ -labeled VSV RNA, provided markers on nitrocellulose paper; unlabeled bands between 40S and G are often found in VSV RNA preparations and may represent aberrant RNA products, breakdown products, or trapping of radioactivity by 28S ribosomal RNA. A measurement of the transfer efficiency from the gel onto nitrocellulose paper was obtained by determining residual radioactivity on the gel after blotting. At least 65% of the 40S RNA and 90% of the L mRNA transferred to nitrocellulose. Therefore, the limit of sensitivity of Northern hybridization for detecting VSV genomic RNA was determined to be at about 150 pg.

**Detecting VSV RNA from mouse brains.** To determine whether VSV RNA from individual mouse brains could be detected by hybridization, we infected 30 mice intranasally with  $10^8$  PFU of VSV. Four mice were sacrificed between 7 and 8 days after infection, when the onset of hind-limb paralysis was observed. Half of each brain was processed for RNA. The hybridizations showed readily detectable 40S

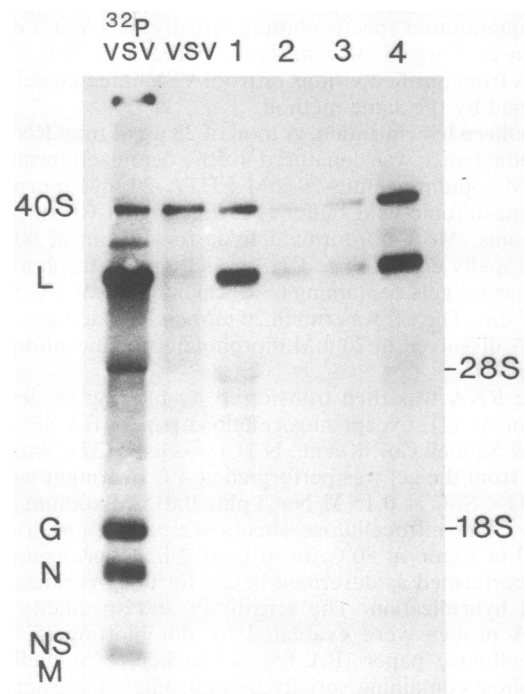


FIG. 4. Northern hybridization of RNA from brains of VSV-infected mice. RNA extracted from the brains of mice infected with  $10^8$  PFU of standard VSV was separated on gels, blotted, and hybridized with clone 6 [ $^{32}\text{P}$ ]DNA. The lane marked " $^{32}\text{P}$  VSV" contains intracellular  $^{32}\text{P}$ -labeled marker viral RNA; the lane marked "VSV" contains unlabeled RNA extracted from purified standard virions and detected by hybridization. Lanes 1 through 4 refer to individual mice.

RNA and L mRNA from all four mice and lack of any specific hybridization to the other RNAs, indicating that the probe contained sequences for the L region of VSV RNA (Fig. 4). The amount of 40S RNA detected correlated well with the infectivity assays from the same brains. Mice 1 and 4 had  $3 \times 10^7$  PFU per brain, whereas mice 2 and 3 contained only  $4 \times 10^6$  to  $8 \times 10^6$  PFU per brain.

A few of the inoculated mice failed to develop paralysis and were subsequently sacrificed on day 14 after infection. Their brains were also processed, but there were no detectable VSV RNA sequences or infectivity (data not shown). These results indicate that, with standard VSV at  $10^8$  PFU, irrespective of whether the mice lived or died, DI RNA sequences were not generated in the brains. However, the presence of detectable standard VSV RNA in brains correlated with paralysis and death.

The lower limit in our hybridization assays correlated with  $2 \times 10^4$  PFU/ml. Since the results (Fig. 3) suggest that 150 pg (or  $10^7$  VSV genomes) is the lower limit of detection, this gives a genome/PFU ratio of 500:1 or greater. Such a

particle/infectivity ratio would be expected for VSV preparations obtained from animals.

