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Inhibition of Cellular DNA Synthesis by Vesicular Stomatitis Virus

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DNA synthesis in mouse myeloma (MPC-11) cells and L cells was rapidly and progressively inhibited by infection with vesicular stomatitis virus (VSV). No significant difference in cellular DNA synthesis inhibition was noted between synchronized and unsynchronized cells, nor did synchronized cells vary in their susceptibility to VSV infection after release from successive thymidine and hydroxyurea blocks. Cellular RNA synthesis was inhibited to about the same extent as DNA synthesis, but cellular protein synthesis was less affected by VSV at the same multiplicity of infection. The effect of VSV on cellular DNA synthesis could not be attributed to degradation of existing DNA or to decreased uptake of deoxynucleoside triphosphates, nor were DNA polymerase and thymidine kinase activities significantly different in VSV-infected and uninfected cell extracts. Analysis by alkaline sucrose gradients of DNA in pulse-labeled uninfected and VSV-infected cells indicated that VSV infection did not appear to influence DNA chain elongation. Cellular DNA synthesis was not significantly inhibited by infection with the VSV polymerase mutant *ts*G114(I) at the restrictive temperature or by infection with defective-interfering VSV DI-011 (5′ end of the genome), but DI-HR-LT (3′ end of genome) exhibited initially rapid but not prolonged inhibition of MPC-11 cell DNA synthesis. DNA synthesis inhibitory activity of wild-type VSV was only slowly and partially inactivated by very large doses of UV irradiation. These data suggest that, as in the effect of VSV on cellular RNA synthesis (Weck et al., J. Virol. **30**:746-753, 1979), inhibition of cellular DNA synthesis by VSV requires transcription of a small segment of the viral genome.

Infection of vertebrate cells with infectious vesicular stomatitis virus (VSV) or certain defective interfering (DI) particles of VSV results in the inhibition of cellular DNA, RNA, and protein syntheses (7, 28, 35, 36, 41), leading eventually to cell death (24-26). Much attention has been paid to the abilities of VSV to shut off cellular RNA and protein syntheses (7, 15, 19, 33, 34). Previous work (37) has shown that VSV inhibits initiation of RNA synthesis primarily by decreasing the number of functional units of RNA polymerases II and III. The inhibitory function(s) has been shown to be highly resistant to UV irradiation (35) and dependent on primary transcription of the viral genome (38).

Inhibition of protein synthesis by low multiplicities of VSV also requires viral transcription (25, 26). However, at high multiplicities of infection (MOIs), protein synthesis can be inhibited in the absence of viral specific transcription or translation (19, 39). Thus, a biphasic inhibition of protein synthesis has been proposed: an early UV-insensitive inhibition and a later UV-sensitive inhibition that requires synthesis of viral mRNA (39). Although the abilities of VSV to inhibit RNA and protein syntheses have been examined extensively, a paucity of information exists on the ability of this virulent virus to inhibit cellular DNA synthesis. A few initial observations by Yaoi et al. (41, 42) indicated that the stimulation of cellular DNA synthesis observed in partially synchronized uninfected cells was inhibited by infection with viable VSV or UV-inactivated VSV.

This study was undertaken to determine the conditions under which VSV inhibits cellular DNA synthesis and the kinetics of the reaction compared with its effect on cellular RNA and protein syntheses. We also report data on the genetic and pathogenetic properties of VSV which result in shutting off cellular DNA synthesis.

MATERIALS AND METHODS

Virus, cells, and media. Mouse myeloma (MPC-11) and mouse L cells were cultured as previously described (36). Wild-type VSV (Indiana serotype, San Juan strain) used in these experiments was grown in BHK-21 cells from clones of virus selected from plaques picked from L-cell monolayers. The resulting

virus stocks were titrated by assay of PFU on monolayers of L cells and were stored at −80°C. The temperature-sensitive mutant *ts*G114(I), restricted in primary and secondary transcription, was originally provided by C. R. Pringle, Institute of Virology, Glasgow, Scotland, and was grown and purified as previously described (14, 20, 28). DI particles VSV DI-011 (5′) and DI-HR-LT (3′) were originally obtained from R. A. Lazzarini, National Institutes of Health, Bethesda, Md., and were grown and purified as previously described (2, 13, 21, 31).

Determination of cellular DNA synthesis. Cell suspensions (1×10^7 to 5×10^7 cells per ml) were infected with wild-type, *ts*G114(I), or defective VSV at various multiplicities. Monolayer cultures of L cells, used in initial studies, were infected by the addition of a viral suspension of 0.05 ml to each well of a 24-well Costar culture tray (Bellco Glass, Inc., Vineland, N.J.). After adsorption of virus for 1 h at room temperature, mock-infected or VSV-infected cells were washed, fresh medium was added, and the cells were incubated at 37°C. Suspension cultures were resuspended to a final cell density of 1×10^5 to 4×10^5 cells per ml, seeded in 30-mm culture dishes, and incubated at the appropriate temperature. The time of dilution was taken as time zero of the infectious cycle. Cells were pulse-labeled by the addition of 1 ml of warm medium containing 4 μCi of [^{3}H]thymidine (New England Nuclear Corp., Boston, Mass; specific activity, 77 Ci/mmol) per ml.

After incorporation of [^{3}H]thymidine, cells were washed once in 2 ml of cold phosphate-buffered saline, suspended in 0.5 ml of ice-cold reticulocyte standard buffer, and precipitated by the addition of 0.5 ml of 10% trichloroacetic acid. After at least 10 min on ice, the acid-insoluble material was pelleted by centrifugation at $800 \times g$ for 5 min; 0.2-ml samples of the supernatant solution were withdrawn for measurement of radioactivity. The resulting precipitate was washed twice with 0.5 ml of ice-cold 5% trichloroacetic acid and solubilized in 0.5 ml of Nuclear-Chicago tissue solubilizer; 0.2-ml samples were taken for the determination of radioactivity in a Beckman LS-230 liquid scintillation system and counted at an efficiency of 40% for tritium (36).

Synchronization of cell cultures. Cells were synchronized with respect to DNA synthesis by the combined use of thymidine and hydroxyurea (12). Suspension cultures (10^5 to 4×10^5 cells per ml) were blocked initially for 12 to 14 h in growth medium containing 2 mM thymidine. Cells were then sedimented at room temperature, resuspended, and washed in growth medium without thymidine. The washed pellet of cells was resuspended in fresh medium (1×10^5 to 4×10^5 cells per ml) and incubated at 37°C. Hydroxyurea was added at 10 h to a final concentration of 1 mM for L cells or 0.75 mM for MPC-11 cells. After further incubation for 11 h in hydroxyurea at 37°C, cells were pelleted and resuspended in hydroxyurea medium at a concentration of 1×10^7 to 2×10^7 cells per ml. VSV was added and allowed to absorb for 1 h at room temperature. After absorption, VSV-infected or mock-infected cells were washed in medium not containing hydroxyurea and resuspended to a cell density of 1×10^5 to 6×10^5 cells per ml.

