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Comparative Inhibition of Cellular Transcription by Vesicular Stomatitis Virus Serotypes New Jersey and Indiana: Role of Each Viral Leader RNA

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We compared the ability of the leader RNAs of the New Jersey and Indiana serotypes of vesicular stomatitis virus to inhibit transcription in infected host cells. The level of cellular RNA synthesis in cells infected with either serotype was drastically reduced by 5 h after infection. Studies with UV-inactivated virus demonstrated that shutoff of cellular RNA synthesis directly correlated with the ability of the infecting virus to transcribe its plus-stranded leader RNA. Although both serotypes inhibited cellular RNA synthesis, the Indiana serotype reduced synthesis to lower levels. In addition, an examination of the kinetics of leader RNA synthesis in vivo indicated that up to four times more leader RNA was produced in cells infected with the Indiana serotype than in those infected with the New Jersey serotype. However, in vivo studies also suggested that the leader RNA of the New Jersey serotype was a more efficient RNA inhibitor than was the Indiana serotype leader RNA. Although up to 2,900 copies of the leader RNA per cell could be detected in infected cells, only 550 copies of the Indiana and 100 copies of the New Jersey leader RNAs per cell were present in infected cells that were demonstrating 50% of the maximal inhibition of RNA synthesis. In an in vitro system, leader RNAs of both serotypes inhibited DNA-dependent transcription of the adenovirus late promoter and adenovirus-associated RNA genes, but the New Jersey serotype leader was also a better inhibitor in this reconstituted system. Data from the dose response of inhibition by each leader suggest that polymerase III transcription was more sensitive to inhibition by viral leaders than was polymerase II transcription. Polyadenylated viral mRNAs and the NS and N gene starts transcribed by both serotypes did not significantly inhibit transcription at levels at which the corresponding leader RNAs were inhibitory. Overall, our results strongly suggest a role for the plus-stranded leader RNAs of the New Jersey and Indiana serotypes of vesicular stomatitis virus in inhibiting cellular transcription in vivo. We discuss differences in the nucleotide sequences of the two leader RNAs in relation to their differencs in biological activity and to potential regulatory sequences.

Infection of many cell types with vesicular stomatitis virus (VSV), the prototype rhabdovirus, results in a drastic reduction in the ability of the host cell to synthesize RNA (6, 39, 40, 48). This effect, along with the reduced ability to synthesize DNA (30, 47) and protein (6, 26, 28, 44), eventually leads to cell death (25–27). The ability of VSV to inhibit cellular RNA synthesis requires transcription by the virion polymerase (41, 46). However, studies with UV-inactivated virus (38) indicate that only a small part of the genome must be transcribed. Weck and Wagner (41) suggested that the RNA shutoff was at the level of initiation of transcription, primarily by decreasing the number of functional units of polymerase. Degradation of cell mRNA was not enhanced in infected cells (31), and there has been no evidence for modification of post-transcriptional events such as polyadenylation or transport of RNA (39).

The VSV genome is transcribed sequentially (1, 4), producing a 47- to 48-nucleotide leader RNA and five monocistronic mRNAs. The leader RNA is transcribed from the exact 3′-end of the genome both in vitro (8, 9) and in vivo (20) and is produced in molar amounts higher than any of the viral mRNAs. Because only a small portion of the genome must be transcribed to affect cell RNA synthesis, this leader RNA has been suggested as the inhibitor molecule (38). Recently, Kurilla et al. (19) demonstrated that the leader RNA was present in the nucleus of

infected cells, and studies by McGowan et al. (29) showed that the leader RNA could inhibit DNA-dependent RNA synthesis in an in vitro transcription system. In view of these observations, the VSV leader RNA appears to provide a unique model for studying regulation of transcription by a small RNA.

With the exception of the limited study by Yaoi et al. (48), information on inhibition of cellular macromolecular synthesis by VSV has been obtained for the Indiana serotype. As an approach to further studying the role of the VSV leader RNA in transcription inhibition, we examined the inhibitory effect of two leader RNAs that have slightly different RNA sequences. We report certain similarities and differences in the ability of these two leader RNAs to inhibit transcription, and by examining the kinetics of leader production in vivo, we provide additional evidence that leader RNAs play a key role in inhibiting RNA synthesis in VSV-infected cells.

MATERIALS AND METHODS

Viruses and cells. The VSVs used in this study were the San Juan strain of the Indiana serotype (VSV_{Ind}), originally obtained from the U.S. Agricultural Research Center, Beltsville, Md. (37), and the Hazelhurst strain of the New Jersey serotype (VSV_{NJ}), obtained from the American Type Culture Collection, Rockville, Md. (ATCC 159). Clones of each virus were selected from plaques on L-cell monolayers. Virus stocks were prepared by growing virus in BHK-21 cells at a multiplicity of infection (MOI) of $\simeq$0.1 PFU per cell and were titrated by assay of PFU on monolayers of L cells. Virus was purified by sucrose and potassium tartrate gradient centrifugation as described by Barenholz et al. (5). The mouse myeloma cells (MPC-11), BHK-21 cells, and L cells were grown as described previously (28). The culture medium, Dulbecco Modified Eagle Medium (DMEM), calf serum, and horse serum were obtained from GIBCO Laboratories Grand Island, N.Y.

Tritiated uridine labeling of RNA in infected and uninfected cells. MPC-11 cells were grown in suspension culture to a cell density of 5×10^5 cells per ml and were centrifuged at 1,000 $\times$ *g* for 5 min. The cell pellets were suspended in DMEM, containing 1% horse serum, to a cell density of 1×10^7 to 2×10^7 cells per ml. Samples were infected at an MOI of either 1, 10, or 50 PFU per cell and were incubated for 30 min at 37°C. After the adsorption period, cells were diluted in DMEM and inoculated into 24-well culture plates at 5×10^5 cells per well. For labeling of BHK-21 cells, monolayer cultures were prepared in 24-well plates and infected at the above multiplicities. Cells were pulse-labeled for 10 min by the addition of [^{3}H]uridine to a final concentration of 5 μCi/ml. After labeling, the cells were lysed by the addition of one-tenth volume of 10% sodium dodecyl sulfate (SDS), and after 5 min, an equal volume of 10% trichloroacetic acid was added. The acid-insoluble material was collected by filtration through glass fiber filters (GSA; Whatman, Inc., Clifton, N.J.) and the radioactive content was determined by counting dried filters by scintillation spectroscopy. The protein content of labeled cells was determined by the method of Lowry et al. (23).

UV irradiation of virus. As described previously (38), 25-μl samples of purified VSV (5×10^9 PFU/ml) were dispensed into petri dishes (diameter, 100 mm) and placed at a distance of 10 cm from a UV light source. Virus samples, at 4°C, were exposed at various times to irradiation at a wavelength of 254 nm and a dose rate of 85 ergs per mm^2 per s. Cells were infected with UV-irradiated virus at an MOI of $\simeq$10, based on the original titer of the virus before irradiation.

In vitro VSV transcription assay. VSV plus-stranded RNAs were produced in a transcription assay (12) containing 0.1 mg of VSV per ml, 0.14 M NaCl, 0.2% Triton X-100, 7.5 mM $MgCl_2$, 10 mM Tris-hydrochloride (pH 8.0), 1 mM dithiothreitol, 1 mM each of ATP, GTP, and CTP, and 0.1 mM UTP containing 10 to 100 μCi of [α-^{32}P]UTP (400 Ci/mmol). Transcription mixtures were incubated for 3 h at 31°C, and the reactions were stopped by adding one-tenth volume of 10$\times$ SET (1$\times$ SET is 0.15 M NaCl–5 mM EDTA–50 mM Tris-hydrochloride [pH 8.0]) and extracting with an equal volume of water-saturated phenol. The resulting aqueous phase was extracted from one to three times with chloroform-isoamyl alcohol (24:1 [vol/vol]) and adjusted to 0.3 M with sodium acetate, and the RNAs were precipitated at −80°C for 2 h after the addition of three volumes of 95% ethanol. The precipitate was collected by centrifugation at 10,000 $\times$ *g* for 10 min and suspended in 1$\times$ SET.

