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Effect of Defective Interfering Particles on Plus- and Minus-Strand Leader RNAs in Vesicular Stomatitis Virus-Infected Cells

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Vesicular stomatitis virus-infected cells contain short RNA transcripts, called leader RNAs, which are coded by the exact 3′ end of both the minus-strand and plus-strand nucleocapsid templates. The molar amounts of both the plus-strand leader RNA (which is templated from the minus-strand genome) and the minus-strand leader RNA (which is templated from the plus-strand antigenome) were determined both in standard-virus- and mixed-virus-infected cells by using end-labeled genome probes. The results demonstrate that the presence of defective interfering particles in the infecting virus stock decreases the amount of plus-strand leader RNA but increases the amount of minus-strand leader RNA found in the infected cells. In addition, considerably more minus-strand leader RNA per mole of nucleocapsid template is synthesized in mixed-virus-infected cells than plus-strand leader RNA per mole of nucleocapsid template in both standard-virus- and mixed-virus-infected cells.

Negative-strand viruses such as vesicular stomatitis virus (VSV, rhabdovirus group) contain a large single-stranded RNA genome which serves as a template for the synthesis of five monocistronic mRNA's (15). The viral transcriptase responsible for this synthesis is found both in purified virions and in infected cell cytoplasm associated with the viral nucleocapsids. These nucleocapsids are composed of the single-stranded genome RNA, one major structural protein, the N protein, and two minor viral proteins, L and NS. Analysis of the UV light inactivation kinetics on viral transcription has suggested that the viral mRNA's are transcribed sequentially by the viral polymerase which enters the nucleocapsid template at a single site at or near its 3′ end (1, 2). These experiments have allowed a gene order on the viral genome to be determined, and it was found that the mRNA coding for the N protein, the most abundant viral protein, is the first to be transcribed and the mRNA coding for the L protein, the least abundant viral protein, is the last.

Colonno and Banerjee (3-5) have demonstrated that another RNA species, in addition to the five monocistronic mRNA's, is synthesized in the in vitro virion polymerase reaction. This RNA species, called leader RNA, is defined by the following characteristics: it is extremely short (48 nucleotides long); it is neither capped at its 5′ end nor polyadenylated at its 3′ end like the viral mRNA's; it is the first RNA species to be synthesized in vitro; and it is complementary to the exact 3′ end of the minus-strand genome template. Colonno and Banerjee were unable to detect this leader RNA in virus-infected cells by direct radiolabeling of the RNA in vivo most probably because of the limited amounts of radioactivity that can be incorporated into an RNA chain of such short length. However, if minus-strand genome RNA labeled at its precise 3′ end with 5′-[^{32}P]cytidine 3′,5′-bisphosphate and RNA ligase is used as a probe, leader RNA is clearly detectable in both Sendai virus- and VSV-infected cells (9). In addition, the use of an end-labeled probe which contains the 3′ end of the plus-strand antigenome RNA has allowed the detection of a similar sized leader RNA in VSV-infected cells which is thought to be templated from the 3′ end of the nondefective (ND) antigenome (9). Since VSV-infected cells have now been demonstrated to contain two leader RNAs of similar size but synthesized from different templates, we have defined these leader RNAs by strand polarity: the leader RNA synthesized from the minus-strand genome template is plus-strand leader RNA, and leader RNA synthesized from the plus-strand genome template is then minus-strand leader RNA. In our previous experiments (9), no attempt was made to quantitate the amount of either leader RNA in our standard-virus-infected cells. This communication reports the quantitation of both leader RNAs in cells infected either with virus

stocks containing no detectable amounts of defective interfering (DI) particles (standard virus) or with virus stocks which contain DI particles (mixed virus).