Monolayer cultures of mouse L cells were synchronized by the procedure described above, except that cells were not resuspended and pelleted but remained attached and were washed by covering the monolayer.

Alkaline sucrose gradient analysis of cellular DNA. Linear 36-ml, 5 to 20% sucrose gradients containing 0.3 N NaOH, 0.5 M NaCl, and 0.01 M EDTA were prepared (11). Briefly, 0.8 ml of 0.5 N NaOH–0.01 M EDTA was layered on the surface of the gradient. Radiolabeled cells (10^5 to 4×10^5) were washed in phosphate-buffered saline, resuspended in reticulocyte standard buffer, and layered on top of the gradient. A final 0.6 ml of 0.5 N NaOH–0.01 M EDTA was added. The gradient was placed in the cold (4°C) for 12 to 14 h and then centrifuged in an SW27 rotor at $88,000 \times g$ for 4 h. Fractions (0.8 ml) were collected from the bottom of the tube, and 500 μg of bovine serum albumin was added. Then 10% trichloroacetic acid was added, and the resulting precipitates were collected on glass fiber filters (GF/A; Whatman Scientific Products, Columbia, Md.); filters were washed with 5% trichloroacetic acid and then 95% alcohol, and the radioactivity was determined by liquid scintillation counting.

Autoradiography of whole cells. VSV-infected or mock-infected cells grown either on 5-mm cover slips (Bellco Glass, Inc.) or in suspension were pulse-labeled for 1 h with 4 μCi of [^{3}H]thymidine per ml. After the pulse, cells were washed three times with phosphate-buffered saline, fixed for 5 min with 25% acetic acid in methanol, mounted on slides, and dried. Slides were then dipped for 10 to 20 s in nuclear track emulsion NT-B2 (Eastman Kodak Co., Rochester, N.Y.), prewarmed to a temperature of 40 to 45°C. Excess emulsion was removed, and the slides were dried for 1 h, sealed in boxes, and placed at 4°C for 2 to 3 days. Exposed preparations were developed for 2.5 min in Dektol (D-19), fixed for 3 to 5 min with Kodak acid fixer, and washed carefully for 15 min with chilled water.

Extraction and assay of thymidine kinase and DNA polymerase. The methods for preparation of cytosol fractions of cells for assay of thymidine kinase activity have been described previously (29). Briefly, the enzyme was assayed in a 100-μl reaction mixture containing 0.15 M Tris-hydrochloride (pH 7.5), 2.5 mM ATP, 2.5 mM $MgCl_2$, 0.1 mM [^{14}C]thymidine (78 μCi/mmol), 2.5 mM dithiothreitol, 10 mM NaF, 1.0 mg of bovine serum albumin per ml, 3 mM phosphocreatine, 1.0 U of creatine phosphokinase, and 10 μl of enzyme. After incubation at 37°C for 1 h, 50-μl samples of the reaction mixture were spotted onto Whatman DE81 chromatography paper disks. The amount of [^{14}C]dTMP formed was determined by batch washing of the disks in three changes of 95% ethanol, followed by liquid scintillation counting of the radioactivity. Enzyme activity was linear with time for at least 1 h.

Cellular DNA polymerases were assayed from isolated nuclei in a 200-μl reaction mixture of the following composition: 50 mM Tris-hydrochloride (pH 8.5), 8 mM $MgCl_2$, 0.5 mM dithiothreitol, 100 μg of bovine serum albumin, 50 μg of activated calf thymus DNA, 0.1 mM each dCTP, dGTP, and dATP, 0.01 mM [^{3}H]dTTP (75 cpm/pmol), and 40 μl of enzyme. After incubation for 30 min at 37°C, 100-μl samples of the

reaction mixtures were spotted onto numbered glass fiber disks (Whatman GF/A). The amount of [^{3}H]-dTMP incorporated into acid-insoluble product (DNA) was determined by sequential batch washing of the dried disks in 5% trichloroacetic acid, 95% ethanol, and finally in acetone as described by Allen et al. (3). Radioactivity in the disks was determined by liquid scintillation counting.

RESULTS

Comparative inhibition by VSV of DNA synthesis in MPC-11 and L cells. The kinetics of VSV inhibition of cellular DNA synthesis was examined in exponentially growing mouse myeloma (MPC-11) and mouse L cells. Cultures were infected with VSV at various MOIs, and the rate of cellular DNA synthesis was compared to that in uninfected cells which were pulse-labeled with [^{3}H]thymidine for 10 min at 1-h intervals after infection.

Inhibition of cellular DNA synthesis in unsynchronized MPC-11 cells was more rapid at each MOI tested than that in VSV-infected L cells (Fig. 1). A similar differential susceptibility of these cell lines was previously reported for VSV inhibition of RNA synthesis (36), although yields of progeny virus were found to be equivalent for VSV-infected MPC-11 and L cells (B. H. Robertson and R. R. Wagner, *in* D. H. L. Bishop and R. W. Compans, ed., *Fifth International Congress on Negative Strand Viruses*, in press). The MOI proved to be significant for the inhibition of mouse L-cell DNA synthesis as compared with DNA synthesis in MPC-11 cells. At 4 h after VSV infection at MOI $\simeq$ 10, DNA synthesis in MPC-11 cells was inhibited to a level which was 15% that of control cells, whereas L-cell DNA synthesis had decreased to a rate of not quite 35% that of uninfected cells. With a lower MOI ($\simeq$1), the difference in the susceptibility of the two cell lines was even more striking (Fig. 1), L cells being almost completely resistant at MOI $\simeq$ 1 to the effects of VSV infection. By comparison, MPC-11 cells were almost equally sensitive at all MOIs tested. Therefore, based on the Poisson distribution, which predicts that an MOI $\simeq$ 1 should result in infection of ~63% of the cells, it can be assumed that VSV particles unable to form plaques can readily inhibit cellular DNA synthesis in MPC-11 cells. Clearly, apparent multiplicities of 1, 10, or 100 resulted in similar inhibition of DNA synthesis in MPC-11 cells, a finding similar to that reported by Weck and Wagner (36) for RNA synthesis in VSV-infected cells.

VSV inhibition of DNA synthesis in synchronized cells. Yaoi et al. (42) postulated that VSV shuts off only the onset of DNA synthesis as cells enter the S phase of the growth cycle.