Polyadenylated RNAs, isolated by oligodeoxythymidylic acid cellulose chromatography as described by Kuchler (18), were precipitated with ethanol, and the incorporation of [^{32}P]UMP was determined by Cerenkov counting of the resulting RNA pellets.

Leader RNA was separated from other plus-stranded RNAs by electrophoresis at 1,600 V on 8 M urea–20% polyacrylamide gels in buffer containing 10 mM Tris (pH 8.0), 1 mM EDTA, and 5 mM boric acid. The leader RNA, detected by autoradiography, was eluted from the gel overnight in buffer containing 500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, and 0.1% SDS. Acrylamide fragments were removed by filtration of the eluate through a 0.22-μm Milex-GV filter unit, and the leader RNA was precipitated by the addition of three volumes of 95% ethanol. The precipitate was collected by centrifugation at 12,000 $\times$ *g* for 30 min and was suspended in 1$\times$ SET. After one extraction with phenol and two extractions with chloroform-isoamyl alcohol (24:1 [vol/vol]), the leader RNA was repeatedly precipitated with ethanol (at least three times), and the final RNA pellet was suspended in distilled water. The amount of leader RNA was determined both by relative incorporation of [^{32}P]UMP and by optical density at 260 nm. The ratio of absorbance at 260/280 nm was typically 1.9 to 2.1. The yield was approximately 1 μg of leader RNA per 2 mg of virus used for transcription. Samples stored at −80°C showed no degradation within 2 months as determined by gel electrophoresis.

DNA-dependent transcription with a HeLa cell extract. As described previously (29), HeLa cells were grown in DMEM supplemented with 5% calf serum and 10% tryptose phosphate broth (GIBCO) to a density of 5×10^5 cells per ml, and a transcriptionally active extract was prepared from these cells by the

method of Manley et al. (24). The DNA templates used for transcription were the plasmid pBR322-*Bal*-1-E recombinant of the adenovirus (Ad-2) DNA late promoter (LP; originally obtained from P. A. Sharp, Massachusetts Institute of Technology) and a pBR322 recombinant of the adenovirus-associated (VA) RNA genes (originally a gift from D. F. Bogenhagen, Carnegie Institute). Both recombinant plasmids were propagated in *Escherichia coli* HB101 and were purified by CsCl gradient centrifugation. Before transcription, the Ad-2 late promoter was cleaved with the restriction endonuclease *Sma* I in the buffer specified by the manufacturer (Bethesda Research Laboratories, Bethesda, Md.).

The DNA templates were transcribed in a reaction mixture containing 3 mM $MgCl_2$, 15 mM ammonium sulfate, 0.1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 1 mM each of ATP, CTP, and GTP, 0.1 mM UTP containing 10 μCi of [α-^{32}P]UTP (410 Ci/mmol) and 20% (vol/vol) of HeLa cell extract. After 30 min at 31°C, reactions were stopped by adding 10 volumes of 1× SET and extracting once with SET-saturated phenol. After adding 30 μg of yeast carrier RNA, the transcription products were precipitated from the aqueous phase by the addition of sodium acetate to 0.3 M and three volumes of ethanol. The precipitate was collected by centrifugation at 10,000 × *g*, suspended in 8 M urea containing 0.1% bromophenol blue and xylene cylanol, and the transcription products were separated on an 8 M urea–8% polyacrylamide slab gel in the Tris-borate buffer described above.

Isolation of cellular RNA. Total cellular RNA was isolated from infected and uninfected BHK-21 and MPC-11 cells by a modification of the method of Wold et al. (45). Cells were washed in TE buffer (10 mM Tris-hydrochloride (pH 8.0)–10 mM EDTA) and centrifuged at 1,000 × *g* for 10 min. The cell pellets were suspended in 10 volumes of TE buffer containing 1% SDS and 500 μg of proteinase K per ml and were incubated at 37°C overnight. The cell lysate was extracted twice with phenol-chloroform-isoamyl alcohol (24:24:1 [vol/vol/vol]), and the nucleic acids were precipitated with ethanol in the presence of 0.2 M sodium acetate. After centrifugation, the nucleic acid pellet was lyophilized and suspended in TE buffer containing 50 mM sodium acetate. To remove contaminating DNA, samples were digested for 30 min at 37°C with 50 μg of pancreatic DNase (Worthington Diagnostics, Freehold, N.J.; RNase-free) per ml, followed by phenol extraction and precipitation with ethanol. Alternatively, samples were mixed with CsCl to a final density of 1.56 g/ml and centrifuged at 240,000 × *g* for 24 h. The CsCl-pelleted RNA was suspended in SET containing 0.1% SDS and was precipitated with ethanol. The ratio of absorbance at 260/280 nm was typically 2.0 to 2.1.

Preparation of 3′-end-labeled VSV RNA. The 42S minus-stranded genome was extracted from purified virus by incubating with 0.1% Triton X-100–1% SDS–500 μg of proteinase K per ml for 1.5 h at 31°C. The mixture was extracted twice with SET-saturated phenol, and RNA was precipitated with ethanol. The 42S RNA was specifically labeled by ligation of [5′-^{32}P]cytidine-3′,5′-bisphosphate ([^{32}P]pCp) to its 3′-end by T4 RNA ligase (Bethesda Research Laboratories). The 20-μl reaction mixture contained 3 to 5 μg of 42S RNA, 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 8.1), 10 mM $MgCl_2$, 3 mM dithiothreitol, 10 μM ATP, 10% dimethyl sulfoxide, 10 μg of bovine serum albumin (Boehringer Mannheim Corp., New York, N.Y.; nuclease-free) per ml, 250 μCi of [^{32}P]pCp (3,000 Ci/mmol), and 10 U of T4 RNA ligase. After 18 h at 4°C, 30 μg of yeast RNA carrier was added, and the 42S RNA was precipitated with ethanol. The precipitated RNA was collected, dissolved in SET, and centrifuged through a 5 to 25% sucrose gradient (10 mM Tris-hydrochloride–1 mM EDTA–0.15 M NaCl–0.5% SDS) for 105 min at 125,000 × *g*. The peak at 42S was collected, and the RNA was recovered by ethanol precipitation. The specific activity of the end-labeled probe was typically 2×10^5 to 5×10^5 cpm/μg of 42S RNA.

Detection of VSV leader RNA by hybridization. The 3′-end-labeled virion RNA was hybridized to leader RNA essentially as described by Leppert et al. (20). Briefly, samples of cellular RNA (approximately 12 μg) from infected and uninfected cells were adjusted to contain 10 mM Tris-hydrochloride (pH 8.0) and were boiled for 5 min in the presence of approximately 2×10^4 cpm of 42S RNA probe. In preparing the RNA samples, identical numbers of cells and volumes were used to ensure that differences in hybridization would reflect differences in leader RNA content. After boiling, samples were cooled on ice, adjusted to 0.5 M NaCl, and incubated at 65°C. After 4 h, the samples were digested with 30 μg of pancreatic RNase A (Worthington) per ml and 60 U of RNase T1 (Bethesda Research Laboratories) per ml for 30 min at 37°C. SDS and pronase were then added to final concentrations of 0.4% and 200 μg/ml, respectively, and the incubation was continued for 30 min. After adding 30 μg of yeast carrier RNA and adjusting the mixture to 0.3 M with sodium acetate, the double-stranded RNA was precipitated at −80°C with three volumes of 95% ethanol. The resulting precipitate was collected and lyophilized, and the RNAs were separated by electrophoresis on a 10% polyacrylamide gel. The number of genome equivalents of leader in infected cell RNA samples was determined from the specific activity of the probe and the counts per minute in the double-stranded leader. In all experiments, the amount of end-labeled probe used for hybridization was determined to be in excess.

