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Inhibition of Vesicular Stomatitis Viral mRNA Synthesis by Interferons

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Interferon (IFN) treatment inhibited the replication of vesicular stomatitis virus (VSV) in human GM2767 and mouse JLSV-11 cells. The replication of this virus in either human RD-114 or mouse A402 cells was insensitive to IFN treatment. We analyzed various steps in the VSV life cycle as they occurred under different conditions of IFN treatment to identify the point(s) at which IFN was exerting its inhibitory effect. IFN treatment led to strong inhibition of viral protein synthesis and accumulation of viral RNA in both lines of IFN-sensitive cells. No such effect was observed in the IFN-resistant cells. Using a temperature-sensitive mutant (*ts*G41) and wild-type VSV that were not undergoing protein synthesis, we determined that the major site of action of IFN against VSV replication in JLSV-11 and GM2767 cells was at the level of primary viral transcription. The accumulation of primary viral transcripts was strongly inhibited in these cells by IFN treatment. This effect was not a consequence of any effect of IFN on virus entry and uncoating. Thus, it appears that IFN exerts a direct effect on the VSV transcriptional process in GM2767 and JLSV-11 cells.

Although the antiviral actions of interferons (IFNs) have been studied extensively, the detailed mechanism by which IFN inhibits the replication of a virus remains unclear (13, 28). It is apparent that IFN can prevent virus replication by interfering with various steps in viral life cycles, for example, viral entry, viral macromolecular synthesis, and viral morphogenesis. Which, if any, of these impairments is the major one depends primarily on the virus in question. The particular host cell can also contribute to these effects.

The actions of IFNs are mediated by the products of cellular genes whose expression is affected by IFN treatment. The specific roles of these gene products in the cellular response to IFN are poorly understood. The possibility exists that the replication of a family of viruses with similar replicative strategies may be affected by a product of the same IFN-inducible gene. Only one such IFN-inducible gene has been identified, the Mx gene product of mice, which specifically inhibits influenza virus replication (33).

Vesicular stomatitis virus (VSV) is the prototype rhabdovirus whose replication is inhibited by IFN in many cells. The IFN-inducible gene product(s) responsible for the inhibition of VSV replication has not been identified. In most cases the major or exclusive site of action of IFN against VSV is at the level of viral RNA and protein synthesis, although defects in viral entry and morphogenesis have been demonstrated in some cell lines (1, 3, 5, 14–20, 24, 32, 34–36, 38). Since VSV protein and RNA synthesis are interdependent, a defect in either process could be manifested as inhibition of both (2).

Earlier attempts to identify the primary site of action of IFN against VSV have produced conflicting results. While some investigators concluded that the primary site of IFN action against VSV is at the level of primary transcription (15–17), others concluded that it is at the level of viral protein synthesis (1, 3, 24, 32). It was also suggested that the mechanism may be different in different cell lines (34). These early studies were done with impure IFN preparations, since pure IFN was not available at that time. Since VSV cDNA clones were also unavailable, the methods of measuring viral RNA synthesis were imprecise. These measurements required the use of inhibitors of cellular RNA synthesis such as actinomycin D. Recently, Samuel and his colleagues have reinvestigated the mechanism of inhibition of VSV replication by IFN in human U amnion cells (18–20, 35, 36). They used pure IFN preparations and better methods of quantitating VSV RNA levels. They did not observe any effect of IFN-αA on early or late stages of VSV replication. Inhibition of viral protein synthesis in the absence of any effect of IFN on primary viral transcription was observed. They concluded that IFN-αA treatment of these cells leads to a defect in the cellular translational machinery. This effect may be specific for IFN-α, since IFN-γ treatment of the same cells reduces virus yield about 100-fold without pronounced inhibition of viral protein synthesis.

In contrast to these findings, we report here that in IFN-αA-treated human GM2767 fibroblasts and in IFN-α/β-treated mouse JLSV-11 cells VSV primary transcription was strongly inhibited. Such inhibition of VSV primary transcription was not observed in IFN-treated human RD-114 cells or in IFN-treated mouse A402 cells, in which IFN treatment did not reduce VSV yields appreciably.

(Some of the results reported here were presented at the Annual Meeting of the American Society for Virology at Albuquerque, N. Mex., in 1985 and at a Schering-UCLA Symposium at Steamboat Springs, Colo., in 1986. Part of this work is included in the doctoral thesis of Linda S. Belkowski, Cornell University, New York, N.Y., 1986.)

MATERIALS AND METHODS

Cells and viruses. GM2767, a human fibroblast line trisomic for chromosome 21, was purchased from the Human Genetic Mutant Cell Repository, Camden, N.J. Human RD-114 rhabdomyosarcoma cells were provided by B. K. Pal, California State Polytechnic University, Pomona. These human cells were maintained in minimal essential medium

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(MEM) containing 10% fetal calf serum (7). Mouse JLSV-11 cells were a gift of P. Besmer, Sloan-Kettering Institute (29). A402, an IFN-resistant variant of JLSV-11, was isolated in our laboratory by screening clonal derivatives of this line for IFN sensitivity (unpublished results). These lines were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Wild-type (WT) VSV, Indiana strain, was propagated and quantitated in mouse L929 cells. Virus stocks had an average titer of 8×10^8 PFU/ml. The temperature-sensitive (*ts*) mutant of VSV, *ts*G41, was provided by Philip Marcus, University of Connecticut, Storrs. Like the WT virus, it was propagated in L929 cells, except that virus propagation and titration were carried out at 30°C (the permissive temperature). The virus was also quantitated at 39.5°C (the nonpermissive temperature); this titer was always at least 10^3-fold lower than the corresponding titer at 30°C. The temperature-sensitive lesion in this virus has been localized to the viral N gene (23).