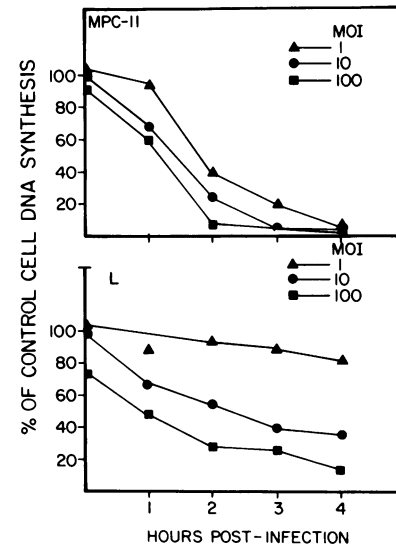

FIG. 1. *Comparative effect of VSV on the overall rate of DNA synthesis in unsynchronized MPC-11 and L cells, plotted as the percentage of incorporation of [^{3}H]thymidine by VSV-infected cells and parallel uninfected cultures of each cell type. After VSV or mock adsorption for 1 h at the multiplicities indicated, duplicate samples of uninfected and infected cells were pulse-labeled for 10 min at 1-h intervals with [^{3}H]thymidine (2 μCi/ml) at 37°C. Cells were harvested after labeling, and trichloroacetic acid-precipitable radioactivity was determined as described in the text. Results are plotted as the average of duplicate sets of cultures. Incorporation of [^{3}H]thymidine by uninfected cells ranged from 5 × 10^3 to 7 × 10^3 cpm per 2 × 10^5 cells.*

These authors had available only the rather inefficient method of medium withdrawal and replacement to synchronize their chicken embryo cells and reached only ~55% cell synchrony; they reported inhibition of onset of DNA synthesis in these partially synchronized cells, compared with no effect on established DNA synthesis in unsynchronized cells (42). We decided to reexamine this question by using a more stringent protocol for establishing cell synchrony to examine more accurately the effect of VSV infection on cells just before or after entering the S phase of the growth cycle. Cells were synchronized by a thymidine block for 12 h and resuspended in fresh medium for 10 h, followed by an additional hydroxyurea block for 12 h before infection (see above). This protocol resulted in >90% cell synchrony at 1 h after release from the hydroxyurea block (data not shown). The degree of synchrony was monitored by autoradiographic assays of [^{3}H]thymidine uptake, the disintegration of which produced tracks over nuclei of cells covered with NT-B2 film emul-

sion; a cell was scored as being in the S phase if 10 or more grains could be counted over the nucleus. Synchronized cells were infected with VSV (60-min adsorption) or mock infected at the time of release from the hydroxyurea block and pulse-labeled with [^{3}H]thymidine for 60 min at various times postinfection. DNA synthesis was assayed by autoradiographic grain counts.

In Fig. 2, representative autoradiographs of uninfected and VSV-infected L cells are shown. At time zero postinfection (after VSV adsorption), the relative intensity of grains over infected and uninfected cells was quite similar. However, at 4 h after release of the hydroxyurea block (also 4 h postinfection), the grain count over VSV-infected cells was greatly reduced when compared with that of uninfected cells.

Quantitative data were obtained on kinetics of DNA synthesis inhibition in S-phase cultures of synchronized MPC-11 and L cells infected with VSV (MOI $\simeq$ 10) at 1 h before release of the hydroxyurea block. This time period allows the infecting virus to express those putative functions necessary for inhibition of DNA synthesis. Figure 3 shows the progressive decline in incorporation of [^{3}H]thymidine into acid-precipitable DNA of synchronized MPC-11 and L cells infected with VSV. Inhibition of DNA synthesis was evident at 1 h postinfection and continued to an inhibition level of ~90% for MPC-11 cells and ~78% for L cells at 4 h postinfection. VSV infection at multiplicities of 5 and 2 resulted in similar patterns of DNA synthesis inhibition in synchronized and unsynchronized MPC-11 and L cells (data not shown).

Subsequent studies were all performed with MPC-11 cells because they provided more consistent patterns of susceptibility to infection with VSV.

A question that arises is whether VSV infection differentially affects DNA synthesis in cells infected at different stages of the cell growth cycle. Yaoi and Amano (41) reported that chicken embryo cells infected in G_1 phase with VSV did not enter the S phase, but DNA synthesis was not inhibited if cells were infected after the S phase had begun. The availability of the more efficient double thymidine-hydroxyurea block to synchronize cells, rather than the inefficient medium replacement techniques (41), enabled us to reexamine this question by infecting MPC-11 cells at different phases of the cell

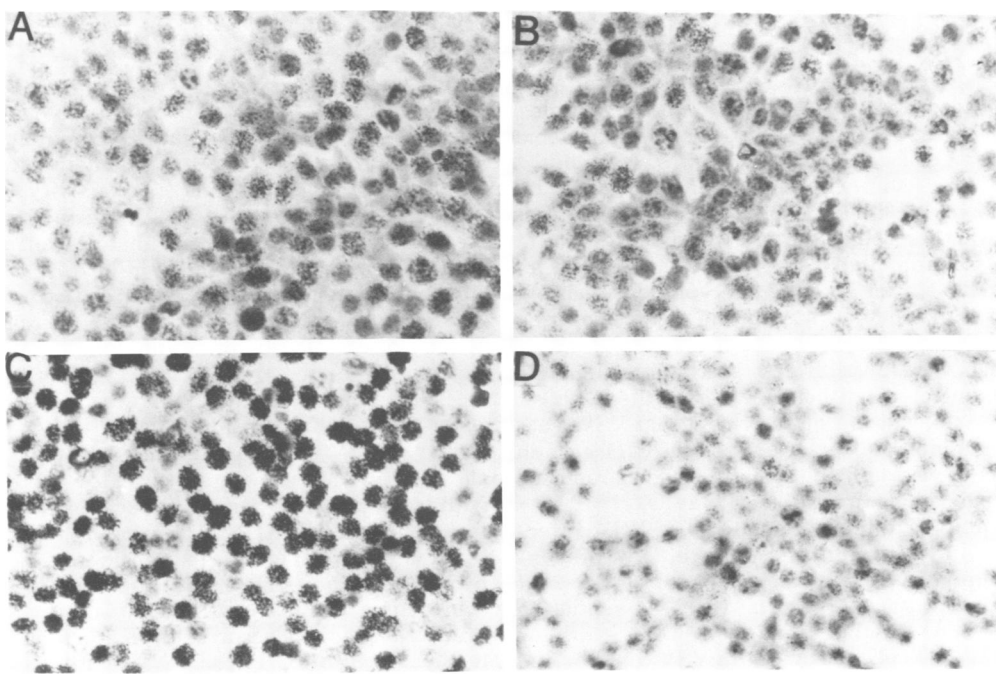

FIG. 2. *Autoradiographic analysis of VSV-infected and uninfected synchronized L cells. Cells were pulse-labeled with 4 μCi of [^{3}H]thymidine per ml for 1 h at various times postinfection, and the amount of incorporation was determined by autoradiographic analysis as described in the text. The relative number of grains per nucleus was determined with the aid of a microscope. Shown are representative fields of mock-infected synchronized L cells at 1 h (A) and 4 h (C) postinfection and VSV-infected synchronized L cells at 1 h (B) and 4 h (D) postinfection. Cells were fixed at the end of the 1-h pulse.*