RESULTS

Growth of VSV in mouse myeloma cells. For most experiments, mouse myeloma cells, MPC-11, were used because they were previously found to be highly susceptible to the inhibitory and cytopathic effects of VSV_{Ind} (39). To determine the growth kinetics of VSV_{NJ} and to compare it with VSV_{Ind} in these cells, suspension cultures were infected with each virus at an MOI of ≈5, and after an adsorption period of 1 h, the cells were washed three times and suspended in fresh medium. The supernatants were assayed at 1-h intervals for release of infectious virus by plaque assay on mouse L cells.

With both serotypes, infectious virion production began approximately 2 h after infection and reached maximal levels by 5 to 6 h. VSV_{NJ}

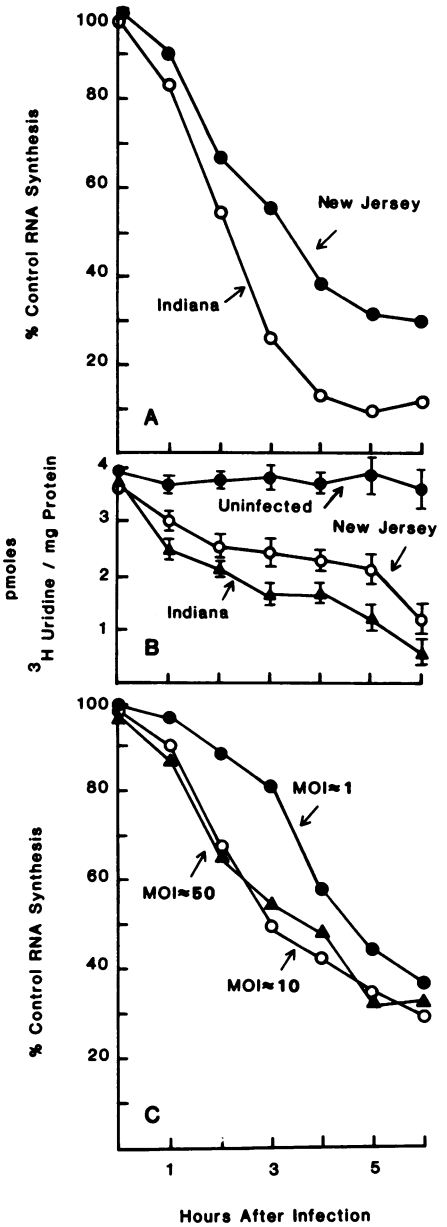

FIG. 1. Inhibition of cellular RNA synthesis by VSV serotypes New Jersey and Indiana. MPC-11 cells were infected with the viruses and were pulse-labeled with [^{3}H]uridine for 10 min at 1-h intervals after infection. The amount of cell RNA synthesis was determined, relative to mock-infected cells, by the amount of acid-precipitable radioactivity in each sample. (A) RNA shutoff in cells infected with either serotypes at an MOI of ≈10. (B) Inhibitory effect of the viruses on RNA synthesis relative to the protein content of the cell. The amount of uridine incorporated into cell RNA was determined from the specific activity of the label and the amount of acid-precipitable radioactivity. The protein content was determined by the method of Lowry et al. (23). (C) Effect of multiplicity on the ability of VSV_{NJ} to inhibit RNA synthesis. In (A), (B), and (C), all data points were the average of three determinations.

infected cells released ~8 PFU per cell, and VSV_{Ind}-infected cells released ~25 PFU per cell. With both serotypes, the viability of infected cells was ~1.0% that of controls 10 h after infection. As an additional indicator of the slower growth of the New Jersey serotype, we found that the viral plaque diameter of VSV_{NJ} was 35% the diameter of VSV_{Ind} plaques (1.4 versus 3.5 mm) when plated on L cells.

Inhibition of cellular RNA synthesis. The effects of VSV_{NJ} and VSV_{Ind} on cellular RNA synthesis were compared by pulse-labeling cells with [^{3}H]uridine at various times after infection with each serotype and determining the amount of acid-precipitable radioactivity as described above. The contribution of viral RNA synthesis to the acid-precipitable counts was less than 5% by 6 h after infection. This was determined by pulse-labeling control and infected cells in the presence of actinomycin D (38).

Figure 1 demonstrates that both serotypes inhibit cell RNA synthesis, although VSV_{Ind} was more effective. The level of RNA synthesis is VSV_{Ind}-infected cells was reduced rapidly to levels approximately 15% that of the control cells by 4 h after infection (Fig. 1A). However, RNA synthesis in cells infected with VSV_{NJ} declined at a somewhat slower rate, and the maximal inhibition by VSV_{NJ} was between 25 and 30% 6 h after infection. The Indiana serotype also was shown to be more effective in inhibiting RNA synthesis when measured relative to the amount of cellular protein (Fig. 1B). In the uninfected cells, the amount of RNA and protein increased proportionally with time as indicated by the relatively constant ratio of ~3.7 pg of uridine incorporated per mg of protein. The decrease in the ratio of the infected cells reflected a steady decrease in uridine incorporation; the cell protein content remained at approximately the same level.

We determined that the difference in inhibitory activity between the two serotypes was not due to slight differences in MOI. Cells were infected with either the New Jersey or Indiana serotypes at MOIs of 1, 10, and 50 PFU per cell and were pulse-labeled with [^{3}H]uridine as described above. There was no significant difference in the inhibition of RNA synthesis at apparent multiplicities of 10 and 50 PFU per cell for both the New Jersey (Fig. 1C) and Indiana (data not shown) serotypes. A similar finding was reported by Weck and Wagner (39) for the Indiana serotype. Although the inhibitory effect at an MOI of ≈1 was less rapid, by 6 h the level of inhibition was equivalent to that seen in cells

infected with a higher MOI. This reduced initial effect could be explained by considering the Poisson distribution; at an MOI of 1, only 63% of the cells should be infected. However, because of the rapid inhibition at a low MOI, it has been suggested that particles unable to form plaques can shut off cellular RNA synthesis (39). All subsequent experiments measuring RNA synthesis inhibition were carried out at an MOI of ≈10.

We also examined the ability of both serotypes to inhibit RNA synthesis in BHK-21 cells. Although these cells are less sensitive to the inhibition of RNA synthesis, as previously reported (39), the VSV_{Ind} was more effective in the shutoff of RNA synthesis than was VSV_{NJ} (data not shown). Thus, the difference between the two viruses does not appear to be a cell-dependent effect.

RNA synthesis inhibition with UV-inactivated virus. The leader RNA sequences are the first to be transcribed from the VSV genome, and because of sequential 3′ to 5′ transcription, these sequences would be the least sensitive of the viral genes to UV inactivation. Weck et al. (38) demonstrated that the UV target size for VSV inhibition of RNA synthesis was small, suggesting a role for the leader RNA. As another approach to test this possibility, we attempted to correlate the ability of UV-inactivated VSV to shut off RNA synthesis with its ability to synthesize the leader RNA.

Samples of VSV irradiated with increasing UV doses, were tested for their capacity to inhibit RNA synthesis in MPC-11 cells and for in vitro transcription activity. Cells infected with UV-irradiated VSV_{NJ} were pulse-labeled with tritiated uridine 4 h after infection, and the trichloroacetic acid-precipitable counts were determined as described above. Products of in vitro transcription of UV-irradiated VSV_{NJ} were analyzed for content of polyadenylated RNA by oligodeoxythymidylic acid chromatography and for content of leader RNA by excising and counting bands after separation on polyacrylamide gels.

As shown in Fig. 2, a dose of approximately 2,000 ergs per mm^2 eliminated the ability of VSV_{NJ} to produce polyadenylated RNA in an in vitro transcription reaction, a dose that only slightly decreased the ability to inhibit RNA synthesis in the cell. A dose of ~12,000 ergs per mm^2 was required to reduce the maximal inhibitory effect of both the New Jersey (Fig. 2) and the Indiana serotypes (data not shown) to 37% (the 37% 1/e survival dose). The UV dose required to reverse the RNA synthesis inhibitory activities of VSV_{NJ} and VSV_{Ind} directly correlated with the UV dose required to reduce synthesis by these viruses of leader RNA; the amount of leader RNA and the ability to inhibit RNA synthesis decreased proportionally with increasing UV dose. At doses greater than 2,500 ergs per mm^2, 90% or more of the total RNA being transcribed was leader RNA (data not shown).