IFNs. Human IFN-αA was provided by S. Pestka of the Roche Institute for Molecular Biology. Human IFN-γ was obtained from Genentech, San Francisco, Calif. Mouse IFN-α/β was supplied by Peter Lengyel, Yale University, New Haven, Conn.

Antiserum. Polyclonal rabbit anti-VSV antiserum was the generous gift of Philip Marcus.

cDNA clones. The plasmids pN4, pNS319, pG1, and pM309 carrying inserts of cDNAs for the VSV N, NS, G, and M proteins, respectively, were provided by J. Rose, Salk Institute, La Jolla, Calif. (6, 26). LH661, a partial cDNA clone of VSV L protein (27), was provided by Manfred Schubert, Bethesda, Md. These cDNAs were propagated in *Escherichia coli* RR1 and radiolabeled by nick translation (25).

Measurement of infectious virus yields. The effect of IFN on infectious VSV yield was measured on the indicated cells with confluent monolayers in 6-well tissue culture dishes. Prior to challenge with VSV, the cells were treated with the indicated doses of IFN (IFN-αA for human cells or IFN-α/β for mouse cells). Cells were then infected with VSV at a multiplicity of 10 PFU/cell. At 18 h postinfection the plates were freeze-thawed twice, and VSV in the medium was quantitated by a plaque-forming assay on mouse L929 cells (29).

Analysis of total viral protein synthesis. Confluent monolayers of cells were treated with IFN as described above. Viral protein synthesis was measured by pulse labeling cells from 3 to 3.5 h postinfection with methionine-free MEM supplemented with 50 μCi of [^{35}S]methionine per ml. After radiolabeling, the monolayers were washed twice with 35 mM Tris (pH 7.5)–146 mM NaCl. Cells were lysed by the addition of 0.5 ml of extraction buffer (10 mM Tris, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.2% Triton X-100) to the dishes. After approximately 2 min on ice (for lysis) the extracts were centrifuged for 10 min at 1,500 × *g* to remove nuclei. The supernatants were analyzed by polyacrylamide gel electrophoresis.

Primary viral translation. Primary translation was analyzed with an anti-VSV antibody and *ts*G41 VSV. Cells (either control or IFN-treated) were infected with *ts*G41 at a multiplicity of infection (MOI) of 10. The infected cells were maintained at 39.5°C (the nonpermissive temperature) for 4 h. The cells were then pulse-labeled with methionine-free MEM for 0.5 h, and cell extracts were prepared. Cell extracts from equal numbers of cells (200 μl final volume) were immunoprecipitated by the addition of 4 μl of anti-VSV serum at 4°C for 16 h; 20 μl of a 1:1 suspension of protein A-Sepharose beads (Pharmacia) in extraction buffer was added, and incubation was continued for 3 h at 4°C. The beads were washed four times with washing buffer, and the bound viral polypeptides were analyzed by electrophoresis.

Analysis of VSV-related RNA. For analysis of total VSV transcripts, cytoplasmic RNA (12) was prepared from VSV-infected cells at 5 h postinfection. WT VSV-infected cultures were maintained at 37°C until harvest, while those infected with the *ts* mutant were maintained at 30°C. To analyze primary transcripts of WT VSV, infection and incubation (3 h) were carried out in the presence of 100 μM anisomycin. Viral RNA synthesis in *ts*G41-infected cultures was restricted to primary transcription by keeping the cultures at 39.5°C during the 3-h incubation.

For RNA dot blotting, 25 μg of each RNA (in water) was evaporated to dryness. The RNA was suspended in 20 μl of H_2O, and 12 μl of 20 × SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 8 μl of 37% formaldehyde were added. The RNA was denatured at 60°C for 15 min and cooled to room temperature. Twofold serial dilutions of the RNA were prepared in 15× SSC and dotted onto nitrocellulose. The blot was air-dried and baked in vacuo at 80°C for 2 h. The dot blots were prehybridized, hybridized with ^{32}P-labeled VSV cDNA clones, and washed as previously described (12). Blots were autoradiographed with Kodak XAR-5 film and Du Pont Cronex intensifying screens.

For Northern analysis, RNA samples were denatured and electrophoresed in 1.5% agarose gels in the presence of 2.2 M formaldehyde (12). After electrophoresis the gel was rinsed for 10 min in 6× SSC prior to capillary transfer to a nitrocellulose sheet (preequilibrated in 20× SSC) with 20× SSC as the transfer buffer. Northern blots were prehybridized, hybridized, washed, and autoradiographed under the same conditions as dot blots. Individual viral RNA bands were quantitated by densitometric scanning.

Analysis of initial events in the VSV life cycle. Preparations of [^{35}S]VSV were used to analyze the processes of VSV binding, internalization, and uncoating in JLVS-11 and GM2767 cells. Virus was prepared from cells labeled with [^{35}S]methionine during the infectious cycle and purified by banding on sucrose gradients (18). Virus titrations were carried out as for unlabeled virus.

Binding was assayed by infecting monolayer cultures with ^{35}S-VSV at an MOI of 10. In each case an equal number of IFN-treated and untreated cells were infected. After adsorption for 1 h at 37°C the monolayers were washed three times with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.2)–150 mM NaCl (HEPES-saline) and lysed in HEPES-saline containing 0.5% sodium dodecyl sulfate. The radioactivity in each lysate was determined with a liquid scintillation spectrometer. Approximately 10% of the input radioactivity was bound under these conditions.