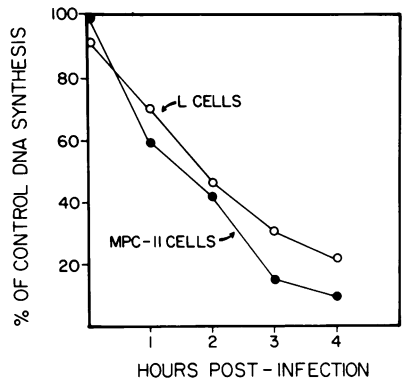

FIG. 3. *Comparative effect of VSV infection on DNA synthesis in thymidine-hydroxyurea synchronized MPC-11 and L cells infected (MOI ≃ 10) 1 h before release from the hydroxyurea block. Results are plotted as the percentage of [^{3}H]thymidine incorporation by control (uninfected) cultures of each type. Acid-precipitable counts were determined by pulse-label for 10 min at 1-h intervals as previously described (see text). Incorporation of [^{3}H]thymidine by uninfected cells ranged from 1 × 10^3 cpm per 5 × 10^5 cells at time zero to 7 × 10^3 cpm per 5 × 10^5 cells at 4 h for mock-infected cells.*

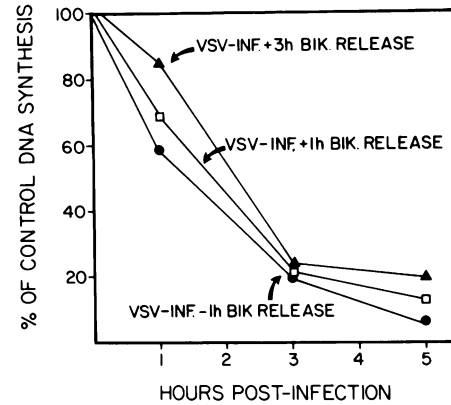

FIG. 4. *Effect of VSV infection on DNA synthesis in synchronized MPC-11 cells relative to the time of release from the hydroxyurea block. Synchronized MPC-11 cells were infected with VSV (MOI ≃ 10) at 1 h before (−1 h), 1 h after (+1 h), or 3 h after (+3 h) release from the hydroxyurea block. At 1-h intervals postinfection, samples were pulsed for 10 min with [^{3}H]thymidine and processed as previously described (see text) for acid-insoluble radioactivity. Results are plotted as the percentage of DNA synthesis in control uninfected cells relative to the time after infection. [^{3}H]thymidine incorporation by uninfected MPC-11 cells ranged from 3 × 10^2 cpm per 6 × 10^5 cells at time zero to 1 × 10^4 cpm per 6 × 10^5 cells by 5 h after mock infection.*

cycle. Cells 95% synchronized by sequential thymidine and hydroxyurea blocks were infected with VSV 1 h before, 1 h after, and 3 h after release from the hydroxyurea block. After a 60-min adsorption of virus at each of these times, cells were pulse-labeled with [^{3}H]thymidine, and acid-precipitable radioactivity was counted.

Figure 4 shows rather similar kinetics of inhibition of MPC-11 cell DNA synthesis regardless of whether cells were infected before or after release from the hydroxyurea block. Cells infected 3 h after entering the S phase were probably somewhat less susceptible to VSV infection than were cells infected 1 h into the S phase, which were possibly less susceptible than the cells infected 1 h before entering the S phase. However, it seems quite evident that VSV readily inhibits DNA synthesis of MPC-11 cells in the S phase, unlike its failure to do so reported for chicken embryo cells (41, 42).

Lack of detectable effects of VSV infection on [^{3}H]thymidine uptake, DNA degradation, DNA-synthesizing enzymes, and DNA chain elongation. It was essential to determine whether inhibition of DNA synthesis of MPC-11 cells infected with VSV could be due to impaired uptake of deoxynucleosides, degradation of preexisting cell DNA, inactivation of cellular DNA-synthesizing enzyme, or premature termination of elongating DNA chains or a combination of these. Four sets of experiments were performed to rule out these possibilities.

We examined the possibility that VSV infection alters host cell membranes, resulting in decreased permeability to [^{3}H]thymidine. Acid-soluble and acid-insoluble radioactivity was measured in uninfected and VSV-infected MPC-11 cells to determine whether thymidine transport was impaired. Table 1 shows that VSV infection did not inhibit uptake of thymidine into the intracellular pool of acid-soluble material, despite inhibition of accumulation of acid-precipitable radioactivity in infected cells.

Experiments were next undertaken to determine the stability of cellular DNA during the period of VSV-induced inhibition of DNA synthesis. MPC-11 cells were prelabeled for 18 h with [^{3}H]thymidine and then mock or VSV infected. Rates of [^{3}H]DNA degradation in prelabeled infected and uninfected cells were assayed by measuring any shift from acid-precipitable to acid-soluble counts. No significant shift in acid-precipitable counts occurred even at 5 h postinfection, when MPC-11 cell DNA synthesis was inhibited by greater than 90% (Table 2).

A previous report (37) showed that RNA polymerase II activity was significantly altered in VSV-infected MPC-11 cells, whereas RNA polymerases I and III were only slightly inhibited. Therefore, the possibility was examined that

TABLE 1. *Comparative uptake of [^{3}H]thymidine into acid-soluble and -insoluble fractions of uninfected and VSV-infected cells at 1 h postinfection*[a]

MPC-11 cells	Time after [^{3}H]thymidine pulse (min)	Radioactivity	
		Acid soluble (cpm × 10^2)	Acid insoluble (cpm × 10^3)
Uninfected	20	3.7	6.0
	40	4.0	15.1
	60	8.4	19.9
Infected	20	3.8	4.9
	40	5.9	8.5
	60	8.6	11.3

[a] After mock infection or infection with VSV (MOI ≃ 10) for 1 h at 37°C, cells were labeled continuously for 60 min with [^{3}H]thymidine. Samples were assayed at the intervals indicated, and the trichloroacetic acid-soluble and -insoluble radioactivity was determined as described in the text.