MATERIALS AND METHODS

Virus infection and isolation of infected-cell RNA. Three sets of eight confluent 10-cm tissue culture plates of BHK cells were either mock infected, infected with 10 PFU of the Mudd-Summers Indiana strain of VSV per cell, which contained either no or undetectable amounts of DI particles, or 10 PFU of the above stock per cell plus an equivalent amount of medium from cells infected with both the ND and the Mudd-Summers defective T particle. After 40 min, the infecting medium was removed and replaced with 3.5 ml of minimal essential medium containing 1% fetal calf serum per plate and 2 μg of actinomycin D per ml. At 2.25 h postinfection, 25 μCi of [^{3}H]uridine (specific activity, 25 Ci/mmol) per ml was added to the medium, and the cells were harvested at 6.5 h postinfection. Cytoplasmic extracts of the mock- and virus-infected cells were prepared, and the extracts were centrifuged through a 20 to 40% CsCl gradient as previously described (9). The visible nucleocapsid bands at a density of 1.30 g/ml were first removed through the side of the centrifuge tube, and the remainder of the gradient was discarded. The pelleted RNAs were then dissolved in 0.5 ml of TNE (25 mM Tris-chloride, pH 7.4; 50 mM NaCl; 1 mM EDTA) containing 0.3% Sarkosyl, precipitated with ethanol, and redissolved in 0.2 ml of ET (10 mM Tris-chloride, pH 7.4; 1 mM EDTA).

Preparation of viral probes. Radioactive probes were made from the purified 55-base pair (bp) VSV DI stem RNA and the VSV 42S minus-strand RNA by the enzymatic addition of 5′-[^{32}P]cytidine 3′,5′-bisphosphate to their 3′-OH ends according to the published procedure of Leppert et al. (9). The substrates for ligation, the DI stem RNA and the ND 42S minus-strand RNA, were prepared as earlier described by Perrault and Leavitt (10) and Leppert et al. (9), respectively. The specific activity was 3.40×10^5 cpm/pmol for the 42S 3′ ^{32}P-labeled probe and 1.04×10^5 cpm/pmol for the DI stem 3′ ^{32}P-labeled probe.

Detection of leader RNAs by hybridization and gel electrophoresis. The two ^{32}P terminally labeled probes (2,000 Cerenkov cpm per reaction) were each annealed separately to RNA isolated from mock-infected, standard-VSV-infected, and mixed-VSV-infected cells. Before annealing, the double-stranded stem probe was denatured by heating for 1 min at 50°C in 90% dimethyl sulfoxide in the presence of CsCl-pelleted RNA, and the RNAs were precipitated with ethanol. Annealings were carried out in a total volume of 15 μl of 2× A solution (300 mM NaCl; 20 mM Tris, pH 7.4; 2 mM EDTA). After annealing for 30 min at 73°C in sealed capillaries, the contents of each capillary were washed into 100 μl of 2.5× buffer A containing 0.1% sodium dodecyl sulfate and 10 μg of pancreatic RNase per ml. Each reaction was incubated for 10 min at 25°C, and the RNase was digested by adjusting the sodium dodecyl sulfate concentration to 0.2% and adding pronase to 500 μg/ml. After a further incubation for 15 min at 25°C, 25 μg of tRNA was added as carrier, and the RNAs were recovered by ethanol precipitation. The precipitated RNAs were then dissolved in 15 μl of TNE containing 12% glycerol and 0.1% bromophenol blue and electrophoresed on a 10% polyacrylamide gel, which was then exposed to X-ray film.