**Detection of VSV DI-T RNA in mouse brains.** To detect DI RNA by hybridization, mice were coinfectd with DI-T and standard VSV. The concentration of standard VSV was held constant at  $10^8$  PFU, and two concentrations of DI-T were used, one at  $10^8$  equivalents and the other at  $10^5$  equivalents. When equal amounts of standard and DI VSV were inoculated, only 40S RNA and L mRNA sequences were detected in the brains of mice (Fig. 5, lanes 1 through 4). DI-T RNA was not found, and the overall mortality in this group of eight mice was 88% by day 14. In contrast, when the lower concentration of DI-T was used in the coinfections, DI-T RNA was detected by hybridization in two of three mice (Fig. 5, lanes 5 through 7), with an overall reduction of mortality to 50% for this group of eight mice. Control mice infected with only standard VSV had variable amounts of 40S RNA and L mRNA but no DI-T RNA in their brains (Fig. 5, lanes 8 through 12). The mortality for this group of 30 mice was 67%.

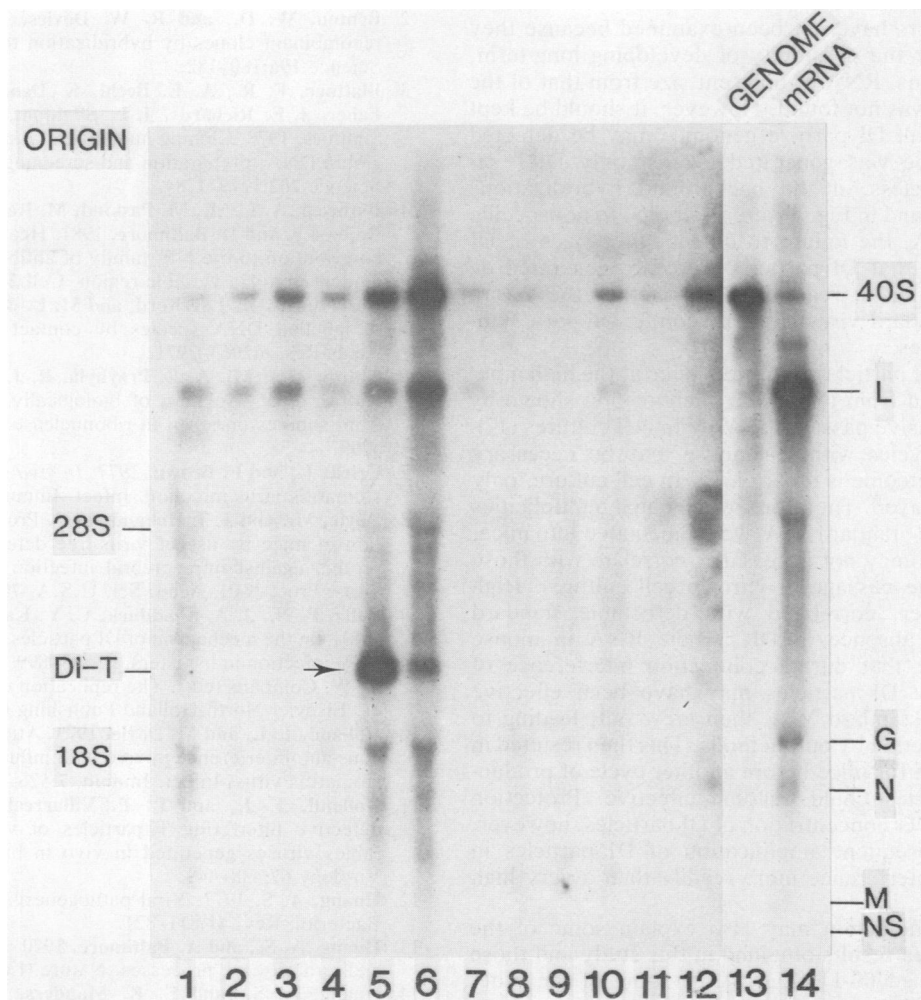


FIG. 5. Northern hybridization of RNA from the brains of mice coinfectd with standard VSV and DI-T or infected with standard VSV alone. RNA from individual mice was separated on gels, blotted, and hybridized with clone 6 [ $^{32}$ P]DNA. Lanes 1 through 4, mice coinfectd with  $10^8$  PFU of standard VSV and  $10^8$ -PFU equivalents of DI-T; lanes 5 through 7, mice coinfectd with  $10^8$  PFU of standard VSV and  $10^5$ -PFU equivalents of DI-T; lanes 8, 10, and 11 and lanes 9 and 12, mice infected with  $10^8$  PFU or  $10^6$  PFU, respectively, of standard VSV alone; lane 13, 250  $\mu$ g of marker VSV genome RNA; lane 14, intracellular viral [ $^{32}$ P]RNA.

## DISCUSSION

These analyses demonstrate the feasibility of using cloned [<sup>32</sup>P]DNA sequences to detect viral RNA by Northern hybridization. This method permits the detection of 150 pg of VSV RNA or 10<sup>7</sup> genome equivalents of DI particle RNA in half of a mouse brain. Previously published limits for detecting VSV DI particles in mouse brains was 5 × 10<sup>6</sup> particles, but the detection system required pooling of several brains and amplification in cell cultures (11). Now single animals can be studied. If necessary, the hybridization assay can be made more sensitive by using larger cDNA probes or by labeling only the excised inserts from the vectors. As has been reported (12, 21), there was considerable variation from mouse to mouse even with the same inoculum. Using hybridizations, many individual mice will have to be examined before a consistent pattern of DI particle synthesis can be obtained.