Virus internalization was determined by binding the labeled virons as described above. After removal of the inoculum and washing with HEPES-saline, fresh medium was added. The cells were incubated at 37°C for 1.5 h. Medium was removed, and the monolayers were washed twice with HEPES-saline. Any virus remaining on the cell surface was removed by incubation with 0.25% trypsin–0.25% EDTA (7 min at room temperature). Trypsinization was stopped by the addition of HEPES-saline containing 10% serum. The cells were harvested by centrifugation and washed twice with HEPES-saline plus 10% serum. The pellet was then solubilized with HEPES-saline containing 0.5% sodium dodecyl sulfate, and the radioactivity was determined by liquid scintillation counting.

Uncoating of VSV was analyzed by infecting control and IFN-treated cell cultures with ^{35}S-VSV at an MOI of 25 to increase the amount of cell-associated radioactivity. At this MOI IFN pretreatment still inhibited VSV replication by at least 10^4-fold (unpublished observations). At different times after infection cells were harvested and homogenized. The extracts were layered onto sucrose gradients, and nucleocapsids and virions were separated by centrifugation (18). The gradients were fractionated, and the radioactivity in the virion and nucleocapsid peaks was determined. The relative radioactivity in the nucleocapsid versus virion peak at 2 h postinfection was used as an index of uncoating.

RESULTS

Effects of IFN on VSV production. The effect of IFN treatment on infectious VSV production is shown in Fig. 1. Pairs of human and mouse cells were used for this analysis. GM2767 cells were extremely sensitive to the inhibition of VSV replication by IFN. Treatment of these cells with 200 U of IFN-αA per ml reduced infectious VSV production about 10^6-fold. As little as 1 U of IFN-αA per ml led to 10^3-fold reduction in virus production in these cells. In contrast, RD-114, a human fibroblastic sarcoma line, was insensitive to inhibition of VSV replication by IFN.

Similar differential results were obtained with the pair of mouse cell lines (Fig. 1B). Treatment of JLSV-11, a mouse bone marrow line, with IFN led to a reduction in infectious VSV yield. A derivative of this line, A402, was resistant to IFN in this regard. Within each pair (human and mouse) of cell lines tested, virus yields in untreated cells were similar. The absolute degree of inhibition in GM2767 cells (10^6-fold), however, was greater than that seen in the sensitive mouse line JLSV-11 (10^4-fold).

VSV protein synthesis in IFN-treated cells. Our goal was to determine the point(s) in the VSV life cycle at which IFN was exerting its inhibitory effects. With the previously described cell lines, a step-by-step analysis of events in the VSV life cycle was carried out.

The rate of VSV protein synthesis as a function of IFN treatment was analyzed (Fig. 2 and 3). Cells were pretreated with the indicated doses of IFN and challenged with VSV. At 3.5 h postinfection the cells were pulse-labeled with [^{35}S]methionine for 0.5 h. The labeled polypeptides were

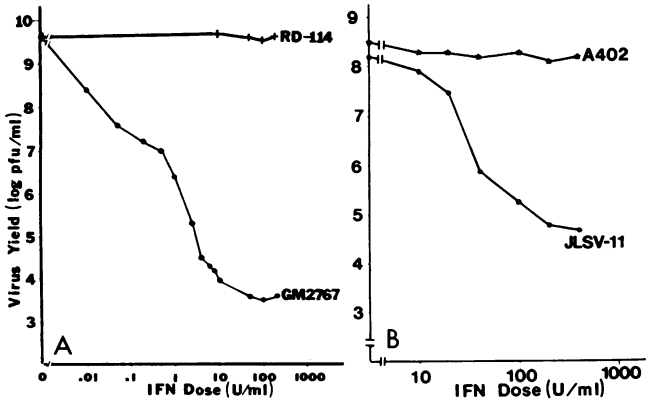

FIG. 1. Effects of IFN on VSV production. Human (A) and mouse (B) cells were treated with the indicated doses of IFN (IFN-αA for human cells, IFN-α/β for mouse cells) for 18 h prior to challenge with WT VSV at an MOI of 10 PFU/cell. At 18 h postinfection the yield of infectious virus was determined by a plaque-forming assay on mouse L929 cells.