RESULTS

To quantitate the amount of plus- and minus-strand leader RNAs present in cells infected with standard virus (i.e., virus stocks containing either no or undetectable amounts of DI particles) and to investigate the effect of DI particles in the infecting virus stock (mixed-virus infection) on the intracellular amounts of these leader RNAs, cytoplasmic RNA free of viral nucleocapsids was isolated from standard-virus- and mixed-virus-infected BHK cells. The total amounts of the CsCl-pelleted RNAs were measured by optical density at 258 nm, and the amount of plus-strand leader RNA present in these preparations was determined by annealing increasing amounts of the CsCl-pelleted RNA with 42S minus-strand virion RNA which had been labeled at its precise 3′ end with 5′-[^{32}P]-cytidine 3′,5′-bisphosphate and RNA ligase. The annealed RNAs were then digested with RNase A in high salt, and the remaining RNAs were electrophoresed on a 10% polyacrylamide gel. As previously demonstrated (9), only a single band of radioactivity, which migrates at the position expected of a 48-bp hybrid, was detected (Fig. 1a). To accurately measure the radioactivity present in these bands, the bands were cut out of the gel, and their Cerenkov radiation was determined by liquid scintillation. As shown in Fig. 1b, a linear relationship exists between the amount of CsCl-pelleted RNA added to the annealing reaction and the amount of 48-bp hybrid recovered from the gel. From the slope of the lines, it can be determined that CsCl-pelleted RNA from standard-virus-infected cells contained 258 fmol of plus-strand leader RNA per optical density unit, whereas RNA from mixed-virus-infected cells contained only 6.3 fmol per optical density unit. This large decrease in the amount of plus-strand leader RNA in cells infected with both ND and DI particles is apparently the result of the concomitant decrease in the amount of 42S minus-strand genome template present in these cells due to the interference with ND genome replication by DI genomes (Fig. 3b and Table 1). When calculated on a product-to-template basis, mixed-virus-infected cells were actually found to contain twice the amount of plus-strand leader RNA per 42S minus-strand genome template as standard-virus-infected cells (Table 1). The significance of this twofold difference is unclear.

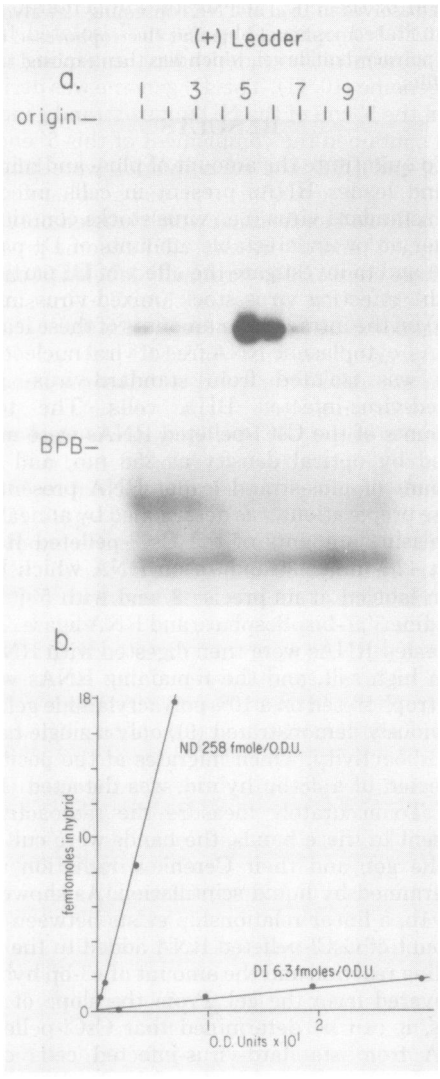

FIG. 1. *Quantitation of plus-strand leader RNA in standard-virus- and mixed-virus-infected cells. Ligated 42S minus-strand virion RNA (2,000 Cerenkov cpm) was added to either 15, 10, 5, or 1 μl of mixed-virus-infected BHK RNA (lanes 1 to 4, respectively); to 15, 10, 5, 0.5, or 0.1 μl of standard-virus-infected BHK RNA (lanes 5 to 9, respectively); or to 10 μl of mock-infected BHK RNA (lane 10). The RNAs were annealed, treated with RNase, digested with pronase, and electrophoresed for 16 h at 45 mA as described in the text. (a) Autoradiograph of the gel. After autoradiography the bands were excised from the gel and counted by Cerenkov radiation. (b) Plot of the optical density of CsCl-pelleted RNA hybridized to the ligated probe versus the amount of plus-strand leader RNA in the RNase-resistant hybrid. ODU, Optical density units.*