Detection of leader RNA produced in vivo. As shown in Fig. 1A, the New Jersey serotype was less effective than the Indiana serotype in inhibiting cellular RNA synthesis. Assuming a role for the leader RNA in the inhibition process, one reason for this observation could be that less leader RNA is produced in VSV_{NJ}-infected cells than in cells infected with the VSV_{Ind} serotype. To test this hypothesis, we analyzed infected

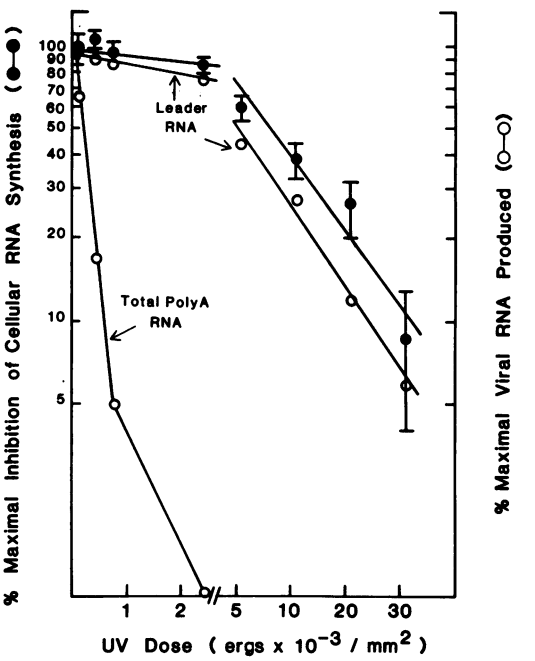

FIG. 2. Comparative effect of UV irradiation on the ability of VSV_{NJ} to transcribe polyadenylated (polyA) and leader RNA in vitro and to inhibit cellular RNA synthesis. VSV_{NJ} was UV irradiated (254 nm) with various doses, and samples were used to infect MPC-11 cells (MOI, ≈10). After 4 h, the level of cell RNA synthesis was determined by pulse-labeling with [^{3}H]uridine; data points represent the average of triplicate determinations. VSV_{NJ} irradiated at each UV dose was also transcribed in an in vitro assay system containing [^{32}P]UTP. The plus-stranded polyadenylated RNAs were separated by oligodeoxythymidylic acid cellulose chromatography and were quantitated by the amount of [^{32}P]UMP incorporation. Leader RNA was separated from other transcription products by electrophoresis on 8 M urea–20% polyacrylamide gels and quantitated by Cerenkov counting of gel slices. Each data point for polyadenylated or leader RNA represents the average of two determinations.

cells for the presence of leader RNA transcript by hybridization to 3'-end-labeled virion RNA.

Cells were infected with the two serotypes (MOI, ≈10), and at various times after infection, the total RNA was extracted as described in Materials and Methods. Samples of RNA were hybridized to the 42S virion RNA probe, digested with ribonuclease, and the double-stranded RNA (dsRNA) products separated by gel electrophoresis. Only products contiguous with the 3'-end of the genome can be detected by this procedure. To assess the specificity of the hybridization reactions for the leader RNA of each serotype, we also hybridized the 42S genome probe of VSV_{NJ} with purified leader of VSV_{Ind} and vice versa. The 42S probes prepared from both serotypes only hybridized to the homologous leader under the annealing conditions of these experiments (data not shown). Therefore, each probe was specific for leader of the same serotype.

Two leader transcripts were detected after hybridization of 42S virion probes to a sample of RNA from infected cells (Fig. 3A and B). It has been suggested that the larger species results from imprecise termination or processing in vivo (19). In cells infected with both serotypes, the amount of leader RNA increased with time, but to much higher levels in the cells infected with the Indiana serotype (Fig. 3A and C). By 5 h after infection, levels of intracellular leader reached 2,400 and 675 copies of leader per cell genome equivalent in the VSV_{Ind}- and VSV_{NJ}-infected cells, respectively. This difference in the quantity of leader produced was not restricted to the MPC-11 cell system. BHK-21 cells, infected with each virus, were also analyzed for their content of leader RNA 4 h after infection; levels of leader reached 1,600 copies per cell in the VSV_{Ind}-infected cells and 400 copies per cell in cells infected with VSV_{NJ} (data not shown).

Production of leader RNA in vivo by UV-inactivated virus. The above results demonstrated that 5 h after infection, there was approximately four times less leader RNA produced in cells infected with the New Jersey serotype than in cells infected with the Indiana serotype. In support of a role for the leader RNA, this result might be expected since we found that the New Jersey serotype also was less effective in inhibiting RNA synthesis. However, the level of inhibition by VSV_{NJ} was not four times less than that of VSV_{Ind} (Fig. 1A). Even though this suggests a difference in the efficiencies of the two leader RNAs in their ability to shut off RNA synthesis, a comparison of the levels of leader RNA in cells that are inhibited in RNA synthesis to maximal levels might be misleading. We cannot assume that the levels of leader RNA present 5 h after infection represent the minimal amount of

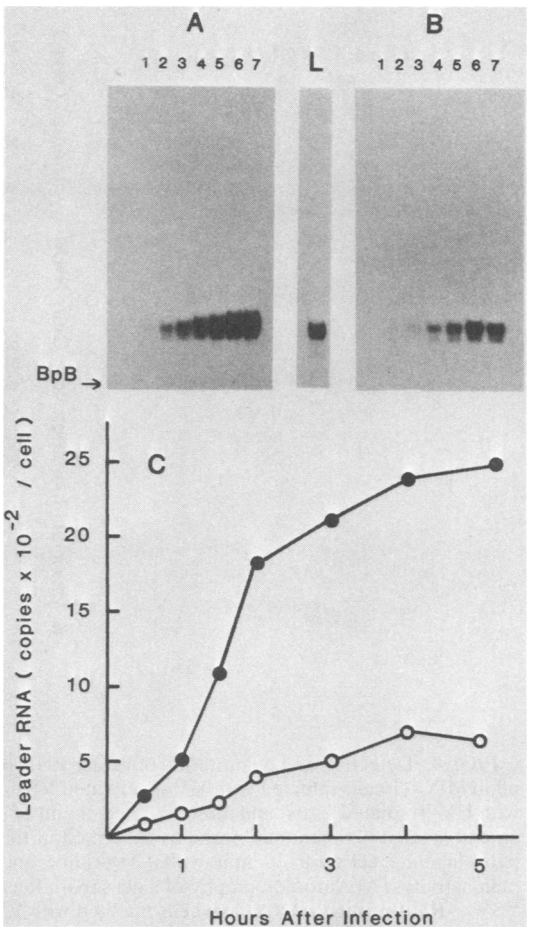

FIG. 3. Kinetics of VSV_{Ind} and VSV_{NJ} leader RNA production in infected MPC-11 cells. Cells were infected with the VSV serotypes New Jersey and Indiana (MOI, ≈10), and at various times after infection, the total RNA was extracted from the cell as described in the text. To detect the plus-stranded leader RNA, cell RNA samples were hybridized with a 42S VSV genomic RNA that had been 3'-end-labeled with [^{32}P]pCp, followed by digestion with pancreatic and T1 ribonucleases. Double-stranded products were separated on 10% polyacrylamide gels, and the amount of leader RNA was determined from the number of Cerenkov counts hybridized and the specific activity of the probe. (A) Autoradiography of a gel separation of double-stranded products after hybridization of end-labeled 42S serotype VSV_{Ind} probe to RNA from VSV_{Ind}-infected cells. Lanes 1 through 7, Hybridization to RNA extracted at the following times after infection: lane 1, 0.5 h; lane 2, 1 h; lane 3, 1.5 h; lane 4, 2 h; lane 5, 3 h; lane 6, 4 h; and lane 7, 5 h. (B) Same as (A) except hybridization of 42S serotype VSV_{NJ} probe to RNA from VSV_{NJ}-infected cells. Lane L, Purified leader RNA which was produced in the in vitro transcription assay and included as a marker. (C) Quantitation of leader RNA produced in cells at various times after infection with VSV_{Ind} (●) and VSV_{NJ} (○).