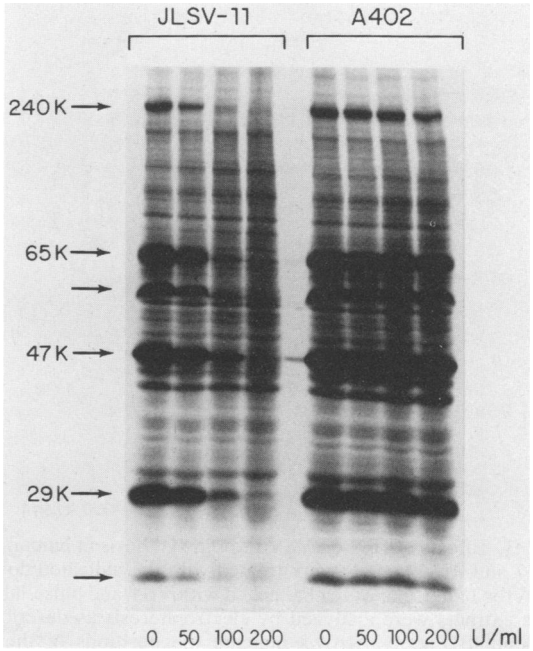

FIG. 2. Effects of IFN on VSV protein synthesis in mouse cells. JLSV-11 and A402 cells were treated with the indicated doses of IFN-α/β for 18 h prior to VSV challenge at an MOI of 10 PFU/cell. At 3.5 h postinfection the medium was replaced with methionine-free medium containing 50 μCi of [^{35}S]methionine per ml. The cells were pulse-labeled for 0.5 h. Extracts were prepared and analyzed by electrophoresis in 15% polyacrylamide gels at 15 mA (constant current) for 16 h. The positions of the viral proteins are indicated by arrows in the figure: 240K = L, 65K = G, 47K = N/NS (unresolved in this gel system), and 29K = M. The arrow below 65K shows the position of the precursor for G protein, and the one below 29K shows the position of a specific degradation product of M protein.

analyzed by polyacrylamide gel electrophoresis. IFN treatment led to a strong inhibition of VSV protein synthesis in both sensitive cell lines JLSV-11 (Fig. 2) and GM2767 (Fig. 3). No such inhibition was observed in the resistant mouse cell line A402 (Fig. 2), while a slight inhibition was observed in RD-114 cells at 100 U of IFN per ml (Fig. 3). In neither sensitive line did IFN treatment appreciably reverse the viral suppression of host protein synthesis.

VSV-related RNA levels. The steady-state levels of VSV-related RNAs as a function of IFN treatment were determined in the four cell lines by an RNA dot hybridization technique. Total cytoplasmic RNA was prepared from VSV-infected cells at 5 h postinfection. Serial dilutions of these samples were denatured and immobilized on nitrocellulose prior to hybridization with a nick-translated VSV cDNA probe. Known quantities of purified VSV mRNA were used as standards. In both GM2767 and JLSV-11 cells IFN treatment strongly inhibited the accumulation of VSV-related RNAs. The steady-state levels of VSV RNAs were comparable in IFN-treated and untreated A402 and RD-114 cells (data not shown). At this point it was necessary to determine the basis of the observed inhibition of viral protein synthesis and accumulation of viral RNAs in IFN-treated sensitive cells. Since VSV protein and RNA synthesis are interdependent, IFN-mediated effects at either level could lead to the observed effects at both. We took advantage of a *ts* mutant of VSV, *ts*G41, to analyze the process of primary viral translation.

Primary VSV translation. We analyzed viral protein syn-

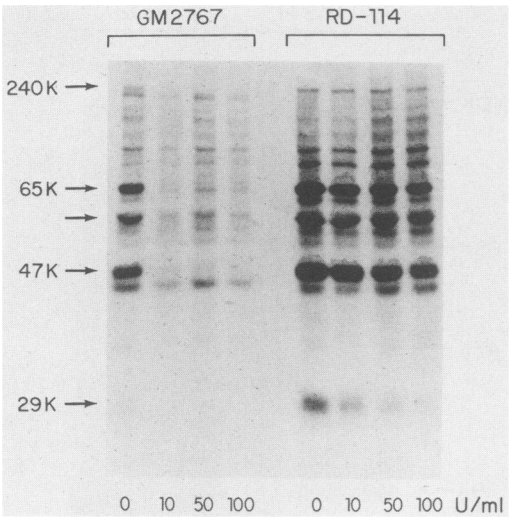

FIG. 3. Effects of IFN on VSV protein synthesis in human cells. GM2767 and RD-114 cells were treated with the indicated doses of IFN-αA for 18 h. Cells were challenged with VSV and pulse-labeled, and the extracts were analyzed by electrophoresis as described in the legend to Fig. 2. Arrows indicate the positions of the viral proteins.

thesis with *ts*G41 at the nonpermissive temperature (39.5°C). Under these conditions viral mRNAs are transcribed, using the input virion RNA (vRNA) as a template. Since replication of the *ts*G41 genome does not occur at this temperature (10, 37), viral RNA and protein syntheses are not amplified and are restricted to that derived from input virions (primary transcription and translation). This virus behaved similarly to WT VSV in its IFN sensitivity (Table 1). At the permissive temperature replication of this virus was inhibited by IFN treatment in GM2767 and JLSV-11 cells but not in RD-114 or A402 cells. In the sensitive cells IFN treatment lowered *ts*G41 yields by the same order of magnitude as the WT virus. At the nonpermissive temperature the residual yields were lowered further in GM2767 and JLSV-11 cells by IFN treatment. No such effect was observed in either resistant cell line.

Figure 4 shows the results of the primary translation analysis in these four cell lines. IFN-treated and untreated cells were infected with *ts*G41 at the nonpermissive temperature and pulse-labeled with [^{35}S]methionine from 4 to 4.5 h postinfection. Newly synthesized VSV proteins were analyzed electrophoretically after immunoprecipitation with anti-VSV antiserum. This antiserum recognized primarily VSV G protein. The other prominent band (lower-molecular-weight band) was of unknown origin, and it was also brought down by nonimmune serum (data not shown). IFN treatment inhibited primary VSV translation in GM2767 and JLSV-11 cells but not in RD-114 or A402 cells.