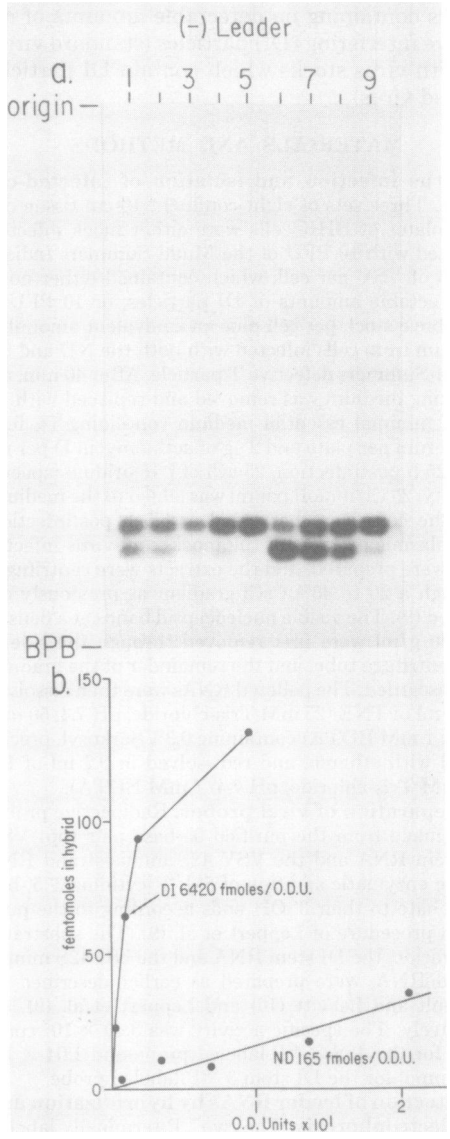

FIG. 2. *Quantitation of minus-strand leader RNA in standard-virus- and mixed-virus-infected cells. Ligated double-stranded stem RNA (2,000 Cerenkov cpm) was added to either 30, 20, 10, 5, or 1 μl of standard-virus-infected BHK RNA (lanes 1 to 5, respectively) or to 5, 1, 0.5, or 0.1 μl of mixed-virus-infected BHK RNA (lanes 6 to 9, respectively). The RNAs were annealed, treated with RNase, digested with pronase, and electrophoresed for 16 h at 45 mA as described in the text. (a) Autoradiograph of the gel. After autoradiograph the bands were excised from the gel and counted by Cerenkov radiation. (b) Plot of the optical density amount (optical density units, ODU) of CsCl-pelleted RNA hybridized to the ligated probe versus the amount of minus-strand leader RNA in the 46-bp RNase-resistant hybrid (lower band in gel).*

TABLE 1. *Vital statistics of VSV standard-virus- and mixed-virus-infected cells*

Infection	CsCl-pelleted RNA		NC-RNA						Plus-strand leader			Minus-strand leader		
	Virus specific[c] (^{3}H cpm × 10^{-6})	Total (ODU[d])	42S, total[a]				19S, total[b]		fmol/ ODU	Total (fmol)	mol/mol of template	fmol/ ODU	Total (fmol)	mol/ mol of template
			^{3}H cpm × 10^{-1}	fmol	Minus strand[e] (fmol)	Plus strand[e] (fmol)	^{3}H cpm × 10^{-1}	fmol						
Standard	2,810	1.38	1,520	686	635	51.5	—	—	256	353	0.56	165	228	4.42
Mixed	230	3.90	56.5	25.5	21.6	3.8	303	588	6.3	24.6	1.14	6,420	25,000	42.3

[a] The 42S RNA, as indicated in Fig. 3a, was recovered from the gradient by ethanol precipitation, and the specific activity of this RNA was directly determined from the UV absorption spectrum, assuming that 1 optical density unit at 258 nm is equivalent to 45 μg of RNA.

[b] The DI 19S self-anneals to 87%. The chain length of the 19S DI genome was measured at 2,600 N by HCHO-agarose gel electrophoresis.

[c] A background of 153 × 10^3 ^{3}H cpm from mock-infected cells has been subtracted.

[d] ODU, Optical density units at 258 nm.

[e] The 42S RNAs from the ND and ND-DI infections were self-annealed to equilibrium as previously described (7) and found to self-anneal to 15% and 30%, respectively.