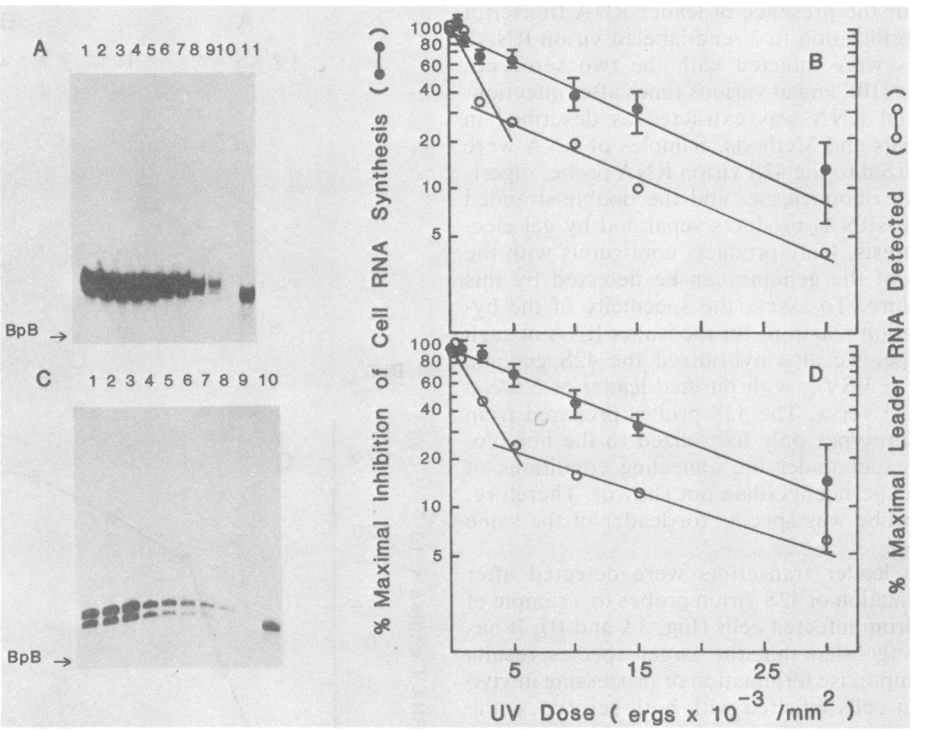

FIG. 4. Detection and quantitation of leader RNA production and determination of the level of cell RNA shut off in MPC-11 cells infected with UV-inactivated VSV_{Ind} (A) and (B) and VSV_{NJ} (C) and (D). Cells were infected with UV-irradiated virus, and after 5 h, the amount of leader RNA produced was determined by hybridization to an end-labeled 42S genomic probe as described in the text. The level of RNA shut off at 5 h was assessed by pulse-labeling cells for 10 min with [^{3}H]uridine and quantitating the amount of resulting acid-precipitable radioactivity. (A) Autoradiography of a gel separation of dsRNA products after hybridization of 42S end-labeled VSV_{Ind} RNA probe to RNA from cells infected with VSV_{Ind} UV-irradiated at (in ergs per mm^2): lane 1, 0; lane 2, 85; lane 3, 425; lane 4, 850; lane 5, 2,550; lane 6, 5,100; lane 7, 10,200; lane 8, 15,300; lane 9, 30,600; and lane 10, hybridization to uninfected control cell RNA; lane 11, purified VSV leader RNA from in vitro transcription. (B) Comparison of the progressive loss of ability of VSV_{Ind} to synthesize leader RNA in the infected cell and to shut off cell RNA synthesis with increasing UV doses. (C) Autoradiograph of a gel separation of dsRNA products after hybridization of 42S end-labeled VSV_{NJ} RNA probe to RNA from cells infected with VSV_{NJ} UV irradiated at (in ergs per mm^2): lane 1, 85; lane 2, 425; lane 3, 850; lane 4, 2,550; lane 5, 5,100; lane 6, 10,200; lane 7, 15,300; lane 8, 30,600; and lane 9, uninfected cell RNA; lane 10, purified VSV leader RNA. (D) Same as (B) but data for VSV_{NJ}.

leader needed to get the maximal level of cell RNA shut off, i.e., the amount of leader RNA produced in an infected cell may be more than is necessary for a maximal inhibitory effect. To compare relative efficiencies in vivo, it would be necessary to compare levels of leader RNA produced at the same time during infection that result in a fixed level of inhibition of cellular RNA synthesis, e.g., 50% maximal inhibition. These conditions were not met by the kinetic data shown in Fig. 1 and 3. However, the relative efficiencies of the two leader RNAs in vivo could be assessed by comparing levels of leader RNA synthesis and cellular RNA shut off in cells infected with UV-irradiated virus.

To this end, MPC-11 cells were infected with VSV_{Ind} or VSV_{NJ} irradiated with increasing UV doses, and after 5 h, the levels of leader RNA and cellular RNA synthesis inhibition were determined as described above. The production of both leader RNAs decreased with increasing UV dose (Fig. 4A and C). In these gels, we observed very small amounts of dsRNA larger than the leader band (Fig. 4A, lanes 1 through 5; Fig. 4C, lanes 1 through 3). These larger RNA transcripts, which must be contiguous with the 3′-end of the genome, have been described previously (19) and likely represent occasional readthrough occurring during transcription of the VSV genome RNA. These RNAs were not observed at doses above 2,500 ergs per mm^2.

The levels of cellular RNA shutoff and leader

RNA synthesis, in cells infected with virus irradiated with increasing UV doses, are shown in Fig. 4B and D. High levels of irradiation were needed to reduce both the level of cellular RNA synthesis inhibition and of leader RNA. Previous work has demonstrated that the 37% (1/e) survival dose for VSV_{Ind} mRNA produced in vivo was approximately 170 ergs per mm^2 and for the N protein, approximately 380 ergs per mm^2 (38). At doses much higher than this, there was no significant effect on leader RNA production or ability to shut off RNA synthesis. In both the VSV_{NJ}- and VSV_{Ind}-infected cells, the level of leader RNA dropped off very rapidly with increasing UV dose, followed by a more gradual decrease that paralleled the reverse of RNA synthesis inhibition. Thus, we have demonstrated that as we progressively inhibit the ability of VSV to shut off RNA synthesis, the level of leader produced in the same cells also decreases. This strongly strengthens the argument that the VSV_{Ind} and VSV_{NJ} leader RNAs are involved in the shutoff in vivo of cellular RNA synthesis.

If both leader RNAs play a role in the shutoff mechanism, their relative efficiencies in vivo could be assessed from the UV inactivation curves by determining levels 5 h after infection when the inhibition of cellular RNA synthesis is 50% of the maximal. At this level of inhibition, there were approximately 550 and 100 copies of leader per cell in the VSV_{Ind}- and VSV_{NJ}-infected cells, respectively (Fig. 4B and D). These values were determined by multiplying the approximate percentage of remaining leader RNA at 50% maximal inhibition, by the maximal leader RNA present in non-irradiated cells (2,600 and 550 copies of leader RNA per cell for VSV_{Ind} and VSV_{NJ}, respectively). These results suggest that even though much less VSV_{NJ} leader is produced in infected cells (Fig. 3C), this virus might shut off cell RNA synthesis by means of an inhibitor more effective than that of VSV_{Ind}. This apparent difference in efficiencies of the two leader RNAs in vivo was confirmed by the in vitro studies described below.