TABLE 1. Effect of IFN on VSV *ts*G41 replication[a]

Cell line	VSV yield (PFU/ml)			
	30°C		39.5°C	
	Control	IFN	Control	IFN
GM2767	3.3×10^8	4.0×10^3	2.7×10^3	2.2×10^1
RD-114	1.2×10^8	5.0×10^7	4.4×10^3	4.7×10^3
JLSV-11	6.8×10^7	5.5×10^4	9.1×10^3	8.8×10^1
A402	1.2×10^8	6.3×10^7	9.7×10^3	5.5×10^3

[a] Cells were treated with IFN for 18 h (200 U of IFN-αA per ml for human cells and 500 U of IFN-α/β per ml for mouse cells). IFN-treated and untreated cells were infected withVSV *ts*G41 at an MOI of 10 for 30 h at 30°C. Progeny virus was quantitated on L929 cells at 30 or 39.5°C.

The degree of inhibition of G protein synthesis in IFN-treated cells was quantitated by densitometric scanning of the autoradiogram shown in Fig. 4. In GM2767 cells G protein synthesis was inhibited by more than 99%, while in RD-114 cells the degree of inhibition was only about 25%. Similarly, IFN treatment inhibited G protein synthesis by about 80% in JLSV-11 cells, whereas no inhibition was observed in the A402 line.

Primary VSV transcription. It was important to determine whether the observed inhibition of primary translation in the IFN-sensitive cells was due to a direct effect of IFN on the synthesis of viral proteins or reflected an effect of IFN on primary viral transcription. Viral RNA synthesis was restricted to primary mRNA transcription in two ways: by using *ts*G41 at the nonpermissive temperature and by using WT VSV in the presence of 100 μM anisomycin (which inhibited protein synthesis by more than 95%; data not shown). The second method exploits the fact that replication of the VSV genome (and hence amplified RNA and protein synthesis) is dependent on ongoing protein synthesis, while primary mRNA transcription is not. The effect of various doses of IFN on the accumulation of primary VSV transcripts is illustrated in Fig. 5. GM2767 and RD-114 cells were treated with the indicated doses of IFN-αA prior to challenge with WT VSV in the presence of 100 μM anisomycin. Total cytoplasmic RNA was prepared at 3 h postinfection and used for RNA dot blot analysis. The total VSV-specific RNA content of these cells was analyzed with a ^{32}P-labeled cDNA probe that contained sequences complementary to all five virally encoded mRNAs. IFN treatment of GM2767 cells inhibited the accumulation of primary VSV transcripts in a dose-dependent fashion. The accumulation of these transcripts in RD-114 cells was unaffected by IFN treatment.

To determine whether the observed inhibition of primary transcription had any selectivity for any particular VSV mRNA, Northern analysis was performed. This analysis also allowed us to determine whether the decreased steady-state levels of primary transcripts observed in the IFN-sensitive cells was a consequence of degradation of the vRNA in the

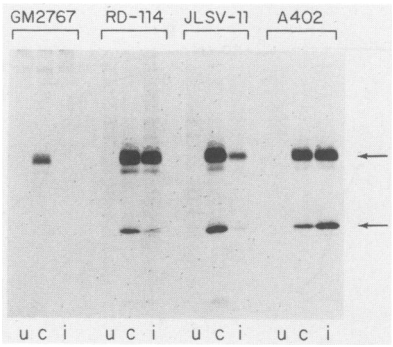

FIG. 4. Effects of IFN on VSV primary translation. Confluent monolayers of GM2767, RD-114, JLSV-11, and A402 cells were infected with *ts*G41 (MOI of 10) at 39.5°C. Cells were pulse-labeled with 50 μCi of [^{35}S]methionine per ml from 4 to 4.5 h postinfection. Cell extracts were prepared and immunoprecipitated as described in Materials and Methods prior to electrophoresis on a 15% polyacrylamide gel. Lanes: u, uninfected control; c, infected control; i, infected and IFN-treated. The upper arrow shows the position of VSV G protein, and the lower arrow shows an unidentified protein.

IFN-treated cells. RNA was extracted from GM2767 cells treated with various doses of IFN-αA and infected with WT VSV in the presence of 100 μM anisomycin. The RNA was electrophoresed through a denaturing gel, transferred to nitrocellulose, and hybridized with the labeled VSV cDNA probe. In this gel system five VSV-related RNA species were observed: the vRNA, L mRNA, G mRNA, N mRNA, and NS/M mRNAs (which comigrate in this gel system). The levels of all VSV-related mRNAs were decreased in a dose-dependent manner by IFN treatment (Fig. 6). The level of the vRNA (which was contributed by input virus) remained relatively unchanged even at the highest IFN dose tested (200 U/ml). This suggests that the inhibition of primary transcription in IFN-treated cells was not a result of degradation of the vRNA which served as the template.

In some of our Northern analyses (such as that shown in Fig. 6), we detected an RNA species that hybridized with the viral probe and whose level did not appear to diminish after IFN treatment. Several lines of evidence suggest that this RNA is probably a cellular rRNA: its length (about 5,000 nucleotides), its presence in uninfected cells, and its presence in the nonpolyadenylated fraction of RNA that had been subjected to oligo(dT)-cellulose chromatography (data not shown). This conclusion is also supported by recent evidence demonstrating sequence homology between VSV and rRNA (21).

Treatment of RD-114 cells with doses of IFN-αA up to 200 U/ml did not appreciably decrease the level of any primary VSV transcript. With the *ts* mutant (*ts*G41) of VSV (at the restrictive temperature) used to analyze primary transcripts in GM2767 and RD-114 cells, the results were similar to those obtained with the WT virus (data not shown).