TABLE 2. *Test for degradation of DNA in host MPC-11 cells infected with VSV*[a]

MPC-11 cells	Time post-infection (h)	Radioactivity	
		Acid soluble (cpm × 10^1)	Acid insoluble (cpm × 10^4)
Uninfected	0	116	2.2
	1	33	1.6
	3	96	1.6
	5	53	1.5
Infected	0	94	2.6
	1	32	1.6
	3	104	1.7
	5	44	1.4

[a] MPC-11 cells were prelabeled with 0.05 μCi of [^{3}H]thymidine for 18 h and then VSV infected or mock infected. After adsorption, cells were harvested at indicated intervals, and the radioactivity was determined as described in the text.

VSV infection might inhibit or lead to the reduction of essential enzymatic activities associated with DNA synthesis. Crude extracts were selectively prepared from VSV-infected and uninfected MPC-11 cells and assayed for thymidine kinase and DNA polymerase activities, as described above. Results (Table 3) revealed no significant reduction in either thymidine kinase or DNA polymerase activity at 4 h postinfection. At this time, cellular DNA synthesis was 90% inhibited. Although these enzymes are but a few of those involved in the DNA replicative process, they are representative indicators of enzymatic activities present in infected cells. However, the possibility that some other key enzymatic activity may be inhibited remains open. Although in vitro DNA polymerase activities from infected and uninfected cells were similar, the reaction catalyzed in vitro may have primarily measured DNA repair or elongation. Any effects of VSV infection on the initiation of DNA synthesis remains speculative.

TABLE 3. *Lack of significant effect on activities of selected enzymes involved in DNA synthesis in VSV- or mock-infected MPC-11 cells*

Enzyme (substrate)	Amt of product formed[a]	
	Uninfected	VSV-infected (MOI ≃ 10)
Thymidine kinase[b] ([^{3}H]-deoxyribosylthymine)	33.7	28.4
DNA polymerase[b] ([^{3}H]-dTTP)	22	19

[a] Data are given as the counts per minute per microgram of extract at 4 h postinfection.

[b] Assays for the respective enzymes were performed as described in the text.

The inability at this time to analyze directly the effects of VSV infection on the initiation of DNA synthesis prompted us to examine more closely the ability of VSV infection to inhibit DNA chain elongation. Suspension MPC-11 cells were either mock infected or VSV infected, as described above. At 1 h after infection, cells were pulsed with [^{3}H]thymidine for 60 min; at 1 h postinfection, the inhibition of DNA synthesis was definitive (Fig. 1, 3, and 4), but [^{3}H]thymidine incorporation into DNA was sufficient for DNA analysis by gradient centrifugation after a 4-h chase. After the 4-h chase, cells were layered onto alkaline sucrose gradients overnight at 4°C to allow gentle lysis of the cells.

Figure 5 shows the comparative analyses by alkaline sucrose gradients of radioactive DNA in VSV-infected and uninfected control cells. Although detectable [^{3}H]thymidine incorporation into DNA from VSV-infected cells was reduced compared with that in the uninfected control cells, most of the label in infected cells chased into DNA lengths similar to those in control cells. These results indicate that although incorporation of [^{3}H]thymidine into DNA is inhibited by VSV infection, the process of DNA chain elongation of already initiated DNA can continue in infected cells.

Comparative inhibition by VSV of cellular DNA, RNA, and protein syntheses. The ability of VSV to shut off host RNA and protein syntheses in unsynchronized cells is well established (7, 8, 24–26), but the patterns of inhibition of macromolecular synthesis have not been examined with highly synchronized cells. To this

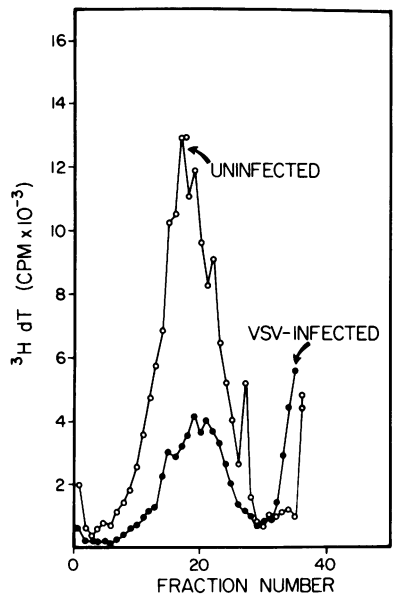

FIG. 5. *Comparative rate zonal analysis of ^{3}H-labeled DNA in VSV-infected and uninfected MPC-11 cells analyzed in alkaline sucrose gradients. Cells were pulse-labeled for 60 min with 5 μCi of [^{3}H]-deoxyribosylthymine per ml and then chased for 4 h. The procedure for analysis on alkaline sucrose gradients is provided in the text.*

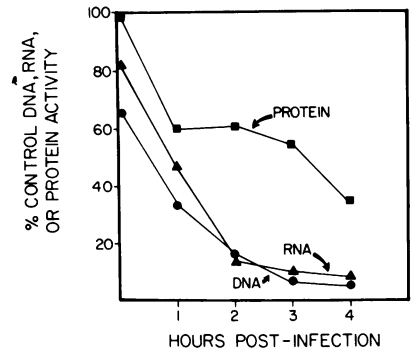

FIG. 6. *Comparative inhibition of DNA, RNA, and protein syntheses in synchronized MPC-11 cells infected with VSV (MOI ≃ 10). Cells were infected as described in the legend to Fig. 1 and pulsed for 10 min at 1-h intervals with [^{3}H]thymidine (2 μCi/ml), ^{3}H-labeled L-amino acids (5 μCi/ml), or [^{3}H]uridine (2 μCi/ml); radioactivity was determined as described in the text. ^{3}H-labeled amino acid incorporation into uninfected cell protein remained constant (1 × 10^3 cpm per 4 × 10^5 cells) relative to the range of [^{3}H]thymidine incorporation into cellular DNA (3 × 10^2 cpm at time zero to 1 × 10^4 cpm per 4 × 10^5 cells by 4 h after mock infection). [^{3}H]uridine incorporation into uninfected cells ranged from 1 × 10^3 to 5 × 10^3 cpm per 4 × 10^5 cells.*

end, MPC-11 cells were synchronized by successive thymidine and hydroxyurea blocks (see above) and infected with VSV by a 60-min adsorption 1 h before release of the hydroxyurea block. Rates of DNA, RNA, and protein syntheses were determined by 10-min pulses of [^{3}H]thymidine, [^{3}H]uridine, or a ^{3}H-labeled amino acid mixture at 1-h intervals after VSV infection or mock infection of control cells.