Minus-strand leader RNA, a similar sized RNA which is thought to be templated from the 3′ end of the ND plus-strand antigenome, was quantitated using 55-bp "stems" isolated from the concentration-independent annealing of a DI genome (10, 11). This DI genome was derived from the 5′ end of the ND minus-strand genome and contained the complement of this 5′ end at its 3′ end (11). The stem hybrids were labeled with 5′-[^{32}P]cytidine 3′,5′-bisphosphate and RNA ligase in a reaction which labeled only the original 3′ end of the intact DI genome present in these stems. This terminally labeled 3′ end is identical to the 3′ end of the ND plus-strand antigenome (see Fig. 9 of reference 9).

Increasing amounts of CsCl-pelleted RNA from standard-virus- and mixed-virus-infected cells were annealed to denatured ligated stems, and the annealed RNAs were digested with RNase in high salt and electrophoresed on a 10% polyacrylamide gel as described in Materials and Methods. Since the minus-strand leader RNA, which is identical to the 2S DI particle polymerase product (13), is only 46 nucleotides long, the resulting stem-leader hybrid has a single-stranded tail which, when digested with RNase in high salt, is converted to a 46-bp hydrid with flush ends. The results of such an experiment, shown in Fig. 2a, demonstrate that the resulting 46-bp hybrid is clearly separable from the original 55-bp hydrid by polyacrylamide gel electrophoresis. To accurately measure the amount of radioactivity in the 46-bp hydrids, these bands were similarly cut out of the gel and their Cerenkov radiation was determined. Figure 2b demonstrates that a linear relationship also exists between the amount of CsCl-pelleted RNA added to the annealing reaction and the amount of 46-bp hybrid recovered from the gel, except when the amount of ligated 55-bp probe becomes limiting in the reaction. From the initial linear portion of these curves, it can be determined that cells infected with standard virus contain 165 fmol of minus-strand leader RNA per optical density unit, whereas cells infected with both ND and DI particles contain 6,420 fmol per optical density unit. This large increase in the amount of minus-strand leader RNA present in mixed-virus-infected cells is due only in part to the increased molar amount of genomes which serve as templates for the minus-strand leader RNA, i.e., both complementary strands of the DI genomes as well as the ND plus-strand antigenome. When calculated on a product-to-template basis, standard-virus-infected cells contain 4.42 mol of minus-strand leader RNA per mol of template, whereas mixed-virus-infected cells contain almost 10 times this amount (Table 1).

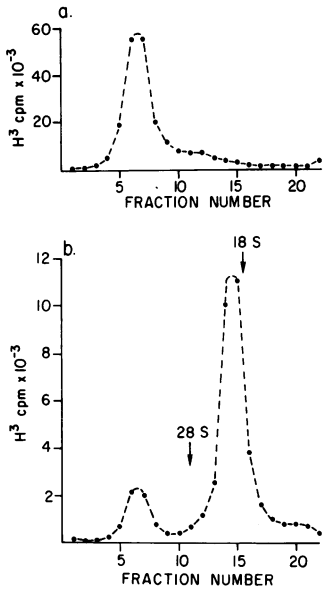

FIG. 3. *Sucrose gradient analysis of [^{3}H]uridine-labeled nucleocapsid RNA in (a) standard-virus- and (b) mixed-virus-infected cells. RNA was obtained from nucleocapsids banded to equilibrium in 20 to 40% CsCl gradients as described in the text. Samples of 100 μl of nucleocapsids (out of a total of 1.0 ml and 0.92 ml from standard- and mixed-virus-infected cells, respectively) were diluted with TNE and pelleted for 60 min at 48,000 rpm in an SW50.1 rotor. The pelleted nucleocapsids were dissolved in 100 μl of TNE containing 1.0% sodium dodecyl sulfate and sedimented on a 5 to 23% sodium dodecyl sulfate-sucrose gradient for 85 min at 58,000 rpm (10°C) in an SW60 rotor.*