Figure 7 shows the results of a Northern blot analysis of primary VSV transcripts in the two mouse cell lines, JLSV-11 and A402. This blot shows the results with WT VSV plus 100 μM anisomycin (lanes 1 and 2) and *ts*G41 at 39.5°C (lanes 3 and 4). As in the human G2767 cells, IFN treatment of JLSV-11 cells led to a reduction in the steady-state levels of

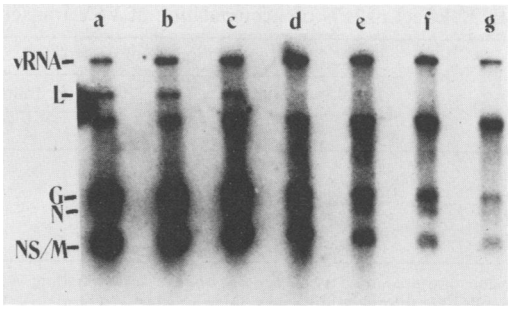

FIG. 6. Northern analysis of VSV primary transcripts in GM2767 cells treated with different doses of IFN. GM2767 cells were treated with IFN-α_2 for 18 h prior to challenge with WT VSV in the presence of 100 μM anisomycin. At 3 h postinfection RNA was prepared, and 25 μg of each sample was denatured and electrophoresed in a 1.5% agarose gel at 45 V for 6 h. The RNA was transferred to nitrocellulose and hybridized with a ^{32}P-labeled probe containing cDNAs to VSV L, G, N, NS, and M mRNAs. The dose of IFN used to treat the cells prior to virus challenge was 0, 0.01, 0.1, 1.0, 10, 100, or 200 U/ml (lanes a through g, respectively). The positions of the viral RNAs are indicated on the left.

primary VSV transcripts when analyzed by either method. In A402 cells as in RD-114 cells, IFN treatment caused little reduction in the levels of these transcripts. The level of genomic RNA was not greatly altered by IFN treatment of either mouse cell line.

To quantitate the relative levels of the various VSV-related RNAs in control and IFN-treated cells, the autoradiograms of the Northern blots were scanned with a densitometer (Table 2). The values shown are averages of those obtained for all of the VSV-related mRNAs. The degrees of decrease of the different mRNA species in IFN-treated GM2767 and JLSV-11 cells ranged from about 77% inhibition for N mRNA to 94% for L mRNA (when measured with WT virus and anisomycin). IFN treatment of GM2767

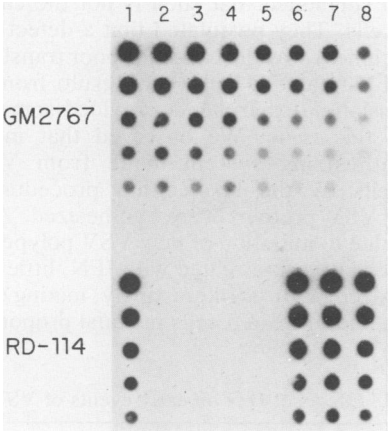

FIG. 5. Effects of IFN on accumulation of VSV primary transcripts in human cells. Cells were treated with IFN-α_2 for 18 h prior to challenge with WT VSV in the presence of 100 μM anisomycin. RNA was extracted from cells at 3 h postinfection, and 50 μg of each RNA sample was denatured, serially twofold diluted, and dotted onto nitrocellulose. The blot was probed with ^{32}P-labeled cDNA probe that was a mixture of sequences complementary to all five viral mRNAs. The IFN dose was 0, 0.01, 0.1, 1.0, 10, 50, 100, or 200 U/ml (lanes 1 through 8, respectively). For RD-114, experiments corresponding to lanes 2 through 5 were not done.

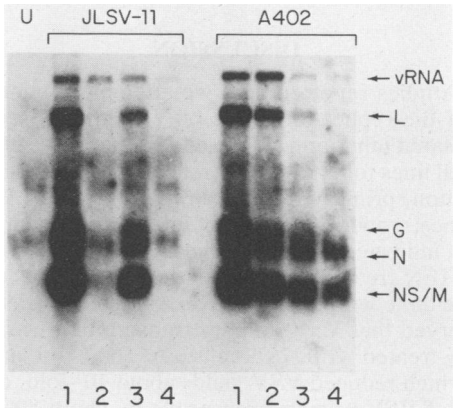

FIG. 7. Northern analysis of VSV primary transcripts in IFN-treated mouse cells. JLSV-11 and A402 cells were infected with either WT VSV in the presence of anisomycin (lanes 1 and 2) or *ts*G41 at the nonpermissive temperature (lanes 3 and 4). RNA extraction, electrophoresis, transfer, and hybridization were done as described in the legend to Fig. 6. Lanes 2 and 4 contain 25 μg of RNA from cells treated with mouse IFN-α/β (500 U/ml) prior to virus infection, while lanes 1 and 3 are RNA from control cells. Lane U is 25 μg of nonpolyadenylated RNA from uninfected JLSV-11 cells. The positions of the virally derived RNAs are indicated.

TABLE 2. Effect of IFN on accumulation of VSV transcripts[a]

Cell line	Accumulation (% of control)			
	Total transcripts		Primary transcripts	
	WT	*ts*G41	WT	*ts*G41
GM2767	<1	<3	10	<1
RD-114	100	100	100	90
JLSV-11	<1	6	10	10
A402	75	80	65	60

[a] The values are presented as percentages of the corresponding values for untreated cells. Total transcription was determined at 37°C for VSV WT and at 30°C for VSV *ts*G41. Primary transcription was determined at 37°C in the presence of anisomycin for VSV WT and at 39.5°C for VSV *ts*G41.

cells led to greater than 99% inhibition of the accumulation of total VSV transcripts and about 90% inhibition of the accumulation of WT VSV primary viral transcripts. The degree of primary transcription inhibition was somewhat greater with *ts*G41. Essentially the same results were obtained with JLSV-11. Little inhibition was observed in RD-114 cells under any conditions. Primary transcription was slightly inhibited in A402 cells but not to a degree comparable with that seen in JLSV-11 cells.