Syntheses of cellular DNA and RNA in synchronized MPC-11 cells were severely compromised by VSV infection than was cellular protein synthesis (Fig. 6). Cellular DNA and RNA syntheses were rapidly shut off to almost a maximal extent by 2 h. On the other hand, although protein synthesis declined rapidly during 1 h, it then leveled off or declined slowly to a final level which was 40% that of the control by 4 h. At 4 h postinfection, viral protein synthesis represents less than 15% of the total protein synthesis in VSV-infected cells (28). Quite clearly, cellular nucleic acid synthesis in synchronized MPC-11 cells is more susceptible to VSV infection than is host protein synthesis.

Requirement for VSV transcription to shut off cell DNA synthesis tested with temperature-sensitive mutant and DI particles. Genetic evidence supports the hypothesis that VSV transcription is required to shut off syntheses of cell protein (25, 28) and RNA (38, 40). Group I temperature-sensitive mutants restricted in transcription (20) and DI particles derived from the 5′ end of the VSV genome (2, 13) fail to inhibit cellular RNA synthesis (34) or to kill cells (24), but 3′-end DI particles which retain primary transcription activity (21) still function in cell killing (26). To determine whether functional VSV transcription, necessary for shutting off cell RNA and protein syntheses, also applies to inhibition of cell DNA synthesis, we tested the effects of a temperature-sensitive mutant in complementation group I and DI particles derived from the 5′ and 3′ ends of the VSV genome.

In Fig. 7, the capacity of the transcription mutant *ts*G114(I) and wild-type VSV to shut off DNA synthesis in MPC-11 cells at temperatures restrictive (39°C) and permissive (31°C) for the mutant are compared. As noted, *ts*G114(I) did not significantly affect host DNA synthesis at 39°C but did so quite efficiently at 31°C, as did the wild-type VSV at both temperatures and at equivalent MOIs. These data appear to support the hypothesis that transcription of VSV is required to inhibit cell DNA synthesis. It has not been possible to test whether the inhibitory product is viral RNA or protein because all previous attempts to block protein synthesis by

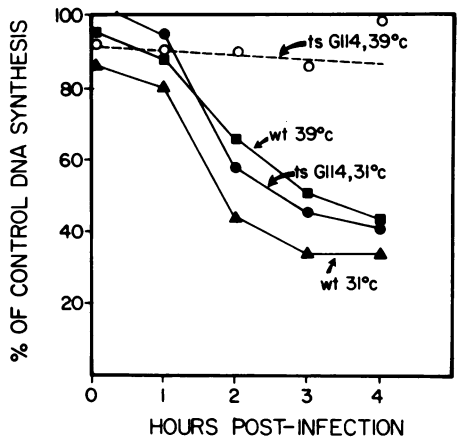

FIG. 7. *Comparative effects on DNA synthesis in MPC-11 cells infected with VSV wild type (wt) or mutant tsG114(I) at permissive (31°C) or restrictive (39°C) temperatures (MOI ≃ 10). The virus was allowed to adsorb for 1 h at room temperature. Replicate cultures were then incubated at 31 and 39°C and pulsed with [^{3}H]thymidine for 10 min at 1-h intervals. Trichloroacetic acid-precipitable counts were measured, and the average activity from duplicate samples was plotted as the percentage of control cell activity. The range of [^{3}H]thymidine incorporation by uninfected cells was 2 × 10^3 to 4 × 10^3 cpm per 2 × 10^5 cells.*

inhibitors such as cycloheximide or puromycin have also inhibited cellular nucleic acid synthesis (38).

These hypotheses were tested further by comparing the DNA inhibitory action of two DI particles, VSV DI-011, derived from the 5′ end of the genome and ~100% self-annealing (31), and VSV DI-HR-LT, derived from the 3′ half of the VSV genome (13, 21). Unlike DI-011, the genome of DI-HR-LT can undergo primary transcription, and the mRNA synthesized can be translated into functional VSV proteins N, NS, M, and G, but no L mRNA or protein is synthesized because ~6,000 nucleotides are deleted from the 5′ end of the genome (21). MPC-11 cells were infected with these DI viruses at multiplicities equivalent to those of wild-type VSV, based on protein determinations (23) of purified viruses and taking into consideration that DI-011 is about one-fourth the size and DI-HR-LT is about one-half the size of wild-type VSV.

In Fig. 8, the effect of purified preparations of DI-011, DI-HR-LT, and wild-type VSV on DNA synthesis of MPC-11 cells infected at equivalent multiplicities is compared. DI-011 did not inhibit cell DNA synthesis to any significant extent (78% that of uninfected cells by 4 h postinfection); this slight inhibition of cell DNA synthesis could be due to undetectable contamination of DI-011 with wild-type full-length infectious B particles. DI-HR-LT inhibited cell DNA synthesis almost as well as did wild-type B virus for the first 2 postinfection, but inhibition then tapered off to ~57% by 4 h, compared with >90% inhibition by wild-type B virus at 4 h. This latter observation was not surprising since DI-HR-LT is unable to synthesize new L protein, which is required for secondary transcription and replication (30, 33) and which may well be required for late cellular effects of VSV infection.

Taken together, these restricted effects on cell DNA synthesis by the temperature-sensitive transcriptase mutant, the nontranscribing DI-011 virus, and DI-HR-LT restricted in secondary transcription strongly support the hypothesis that inhibition of cellular DNA synthesis requires at least primary transcription of VSV.