Inhibition of transcription in vitro by the leader RNAs. To characterize more fully the inhibitory effect of the VSV_{NJ} and VSV_{Ind} leader RNAs on transcription, we utilized the in vitro HeLa cell transcription system described by Manley et al. (24). The leader RNA of the Indiana serotype was previously shown to be inhibitory in this system (29). Two DNA templates were used in this system: a plasmid pBR322 recombinant of the adenovirus (Ad-2) LP gene, transcribed by RNA polymerase II (24, 42) and a cloned VA RNA gene, transcribed by RNA polymerase III (2). HeLa cell extracts were mixed with either the *Sma*I-cleaved LP gene or the VA gene and incubated for 30 min at 31°C as described in Materials and Methods. Transcripts labeled with [^{32}P]UMP were separated by gel electrophoresis and visualized by autoradiography. As previously noted (29), transcription of the *Sma*I-cleaved LP gene resulted in a major transcript of 560 nucleotides (Fig. 5, lane 1); its synthesis was directed by polymerase II as indicated by sensitivity to low concentrations (0.5 μg/ml) of α-amanitin (Fig. 5, lane 2). The origin of the larger transcript is unknown but, because it is also sensitive to low doses of α-amanitin, it is likely synthesized by *pol*II. The VA gene transcript was ~150 nucleotides (29) (Fig. 6, lane 1), and its synthesis was resistant to 0.5 μg of α-amanitin per ml (Fig. 6, lane 8); however, levels of 5 μg of α-amanitin per ml (Fig. 6, lane 9) inhibited its transcription indicating that polymerase III directed synthesis of the VA gene.

To determine any differential effect of the VSV_{NJ} and VSV_{Ind} plus-stranded leader RNAs on transcription in vitro, purified leader RNAs were added to transcription reactions containing the LP and VA DNA templates. Leader RNA

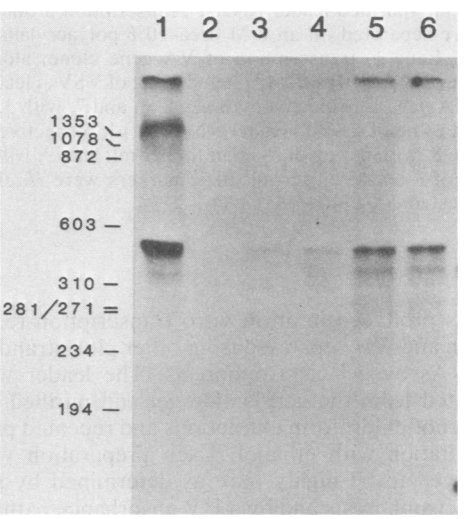

FIG. 5. DNA-dependent transcription in vitro of the Ad-2 LP and inhibition of its transcription by VSV_{NJ} and VSV_{Ind} leader RNAs. An *Sma*I-cleaved pBR322-Ad-2 LP recombinant (90 ng/μl) was incubated alone or with purified leader RNA in the presence of a soluble HeLa cell extract. Transcription products were separated on an 8 M urea–10% polyacrylamide gel and were visualized by autoradiography. Lane 1, products after transcription of the Ad-2 LP alone; lane 2, with 0.5 μg of α-amanitin per μl; lanes 3 and 4, with 18.5 ng of VSV_{NJ} leader RNA per μl; lanes 5 and 6, with 19.9 ng of VSV_{Ind} leader RNA per μl. Size markers were *Hae*III-cleaved φX-174 replicative form DNA (Bethesda Research Laboratories), boiled for 5 min in 8 M urea.

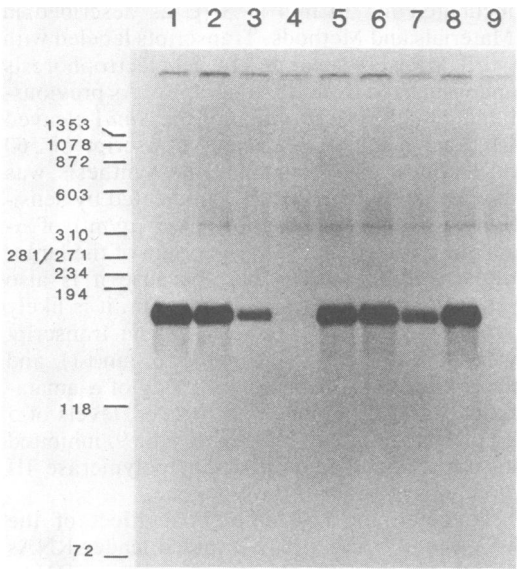

FIG. 6. DNA-dependent in vitro transcription of the VA RNA genes and the inhibition of transcription by VSV_{NJ} and VSV_{Ind} leader RNAs. A pBR322-VA gene recombinant (90 ng/μl) was incubated in a soluble Hela cell extract alone or with the VSV_{NJ}- and VSV_{Ind}-purified leader RNAs. Transcription products were separated on an 8 M urea–10% polyacrylamide gel. Lane 1, transcription of VA gene clone, alone; lanes 2, 3, and 4, with 4, 13, and 21 ng of VSV_{NJ} leader RNA per μl, respectively; lanes 5, 6, and 7, with 3, 8, and 24 ng of VSV_{Ind} leader RNA per μl, respectively; lane 8, with 0.5 μg of α-amanitin per ml; lane 9, with 5 μg of α-amanitin per ml. Size markers were *Hae*III-digested φX-174 DNA.

was produced in an in vitro transcription reaction and was separated from other plus-stranded RNAs by gel electrophoresis. The leader was eluted from the acrylamide gel and purified by phenol-chloroform extractions and repeated precipitation with ethanol. Each preparation was judged to be highly pure as determined by gel electrophoresis and by a UV absorbance pattern between 320 and 220 nm. As controls, regions of each gel containing no transcripts ("mock" leader preparations) or high-molecular-weight transcripts (mRNA size) were excised and treated in the same manner as the leader transcripts. We also tested the inhibitory ability of two small in vitro transcripts of 28 and 42 nucleotides in length, said to represent the N and NS gene starts, respectively (34). To assay for inhibition, samples of leader RNA or control preparations were reprecipitated with ethanol (or lyophilized), suspended in distilled water, and added to the reconstituted HeLa cell transcription system. The level of inhibition was measured by relative incorporation of [^{32}P]UMP and quantitated by Cerenkov counting of gel slices.

The relative effects of the two leader RNAs on transcription of the LP gene are shown in Fig. 5. At equimolar concentrations, the VSV_{NJ} leader RNA was more effective than the VSV_{Ind} leader in inhibiting transcription of this gene; lanes 3 to 4 and 5 to 6 in Fig. 5 show the level of transcription with approximately the same amount of the VSV_{NJ} and VSV_{Ind} leaders, respectively, compared with the level of transcription with no leader added (Fig. 5, lane 1). The VSV_{NJ} leader also was more effective in inhibiting transcription of the VA gene (Fig. 6); in Fig. 6, lanes 2, 3, and 4 show levels of transcription with increasing concentrations of VSV_{NJ} leader, and lanes 5, 6, and 7 show levels of transcription with similar amounts of VSV_{Ind} leader. The inhibition of transcription was not due to carry-over of impurities or contaminants since mock leader preparations did not significantly reduce transcription. Also, mRNA-sized VSV transcripts and yeast tRNA did not inhibit transcription at concentrations at which leader RNA was inhibitory (Table 1). Large amounts of VSV_{NJ} mRNA did inhibit transcription, in agreement with the data of McGowan et al. (29) for VSV_{Ind}. However, at these high concentrations, even yeast tRNA

TABLE 1. Inhibitory effect of various RNAs on in vitro transcription of the Ad-2 LP and VA genes in a HeLa cell reconstituted system

RNA added (ng)	% Inhibition of RNA transcribed on template[a]	
	LP	VA
VSV_{NJ} leader (216)	69	81
VSV mRNA[b] (274)	1	4
VSV mRNA (4,371)	84	73
Yeast RNA (487)	3	7
Yeast RNA (5,750)	100	100
N + NS starts[c] (374)	−4	2
VSV DI-T leader (394)	5	2
VSV DI-T leader (1,434)	26	31

[a] Results are average of duplicate determinations.