Effects of IFN on initial events in VSV infection. The data presented thus far suggested that in GM2767 and JLSV-11 cells IFN had a direct effect on VSV RNA synthesis at the level of primary viral transcription. The demonstration of similar amounts of vRNA (by Northern blotting) in control and IFN-treated cells suggests that the process of VSV entry was unaffected by IFN treatment. To test this hypothesis directly, we used ^{35}S-labeled VSV to measure virus binding, internalization, and uncoating. The uncoating analysis was particularly important to our hypothesis since it is impossible to distinguish the relative amounts of virion RNA in virions and in uncoated nucleocapsids by Northern blotting. The results of these experiments are summarized in Table 3. The lack of any significant effect of IFN on any event in the VSV life cycle occurring prior to primary transcription supports our hypothesis that IFN has a direct effect on this process.

DISCUSSION

In the studies reported here we attempted to identify the step(s) of the replication cycle of VSV that is sensitive to IFN-mediated inhibition. We used IFN-sensitive human and mouse cell lines for analyzing viral entry, uncoating, primary transcription, primary translation, total viral RNA and protein synthesis, and production of infectious progeny virions. Our data indicate that viral primary transcription was impaired in IFN-treated cells. All of the experiments described here were done with human IFN-αA or mouse IFN-α/β. We also observed that VSV primary transcription was inhibited in IFN-γ-treated GM2767 cells. A 500-U/ml amount of IFN-γ, which reduced VSV yields about 10^3-fold, decreased the level of VSV primary transcripts by about 80% without affecting the level of VSV genomic RNA (unpublished observations).

VSV primary transcription was measured by two independent methods: WT VSV was used in the presence of an inhibitor of protein synthesis, and *ts*G41 was used at the nonpermissive temperature. Each method has its shortcomings. Inhibitors of protein synthesis may affect synthesis of VSV mRNAs directly and thus influence the results by an unknown degree. They can also prevent synthesis of IFN-inducible cellular proteins which are necessary for the anti-VSV effects. On the other hand, *ts*G41 can only be used at a nonphysiological temperature, at which the cellular agent responsible for the anti-VSV effect could be inactive. Since this VSV mutant is to some degree leaky (Table 1), the alleged primary transcripts may also contain some secondary transcripts. For the above reasons we used both methods independently. It was reassuring to obtain similar results by each method.

Although our primary conclusion from these studies is that IFN inhibits VSV primary transcription, the possibility remains that other processes are involved in the synthesis of VSV macromolecules that are inhibited by IFN treatment. It is interesting to compare the results obtained for GM2767 cells with those for JLSV-11 cells in this regard. Although the degree of inhibition of VSV primary transcription by IFN was similar in the two cell lines, VSV yield was reduced at least 100-fold more in GM2767 cells than in JLSV-11 cells. It is conceivable that in addition to inhibition of primary transcription, VSV primary translation is also impaired in GM2767 cells but not in JLSV-11 cells. The results presented in Fig. 4 actually support such a notion. Some VSV G protein was synthesized in IFN-treatd JLSV-11 cells but not in IFN-treated GM2767 cells. Without invoking an additional step, can a 90% inhibition of primary transcription account for the 10^6-fold drop in virus yield? In our judgment it can for two reasons: (i) viral RNA and protein synthesis are amplified through the generation of new viral transcription centers, and (ii) multimolecular cooperativity occurs among different viral proteins and RNAs for formation of secondary transcription centers as well as for final morphogenesis of infectious virions.

The data presented here do not provide any molecular explanation for a putative effect of IFN on VSV mRNA translation in GM2767 cells. We have shown that the residual primary transcripts made in IFN-treated GM2767 cells were of the right size, although no further structural characterization was done. This leaves open the possibility that cap methylation of the mRNAs was impaired, making these transcripts less efficient in translation (5). However, Masters and Samuel concluded that such is not the case in human amnion U cells. They postulated that a defect in the translational machinery was the cause for poor translation of VSV mRNA in IFN-treated U cells (19). Results from preliminary in vitro translation experiments provided some information relevant to this issue. We observed that in an in vitro protein-synthesizing system made from VSV-infected GM2767 cells by the lysolecithin procedure (4), good amounts of VSV proteins were synthesized. About half of these were due to initiation of new VSV polypeptide chains. When the cells were pretreated with IFN, little VSV protein was synthesized in vitro. Interestingly, mixing extracts from IFN-treated and untreated cells in equal proportions did not

TABLE 3. Effects of IFN on early events of VSV infection[a]

Early event	Effect of IFN (% of control value)	
	GM2767	JLSV-11
Adsorption	95	107
Penetration	98	109
Uncoating	92	94

[a] Results are expressed as percent of control (control values all set at 100%). IFN doses were 200 U of IFN-α_2 per ml for GM2767 cells and 500 U of IFN-α/β per ml for JLSV-11 cells. Methods for analyzing early events of VSV infection with [^{35}S]methionine-labeled virus are described in the text.

impair translation of VSV mRNAs present in the extract of infected, untreated cells any more than what was caused by the dilution factor (our unpublished observations). This suggests that IFN-treated VSV-infected GM2767 cells may not contain a dominant inhibitor of VSV protein synthesis.