UV inactivation of the VSV genome region that codes for inhibition of cellular DNA synthesis. The apparent sequential transcription from the 3′ end of the VSV genome provides a basis for mapping by preferential UV inactivation of the linear viral cistrons in the following order: 3′-leader-N-NS-M-G-L-5′ (1, 6, 10, 32). One or more of these genome regions must be responsible for inhibiting cellular RNA (38, 40) and protein (27) syntheses. Previous studies revealed that the capacity of VSV to shut off RNA synthesis in MPC-11 cells appears

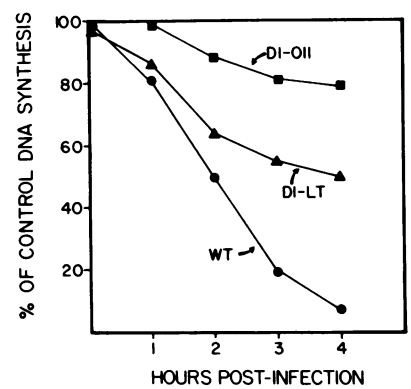

FIG. 8. *Inhibition of MPC-11 cellular DNA synthesis by purified infectious wild type (WT) and purified DI VSV particles (DI-011 and DI-LT). Purified viral preparations were prepared, and protein determinations were made. The amount of virus added was calculated on the basis of the protein equivalent of wild-type virus needed to yield an MOI of 10. The protein concentrations of DI-011 and DI-LT were adjusted for the protein concentrations per particle. Cells were infected and pulsed for 10 min at 1-h intervals thereafter as previously described (see text). Incorporation of [^{3}H]thymidine by uninfected cells ranged from 5 × 10^3 to 7 × 10^3 cpm per 4 × 10^5 cells.*

to be extremely resistant to UV irradiation; UV doses sufficient to inactivate transcription of all VSV mRNA did not impair VSV inhibition of MPC-11 cellular RNA synthesis, but UV doses ostensibly high enough (72,000 ergs/mm^2) to inactivate the leader sequence did (35). Wu and Lucas-Lenard (40) subsequently reported a smaller UV target size (11 to 17% of the genome, equivalent to the N-protein cistron) for inactivation of the VSV inhibition of L cell RNA synthesis. Recent studies in our laboratory indicate that UV inactivation of the VSV inhibitor(s) of cellular RNA synthesis may vary among different cell types and phases of the cell cycle (Robertson and Wagner, in press). Lower doses of UV irradiation inactivate the VSV genes, probably N or NS, responsible for VSV inhibition of protein synthesis in L cells (27), a finding confirmed in our laboratory (J. R. Thomas and A. R. Carroll, unpublished data). Only very early and not very conclusive data have been reported on UV inactivation of the VSV inhibition of cellular DNA synthesis (41, 42). It was of interest, therefore, to determine the UV target size for inactivation of the VSV genome segment responsible for inhibition of DNA synthesis of the MPC-11 cells used in these experiments.

As previously described (35), stock preparations of VSV (10^9 PFU/ml) were diluted 1:10 in 1 ml of Dulbecco-modified phosphate-buffered saline and dispensed into 60-mm tissue culture dishes, which were placed at a distance of 10 cm from the UV light source and exposed to various doses of radiation at a wavelength of 254 nm at a dose rate of 85 ergs/mm^2 per s. After irradiation, the virus preparations were used to infect MPC-11 cells at an MOI of 10, based on the original PFU titer of the virus stocks. The virus was adsorbed to the cells for 1 h at room temperature. Cells were pelleted, resuspended in warm medium, and incubated at 37°C. At 4 h after infection, duplicate samples of cells infected with unirradiated virus or with virus irradiated at different UV doses were labeled for 10 min with [^{3}H]thymidine and assayed for acid-precipitable radioactivity.

In Fig. 9, the effect of increasing doses of UV irradiation on the relative ability of VSV to inhibit DNA synthesis of MPC-11 cells 4 h postinfection is shown. No significant loss of viral activity was detected until a dose of 20,000 ergs/mm^2 was administered to VSV. The capacity of VSV to inhibit DNA synthesis of MPC-11 cells was extremely resistant to UV inactivation. Even doses greater than 80,000 ergs/mm^2 did not completely abolish the ability of VSV to shut off DNA synthesis (data not shown).

As previously reported by Weck et al. (35), the 37% (1/*e*) survival rates (expressed as the dose of UV irradiation at which 37% of a maximal VSV activity could be detected) were 104 ergs/mm^2 for infectivity, 1,050 ergs/mm^2 for VSV in vitro transcription, 170 ergs/mm^2 for in vivo viral mRNA synthesis, 380 ergs/mm^2 for in vivo N-protein synthesis, and 72,000 ergs/mm^2 for shutoff of MPC-11 cell RNA synthesis. As determined from the data shown in Fig. 9, the comparative value of the 37% (1/*e*) survival rate for inhibition of MPC-11 DNA synthesis was 45,000 ergs/mm^2. When the experiment of Weck et al. (35) was repeated to determine the 37% (1/*e*) survival rate for UV inactivation of VSV inhibition of MPC-11 RNA synthesis, we arrived at a value of 52,000 ergs/mm^2 (data not shown). Our data clearly indicate the similarity in UV target size of the VSV genome region responsible for inhibition of MPC-11 cell RNA and DNA syntheses and the vast difference in the target size for other VSV functions.

DISCUSSION

Research on viral cytopathogenicity has not progressed much beyond the stage of phenomenology. The molecular mechanisms by which virulent viruses inhibit macromolecular synthesis of host cells have been under investigation for many years without great success. Definitive data on cellular targets of viral effects are not generally available, except perhaps for inhibition of cellular protein synthesis caused by picornaviruses, the target for which appears to be spe-

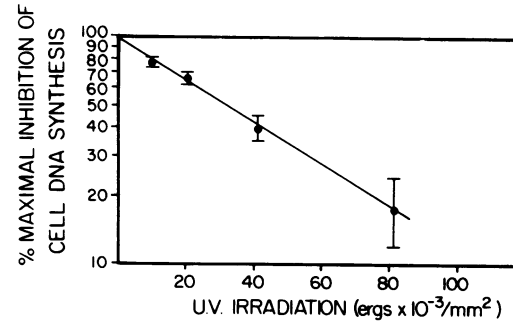

FIG. 9. *Effect of varying doses of UV irradiation on the ability of VSV to inhibit mouse myeloma cellular DNA synthesis at 4 h postinfection. VSV was irradiated as described in the text. Cells were infected at an MOI of 10 and incubated at 37°C. At 4 h postinfection, duplicate samples were pulsed for 10 min with 1 ml of medium containing 2 μCi of [^{3}H]thymidine per ml; the amount of acid-insoluble material was determined as previously described (see text). Data are plotted as the percentage of maximal inhibition; vertical bars represent the range in values from three experiments at each UV dose. Incorporation of [^{3}H]thymidine by uninfected cells averaged 2 × 10^4 cpm per 3 × 10^5 cells.*

cific initiation factors of translation (17). Most virulent viruses appear to shut off both protein and nucleic acid syntheses in the same host cell, but we are still uncertain as to whether these are separate phenomena. In the case of positive-strand picornaviruses, it seems likely that inhibition of cellular RNA synthesis results from reduction in active units of polymerase II that initiate RNA synthesis rather than from inhibition of elongation of pre-initiated RNA chains (4).