[b] mRNA size RNA represents in vitro transcripts of between 1,000 and 3,000 nucleotides that were excised and purified from the same gels used to separate the leader RNA for its purification.

[c] The 28- and 45-nucleotide products, N and NS gene starts, produced in the in vitro VSV transcription assay, were separated by gel electrophoresis and excised from the gel and purified, as was the leader RNA, as described in the text. Because of the small amounts of these transcripts, they were pooled and assayed for inhibitory activity.

nonspecifically inhibited the transcription reaction. Several small RNAs of VSV origin, the N and NS gene starts, and the 46-nucleotide defective interfering (DI)-T leader RNA (29) were not inhibitory at concentrations at which the VSV_{NJ} leader RNA was effective. Even seven times the amount of DI-T leader did not inhibit as well as the VSV_{NJ} leader RNA. As previously indicated (29), the DI-T leader serves as an appropriate control because infection of cells with DI particles from the 5'-end of the VSV genome does not result in the inhibition of cellular RNA synthesis (40).

Dose-response comparisons of the two leader RNAs on transcription inhibition in vitro. The effects of the VSV_{NJ} and VSV_{Ind} leader RNAs at various concentrations were tested in the reconstituted HeLa system. At nearly all concentrations tested, the New Jersey leader was more effective in inhibiting in vitro transcription of both the LP and VA genes (Fig. 7). In the range tested, it appeared that the minimal concentration of Indiana leader needed to inhibit the LP gene was much higher, approximately 40 pmol of leader per pmol of template compared with ~10 pmol of leader per pmol of template for the New Jersey serotype. However, increasing the concentrations of VSV_{Ind} leader above 40 pmol of template resulted in rapid inhibition of transcription within a relatively narrow range of concentration. In contrast, the inhibition of the VA gene by the VSV_{Ind} leader occurred at lower doses. Also, the VSV_{NJ} leader was more effective in inhibiting the VA gene than was the Indiana leader. However, both leader RNAs appeared to be more effective in their ability to inhibit transcription by RNA polymerase III (VA gene) than by polymerase II (LP gene).

Comparison of VSV_{NJ} and VSV_{Ind} leader RNA sequences. The nucleotide sequences of the New Jersey (11) and Indiana (10) leaders are shown in Fig. 8. The sequence of the two 48-nucleotide leader RNAs is highly conserved (~80% homology) with only 10 nucleotide differences (underlined, Fig. 8).

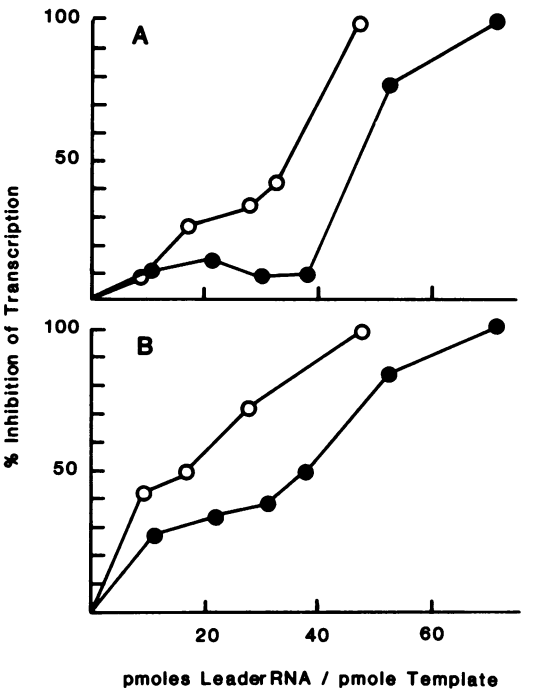

FIG. 7. Dose response to the inhibition of transcription by the New Jersey (○) and Indiana (●) leader RNAs. For both the Ad-2 LP (A) and VA RNA gene (B), 90 ng of template of reaction per μl were used. The extent of inhibition was determined by dividing the amount of transcription of each template from reactions containing leader by the amount from reactions with no leader added. The amount of transcription was determined by Cerenkov counting of the specific transcripts sliced from the gels.

DISCUSSION

Many viruses are able to alter host cell metabolism by interacting in some way with the normal regulatory controls of the cell. With the growing interest in small RNAs as regulatory molecules (for a review, see reference 22), studying the RNA synthesis inhibition by VSV leader RNAs may be useful in understanding transcription regulation by a small RNA as well as enhancing our knowledge of the molecular basis of viral cytopathogenicity. In this study, we provide additional evidence that the VSV leader RNA plays a role in the inhibition of

10 20 30 40

VSV_{Ind} Leader RNA 5' ppACGAAGACAAACAAACCAUUAUUAUCAUUAAAAGGCUCAGGAGAAAAC-OH 3'

VSV_{NJ} Leader RNA 5' ppACGAAGACAAAAAAACCAUUAUUACAAUUAAUUGGCCUAGAGGGAAAC-OH 3'

FIG. 8. Comparison of the nucleotide sequences of the leader RNAs of VSV_{Ind} and VSV_{NJ}. The unique regions of the VSV_{Ind} leader are highlighted by underlining. Data obtained from Colonno and Banerjee (10, 11).

cellular RNA synthesis. Also, we compared two VSV leaders, with differences in RNA sequence, for differences in biological activity.

Previous studies (38) demonstrated that the UV target size for the VSV_{Ind} genome product responsible for shutting off cellular RNA synthesis was small. Although this provided circumstantial evidence for implicating the leader RNA sequence, the correlation between RNA shutoff and the ability of heavily irradiated VSV_{Ind} to produce leader RNA in vitro as well as in vivo was not shown. We demonstrated here that at UV doses at which viral polyadenylated mRNA synthesis was essentially eliminated (~2,500 ergs per mm^2), the inhibition of cellular transcription and the ability to produce leader RNA were not affected. As the UV dose was increased, the synthesis by VSV of leader RNA in vivo or in vitro decreased concomitantly with its ability to shut off RNA synthesis in the cell. In addition, the leader RNA was very effective in inhibiting DNA-dependent transcription in the HeLa cell system, but we were unable to show significant inhibitory activity with other RNAs that were produced in the VSV transcription system. Overall, the data presented strongly suggest that the leader RNA plays a dominant role in the RNA shutoff mechanism.

The value of ~12,000 ergs per mm^2 for the 37% survival of RNA synthesis inhibition is significantly lower than values of 72,000 (38) or 52,000 ergs per mm^2 (30) previously reported. One explanation for this difference is that crude virus stocks, likely containing UV-absorbing impurities, were used in the previous studies in contrast to the highly purified virus used here. Using a value of 104 ergs per mm^2 for the 37% (1/e) survival dose for infectivity (38) and a genome size of 12,000 nucleotides (36), we calculated that the UV target size for 37% survival of cellular RNA synthesis was approximately 85 nucleotides. However, similar calculations for the actual survival of the ability of VSV genome to synthesize the 48-nucleotide leader (from Fig. 2) gave a UV target size of 150 nucleotides. Even though this demonstrates the error in the determination of actual target size by UV inactivation, it clearly indicates that the target size for survival of the RNA synthesis inhibition function approximates the target size of the leader RNA gene. Wu and Lucas-Lenard (46) reported a UV target size of 17% of the genome, or ~2,000 nucleotides, for inhibition of RNA synthesis in mouse L cells. We have no explanation for the difference in our results and theirs, except the remote possibility that the mechanism for RNA synthesis inhibition by VSV is different in mouse L cells and MPC-11 cells.