DISCUSSION

The vital statistics of standard-virus- and mixed-virus-infected BHK cells are summarized in Table 1, which also compares the amount of plus- and minus-strand leader RNAs present per mole of genome template. In cells infected with standard virus, almost eight times as much minus-strand as plus-strand leader RNA was detected per mole of ND genome template. In our previous work on this subject we have assumed that the minus-strand leader RNA present in these standard-virus-infected cells was templated from ND antigenomes, since DI genomes could not be detected in our intracellular nucleocapsid preparations by analytical ultracentrifugation (9). However, the absence of detectable amounts of DI genomes in these experiments may not be sufficient to exclude the possibility that this minus-strand leader RNA was synthesized from undetectable amounts of contaminating DI genomes. From the results reported in Table 1, since ND antigenomes represent only 7.5% of the RNA that sediments at 42S from standard-virus-infected cells, and since mixed-virus-infected cells synthesize almost 10 times as much minus-strand leader RNA per mole of genome template as standard-virus-infected cells, undetectable amounts (less than 1%) of contaminating DI genomes could theoretically account for the minus-strand leader RNA synthesis detected in standard-virus-infected cells. For these reasons, we can see no way at present to unambiguously determine the template for minus-strand leader RNA in our standard-virus-infected cells. However, as mentioned previously (9), we can see no reason why ND antigenomes should not be templates for minus-strand leader RNA.

The origin of the plus-strand leader RNA in both standard-virus- and mixed-virus-infected cells and of the vast majority of the minus-strand leader RNA in mixed-virus-infected cells, on the other hand, is not in question. From the results summarized in Table 1, we estimate that mixed-virus-infected cells contain 37 and 75 times as much minus-strand leader RNA per mole of template as plus-strand leader RNA present in mixed-virus- and standard-virus-infected cells, respectively. This large difference is probably an underestimate since our determination of minus-strand leader RNA depends on the formation of a 46-nucleotide to 55-nucleotide hybrid in the presence of an excess of the complete complement of the 55-nucleotide strand, whereas plus-strand leader RNA is measured by an annealing reaction that can be described by pseudo-first-order kinetics. Since the amount of leader RNA present in virus-infected cells is also a measure of the number of times the viral polymerase has initiated synthesis of an RNA chain on its respective template, it is clear that the viral polymerase has a strong preference for initiating RNA synthesis on DI genomes over both ND genomes and antigenomes. Although the molecular basis for this preference is presently unclear, it does provide an explanation for the strong replicative advantage that DI genomes have over ND genomes. This explanation is also consistent with previous studies which have demonstrated that this replicative advantage is independent of chain length and that the initiation of genome replication, and not chain elongation, is the rate-limiting event in genome replication (6).

In a mixed-virus infection at 6 h postinfection, the intracellular ratio of antigenomes to genomes was double the ratio in a standard-virus infection (Table 1). This effect may simply reflect the lowered amount of 42S RNA made in defective infections, since it has been observed that the ratio of plus- to minus-strand genomes

is also twofold higher early on in standard viral infections at a time, i.e., 2 h postinfection, when the amount of accumulated 42S genome RNA is also relatively small (14). The ratio of plus to minus strands for intracellular DI genomes at 6 h postinfection, however, is about 1:1, for 19S RNA self-anneals to 87% (Table 1). The excess unannealed DI strands are negative because VSV mRNA totally protects these excess strands from RNase digestion whereas 42S negative-strand RNA does not (data not shown). The viral polymerase apparently replicates both DI template strands with nearly equal efficiency, despite the presence of a recombinant cross-over point 55 nucleotides in from the 3′ end of the minus strand, but not the plus strand, of the DI templates (8).