The highly virulent negative-strand VSV has been studied in depth for its capacity to inhibit cellular protein (25–27), RNA (35–38, 40), and, to a much lesser extent, DNA (40, 41) syntheses. There is conflicting evidence as to whether independent VSV functions are responsible for inhibition of cellular RNA and protein syntheses. Although both protein and RNA syntheses are shut off more or less simultaneously and both require viral transcription (25, 38), these two viral functions appear to be differentially inactivated by UV irradiation (27, 35; Thomas and Carroll, unpublished data).

The least studied of the cellular responses to infection with VSV is its effects on cellular DNA synthesis. Although the capacity of VSV to shut off cellular DNA synthesis could be secondary to its effect on RNA synthesis, this seems unlikely, based on the rapid and almost identical rates for inhibition of RNA and DNA syntheses in the same cells (Fig. 6). By the same token, inhibition of cellular DNA synthesis by VSV infection does not appear to be secondary to simultaneous but lesser inhibition of cellular protein synthesis (Fig. 6). Therefore, it is of considerable potential interest that VSV and many other RNA viruses can shut off nuclear DNA synthesis despite a replicative cycle confined to the cytoplasm. Among the cytoplasmic RNA viruses that effectively shut off DNA synthesis are reovirus (12), picornaviruses, such as poliovirus (7, 18) and mengovirus (4, 12), Newcastle disease paramyxovirus (11, 12, 16), and togaviruses, such as Sindbis virus (5) and western equine encephalitis virus (22). Although the mechanisms by which these RNA viruses inhibit DNA synthesis is not understood, significant differences in their ability to inhibit cellular replication have been reported. For example, many of these viruses inhibit RNA and protein syntheses soon after infection (4, 5, 8, 22); however, an exception is reovirus, which inhibits cellular DNA synthesis without detectable inhibition of cellular protein synthesis (12). Moreover, an inhibitory factor for cellular DNA polymerase has been reported to be induced in BHK cells infected with western equine encephalitis virus (22).

There are at last three major unanswered questions concerning the mechanisms by which VSV and other RNA viruses inhibit macromolecular synthesis in host cells. (i) Are there multiple targets in a single cell, or are the multiple effects simply due to a cascading phenomenon? The available data are far from definitive, but they favor the multiple-target hypothesis. (ii) Do different host cells vary widely in the susceptibility of their targets to the action of viral inhibitory factors? There is certainly some evidence that cells of varying origin differ in their responses, but more comparative studies with better controls are required to be certain that the different responses among cell types are significant. (iii) Perhaps the most important question still unanswered is whether multiple viral functions are required for each cellular effect or whether a single viral function (product) is responsible for inhibiting cellular RNA, protein, and DNA syntheses. It is likely that each virus or virus group has a different strategy; it would seem improbable, for example, that positive-strand poliovirus would inhibit cell macromolecular synthesis by a mechanism similar to that of negative-strand VSV.

The studies reported here and in previous papers (35–38) strongly suggest that the same VSV function shuts off both cellular RNA and DNA syntheses. It appears that the kinetics of VSV inhibition of cellular RNA and DNA syntheses are quite similar, as are the requirements for VSV transcription indicated by the results of studies with temperature-sensitive mutants and DI particles. The differences observed in the degree of susceptibility of L-cell RNA synthesis (36) and DNA synthesis reported here are probably not very significant and could be due to multiplicity-dependent effects. Major criteria for a single VSV function being responsible for shutting off both RNA and DNA syntheses are UV inactivation studies that reveal a similar target size for the RNA inhibitor (35) and the DNA inhibition reported here. Our previous UV inactivation studies (35) led us to hypothesize that the products of the structural genes (such as N and NS) of VSV were not likely to be the inhibitors of RNA synthesis in MPC-11 cells and that only the VSV genome region coding for the 48-nucleotide leader (9, 10) was the logical candidate. UV inactivation of the VSV inhibitor of DNA synthesis presented here also would seem to implicate a viral product the size of the leader. We are somewhat at a loss to explain the UV inactivation data of Wu and Lucas-Lenard (40), who interpreted their results as evidence for requiring unimpaired functions of the VSV N or NS genes or both for shutting off RNA synthesis in L cells. The discrepancy in our results could

be due to differences in susceptibility of MPC-11 cells in our experiments as compared with L cells in theirs, but experiments performed in our laboratory by Thomas and Carroll (unpublished data) indicated a similar VSV target size for the RNA synthesis inhibition of L cells and MPC-11 cells. It should also be noted that Wu and Lucas-Lenard (40) calculated a UV dose of 52.2 ergs/mm^2 for 37% survival of VSV infectivity as compared with 104 ergs/mm^2 in our experiments (35). Moreover, they conducted their UV inactivation studies at a VSV input multiplicity of 1, which in our experience only reduces RNA synthesis in L cells by 60% at 4 h postinfection (36).

Available evidence indicates that VSV inhibition of cellular protein synthesis is different from VSV inhibition of cellular RNA and DNA syntheses. As shown here, the kinetics of the inhibitory reactions are sufficiently different to suggest that the two events are unrelated. Admittedly, genetic studies reveal that VSV transcription is required for shutting off cellular protein (25), RNA (38), and DNA syntheses (this report), but different transcriptional or translational products could be responsible for the two cellular events. The data of Marvaldi et al. (27) clearly indicate that UV inactivation of the VSV N or NS genes or both abolishes the capacity of VSV to inhibit cell protein synthesis in L cells; in our laboratory, Thomas and Carroll have confirmed the results that the UV target size of the L-cell protein inhibitor is much larger than that of the L-cell RNA inhibitor (unpublished data).

Studies reported here also indicate, at variance with experiments reported by Yaoi et al. (42), that VSV inhibition of cellular DNA synthesis does not vary significantly with different phases of the cell cycle of synchronized cells. Moreover, autoradiographic studies showed that individual cells do not vary greatly in their response to VSV since thymidine grain counts were uniformly reduced in all cells rather than in a selected cell subset (Fig. 2). We have not yet pinpointed the cellular target for VSV inhibition of DNA synthesis, but studies reported here, unlike studies of the effect of VSV on RNA synthesis in chicken embryo cells (15), rule out of a viral effect on nucleoside transport, DNA degradation, inactivation of DNA polymerase and thymidine kinase, or premature termination of already initiated DNA chains. By exclusion, we hypothesize that VSV inhibits DNA synthesis by blocking initiation of DNA replication, perhaps in a manner similar to its inhibition of RNA transcription (37). The ambiguities inherent in the in vitro DNA polymerase assay make it difficult to rule out conclusively cellular enzymes as targets for VSV inhibition of DNA replication. Further studies are necessary to test the hypothesis that a VSV product inhibits initiation of both RNA transcription and DNA replication.

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