In Fig. 4, the amount of leader RNA produced in cells infected with UV-irradiated virus initially decreased quite rapidly with increasing UV doses, followed by a more gradual decline. This observation may be related to the amount of polymerase available for transcription. In cells infected with nonirradiated virus, or virus irradiated with low doses, virion polymerase is being produced de novo, and maximal transcription of the leader RNA can occur. Because the virion polymerase has a 37% (1/e) survival dose of ~1,000 ergs per mm^2 (38) at increasing doses, the amount of polymerase may become limiting, i.e., transcription may rely only on the polymerase carried into the cell with the infecting virus. Therefore, the initial rapid decline of leader RNA with increasing UV dose may be the result of a rapid loss of polymerase activity. In vitro (Fig. 2), the drop in leader-producing ability with increasing UV dose did not display an initial rapid decline, probably because in the transcription assay, polymerase does not become limiting even at a high UV dose. Efficient transcription need not rely on any de novo production of polymerase due to the large amount of viral protein present in these reactions.

The amount of total leader RNA we detected in VSV_{Ind}-infected cells (both MPC-11 and BHK-21) was significantly higher than that reported by Kurilla et al. (19) after fractionation of BHK-21 cells into soluble nuclear and cytoplasmic fractions. The reason for these discrepancies may be due to differences in the strain of virus used in each laboratory or to losses due to cell fractionation. Also, leader RNA can possibly associate with the nuclear membrane fraction which may have been discarded in the procedure used by Kurilla et al. (19). Since cellular transcription may occur on the insoluble nuclear matrix (32), it would be of interest to examine this nuclear fraction for leader RNA. In addition, the results of Kurilla et al. (19) indicated that in BHK-21 cells infected with VSV, the amount of total cellular leader RNA peaked at approximately 2 h after infection, followed by a drastic decrease by 3 h, and then again increasing in amount by 5 to 6 h. In MPC-11 cells, we did not observe a decrease in total cellular leader RNA with time. The amount of leader RNA increased steadily, reaching a plateau by 5 h after infection (Fig. 3C).

Both the VSV_{NJ} and VSV_{Ind} leader RNAs were able to inhibit transcription of the Ad-2 LP and VA genes, but the VSV_{NJ} leader was more effective (Fig. 5 and 6). From the dose-response data, the inhibition of the LP gene by the two leaders required much more VSV_{Ind} leader to reach a minimal inhibitory concentration (~40 pmol) than did the VSV_{NJ} leader. This different dose response was not evident in the inhibition of the VA gene by the two leaders; both leaders inhibited at the lowest concentration

tested. In addition, the *Pol*III-directed transcription (VA gene) was more sensitive to inhibition by both leaders than was the *Pol*II-directed transcription of the LP gene, a fact previously described for the Indiana leader (29). However, it has been reported that VSV infection inhibits *pol*II transcription to a greater extent than *pol*III (39). The difference in these results is not clear at this time.

These data raise certain questions about the mechanism(s) by which VSV leaders inhibit transcription. The difference in the dose-response curves between the VA and LP gene inhibition and the apparent greater sensitivity of VA gene inhibition by both leaders may simply reflect differences in the optimal conditions for transcription of each template in vitro. However, it may also indicate a difference in the inhibition mechanism of polymerase II and III transcription. If the inhibition of transcription by polymerase II and III were by different mechanisms, we would not necessarily expect that the nucleotide differences in the New Jersey leader that make it more effective in inhibiting polymerase II would also make it more effective in the inhibition of polymerase III transcription. Another factor in considering a possible mechanism is the relatively low levels of leader RNA found in vivo. Our data indicated that even though several thousand copies are present in VSV_{Ind}-infected cells, only several hundred molecules are present in cells whose RNA synthesis is inhibited by 50%. Because the MPC-11 cell contains at least 30,000 active polymerase molecules (41), it would appear that a direct interaction of the leader with the polymerase is an unlikely mechanism for shutting off RNA synthesis, as would a direct binding to the DNA template in vivo. Additional studies will be needed to determine whether leader RNA might be interacting with a minor cofactor common to both polymerases which is possibly more critical to the activity of polymerase III than to that of polymerase II. Moreover, we must be cautious of any interpretation drawn from the in vitro data, as the conditions in the cell and the in vitro transcription system may be quite different.

It is, perhaps, not unreasonable to speculate that the differences in activity of the two leaders studied here are due to the differences in their nucleotide sequence (Fig. 8). Several close similarities of both leaders, as well as several differences, deserve comment. With the exception of one nucleotide (nucleotide 12), the first 24 nucleotides are identical and adenosine rich. However, this change at position 12 results in the perfect palindrome (CAAACAAAC) in the Indiana leader being only partially present in the New Jersey leader. Both leader RNAs contain the palindromic sequence. AUUAUUA, at positions 18 to 25, suggested to be similar to the TAATA homology or Goldberg-Hogness box (3, 13, 16) presumably involved in RNA polymerase II initiation. It is interesting that the New Jersey serotype leader contains an extended AU stretch between nucleotides 26 and 34 which is not entirely present in the Indiana sequence. McGowan et al. (29) also have suggested that the leader sequence of the Indiana serotype between nucleotides 33 and 46 (AGGCUCAG) resembles the deoxynucleotide sequence implicated in RNA polymerase binding (7, 17), as well as being very similar to the consensus sequence (AG/GURAG) obtained for the 5'-end splice sites of intervening sequences (21, 33). The 3'-end of the New Jersey leader also is purine rich and has similar sequences. However, 5 of the 10 differences between the two leaders are within

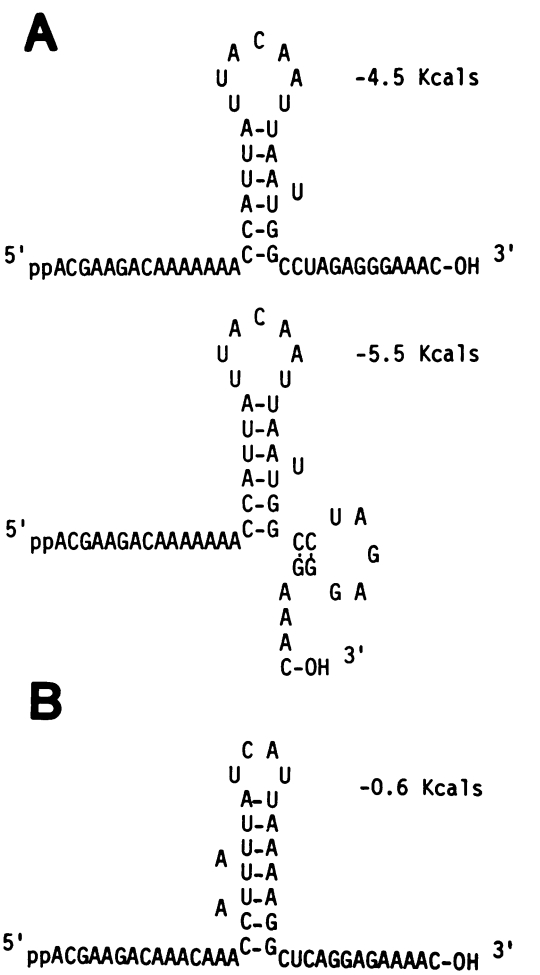

FIG. 9. Potential secondary structure in the VSV_{NJ} (A) and VSV_{Ind} (B) leader RNAs. The free energy of formation is indicated.

12 nucleotides of the 3'-end. Another difference of potential importance is the possibility of a secondary structure in the two leader RNAs. As examples, predicted structures of each leader are shown in Fig. 9, with the free energy of formation calculated by the method of Tinoco et al. (35); only potential structures with a negative free energy are shown. It would appear that the New Jersey leader has the potential for forming a more stable stem and loop structure. Such structures have been implicated as control elements in both procaryotic (for a review, see reference 43) and eucaryotic transcription (14, 15).

Although we can speculate about sequences potentially capable of interacting with or acting as regulatory elements in the cell, at this time no conclusions can be drawn as to the significance of the sequence differences between the VSV_{Ind} and VSV_{NJ} leaders. Additional studies are needed to (i) better define the specific nucleotides of the leader or its overall structure needed for the inhibitory activity and (ii) determine what cellular factor(s) the leader RNA interacts with or alters or both.

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