Nonetheless, in these preliminary studies with infected mice, equal multiplicities of DI-T and standard VSV failed to protect mice; in fact, mortality was slightly higher than with standard virus alone. A DI/standard virus ratio of 1:100 afforded some protection, and this protection correlated with the detection of DI RNA sequences in mice with obvious paralysis. Survivors have not been examined because they are being kept for the possibility of developing long-term, persistent infections. RNA of different size from that of the input DI particle was not found. However, it should be kept in mind that not all DI particle genomes may be detected because our probe was generated against only DI-T sequences. Nonetheless, if the background hybridizations around the DI-T band in Fig. 5 were attributed to nonspecific trapping by rRNA, the failure to detect other sizes of DI particles suggests that DI particles were not generated *de novo* in these animals, regardless of whether they were infected with standard virus alone or coinfecting with standard and DI viruses.

The failure of DI particles to protect mice at the high input may be interpreted from the cyclic phenomenon shown by continuous successive passages of VSV in cell cultures (17). Several growth cycles within a mouse may be necessary before the final outcome is determined. In cell culture, only one passage is assayed. Therefore, when equal multiplicities of DI particles and standard VSV were inoculated into mice, the *in vivo* results may not necessarily correlate with those expected from one passage *in vitro* in cell cultures. High mortality, however, correlated with detectable standard virion RNA and absence of DI particle RNA in mouse brains, suggesting that during coinfection interference of standard VSV by DI particles may have been effective initially and that standard VSV then grew out, leading to paralysis and detection by our methods. This then resulted in the lethal outcome for mice before another cycle of production of DI particles could become effective. Protection afforded at the lower concentration of DI particles, however, suggests that subsequent amplification of DI particles in mice may elicit interference more readily than a very high initial input.

The cyclic phenomenon may also explain some of the divergence between results obtained in this study and those previously published (8–11, 15, 18). In some of the other cases, however, the site of inoculation and the animal species also differed. To determine whether the above interpretation is correct, more mice will be infected and assayed; in certain cases, mice should be sacrificed at different times after infection, irrespective of whether they

develop paralysis. Different sites of inoculation, as well as animals of different ages, should be tried. Moreover, assays for interferon, as well as humoral and cellular immunity, should be pursued concomitantly to differentiate between the relative roles played by these host defenses and DI particles.

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