During infection of cells with both ND and DI particles, the selective replication of DI genomes at the expense of ND genomes also results in a lowered production of viral mRNA's (Table 1) and, thus, fewer viral proteins than found in standard-virus-infected cells. Nevertheless, mixed-virus-infected cells still contain adequate amounts of viral replicase, since the number of genome synthesis initiation events is also a measure of the availability of this enzyme. Viral N protein, in contrast to the viral replicase, is required in stoichiometric amounts for genome replication since this protein is the major structural component of the viral nucleocapsids, the products of genome replication. Although, in the experiments reported here, we have not directly measured the amount of N protein, it appears that mixed-virus-infected cells contain less N protein than standard-virus-infected cells because of both the diminished amount of viral mRNA synthesized and the lowered total amount of viral nucleocapsids that are recovered from mixed-virus-infected cells. This lower amount of viral N protein in mixed-virus-infected cells may explain the large amount of minus-strand leader RNA relative to genome template detailed in Table 1 and noted by Rao and Huang (12). We have previously suggested that initiation of RNA synthesis on genome templates is not sufficient for genome replication since, approximately 50 nucleotides in from the 3′ ends of the genome templates, the viral polymerase encounters a strong stop signal which terminates the RNA chain and leads to the production of leader RNA (9). In this model, the attachment of N protein to the nascent RNA chain has the dual function of initiating nucleocapsid assembly on the nascent chain and promoting read-through of the stop signal by the viral polymerase. The fact that mixed-virus-infected cells contain an unusually large number of leader RNAs relative to genome templates is consistent with a model in which viral N protein modulates the frequency of chain termination and read-through at this stop signal.

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LITERATURE CITED

1. **Abraham, G., and A. K. Banerjee.** 1976. Sequential transcription of the genes of vesicular stomatitis virus. Proc. Natl. Acad. Sci. U.S.A. **73**:1504–1508.
2. **Ball, L. A., and C. N. White.** 1976. Order of transcription of vesicular stomatitis virus. Proc. Natl. Acad. Sci. U.S.A. **73**:442–446.
3. **Colonno, R. J., and A. K. Banerjee.** 1976. A unique RNA species involved in initiation of vesicular stomatitis virus RNA transcription *in vitro*. Cell **8**:197–204.
4. **Colonno, R. J., and A. K. Banerjee.** 1977. Mapping and initiation studies on the leader RNA of vesicular stomatitis virus. Virology **77**:260–268.
5. **Colonno, R. J., and A. K. Banerjee.** 1978. Complete nucleotide sequence of the leader RNA synthesized *in vitro* by vesicular stomatitis virus. Cell **15**:93–101.
6. **Kolakofsky, D.** 1979. Studies on the generation and amplification of Sendai virus defective-interfering genomes. Virology **93**:589–593.
7. **Kolakofsky, D., F. Boy de la Tour, and A. Bruschi.** 1974. Self-annealing of Sendai virus RNA. J. Virol. **14**: 33–39.
8. **Leppert, M., L. Kort, and D. Kolakofsky.** 1977. Further characterization of Sendai virus DI-RNAs: a model for their generation. Cell **12**:539–552.
9. **Leppert, M., L. Rittenhouse, J. Perrault, D. F. Summers, and D. Kolakofsky.** 1979. Plus and minus strand leader RNAs in negative strand virus-infected cells. Cell **18**:735–747.
10. **Perrault, J., and R. W. Leavitt.** 1977. Inverted complementary terminal sequences in single-stranded RNAs and snap-back RNAs from vesicular stomatitis defective interfering particles. J. Gen. Virol. **38**:35–50.
11. **Perrault, J., B. L. Semler, R. W. Leavitt, and J. J. Holland.** 1978. Inverted complementary sequences in defective interfering particle RNAs of vesicular stomatitis virus and their possible role in auto-interference, p. 527–538. *In* B. Mahy and R. Barry (ed.), Negative strand viruses and the host cell. Academic Press, Inc., New York.
12. **Rao, D. D., and A. S. Huang.** 1979. Synthesis of a small RNA in cells coinfected by standard and defective interfering particles of vesicular stomatitis virus. Proc. Natl. Acad. Sci. U.S.A. **76**:3742–2745.
13. **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.
14. **Simonsen, C. C., S. Batt-Humphries, and D. F. Summers.** 1979. RNA synthesis of vesicular stomatitis virus-infected cells: in vivo regulation of replication. J. Virol. **31**:124–132.
15. **Wagner, R. R.** 1975. Reproduction of rhabdoviruses. Compr. Virol. **4**:1–94.