Our conclusion that VSV primary transcription is a major site of IFN action is in agreement with some previous studies (15–17) but not with others (1, 3, 24, 32). It is difficult to compare our results with most of the early investigations because of the inherent differences in the reagents and techniques used for measuring VSV transcription. The above reservation does not apply to the studies by Masters and Samuel (18–20), who used techniques and reagents similar to ours. Our studies do differ in that we used two independent methods of measuring VSV primary transcription. We also measured transcription of all five VSV mRNAs and directly examined the effects of IFN on progeny virus yield of *ts*G41 rather than assuming that replication of *ts*G41 and WT VSV was affected equally by IFN in our cell lines.

How do we reconcile the apparently contradictory conclusions of different investigators that the action of IFN against VSV is brought about by impairment of different stages of the viral life cycle? We suggest that different modes of action may operate in different cell lines and more than one such mechanism may operate in the same cell line. Support for the above line of thinking comes from what is known about the mechanism of action of IFN against influenza virus. It has been elegantly demonstrated that the IFN-inducible Mx protein inhibits influenza virus replication (33) and functions at the level of primary transcription (11). However, influenza virus is sensitive to IFN to some degree in Mx^- cells, in which this type of inhibition is at the level of viral protein synthesis (11). In a similar manner, IFN may impair both VSV RNA synthesis and protein synthesis. In JLSV-11 cells the former mechanism was prevalent, in U cells the latter one was prevalent, and in GM2767 cells both were functional. As a consequence, VSV yield was diminished by IFN treatment of JLSV-11 and U cells by about 10^3-fold only, whereas in GM2767 cells it was reduced by 10^6-fold.

Although all actions of IFN, including its antiviral actions, are mediated by products of different IFN-inducible genes, identification of the gene responsible for a specific action has only been possible in the case of the Mx gene. We have been using partially IFN-responsive cell lines to determine whether certain IFN-inducible gene products are likely to be reponsible for specific antiviral actions of IFNs (7, 28–31). For example, we have previously shown that the 2′,5′-oligoadenylate synthetase-RNase L pathway is defective in JLSV-11 cells (29). This suggests that VSV replication can be inhibited via other IFN-inducible pathways. Similarly, the double-stranded RNA-dependent protein kinase pathway is unlikely to have a role in this function since it is not induced in IFN-treated human fibroblasts (9). Recently we have observed that in RD-114 cells several IFN-inducible mRNAs are induced normally (J. Kusari and G. C. Sen, unpublished observation), making it highly unlikely that the products of these mRNAs are by themselves responsible for anti-VSV actions. As cDNA probes for more IFN-inducible human and mouse mRNAs become available, our partially responsive cell lines would be increasingly useful for correlating different antiviral actions with the expression of different IFN-inducible genes.

The mechanism of inhibition of VSV primary transcription in IFN-treated GM2767 and JLSV-11 cells remains to be understood. The synthesis of VSV mRNAs could be impaired directly or the degradation of these mRNAs could be enhanced in IFN-treated cells. In vitro VSV transcriptional systems would be useful for clarifying these issues. Recent observations by two groups that tubulin (22) or tubulin-associated proteins (8) may affect VSV transcription offer, in principle, attractive sites of host-virus interactions where the IFN system may intervene.

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LITERATURE CITED

1. **Ball, L. A., and C. N. White.** 1978. Effect of interferon pretreatment on coupled transcription and translation in cell-free extracts of primary chick embryo cells. Virology **84**:496–508.
2. **Banerjee, A. K., G. Abraham, and R. J. Colonno.** 1977. Vesicular stomatitis virus: mode of transcription. J. Gen. Virol. **34**: 1–8.
3. **Baxt, B., J. A. Sonnabend, and R. Bablanian.** 1977. Effects of interferon on vesicular stomatitis virus transcription and translation. J. Gen. Virol. **35**:325–334.
4. **Brown, G. D., R. W. Peluso, S. A. Moyer, and R. W. Moyer.** 1983. A simple method for the preparation of extracts from animal cells which catalyze efficient *in vitro* protein synthesis. J. Biol. Chem. **258**:14309–14314.
5. **De Ferra, F., and C. Baglioni.** 1981. Viral messenger RNA unmethylated in the 5′-terminal guanosine in interferon-treated HeLa cells infected with vesicular stomatitis virus. Virology **112**:426–435.
6. **Gallione, C. J., J. R. Greene, L. E. Iverson, and J. K. Rose.** 1981. Nucleotide sequences of the mRNAs encoding the vesicular stomatitis virus N and NS proteins. J. Virol. **39**:529–535.
7. **Herz, R. E., B. Y. Rubin, and G. C. Sen.** 1983. Human interferon-α and -γ-mediated inhibition of retrovirus production in the absence of an inhibitory effect on vesicular stomatitis virus and encephalomyocarditis virus replication in RD-114 cells. Virology **125**:246–250.
8. **Hill, V. M., S. A. Harmon, and D. F. Summers.** 1986. Simulation of vesicular stomatitis virus *in vitro* RNA synthesis by microtubule-associated proteins. Proc. Natl. Acad. Sci. USA **83**:5410–5413.
9. **Holmes, S. L., and S. L. Gupta.** 1982. Interferon action in human fibroblasts: induction of 2′,5′-oligoadenylate synthetase in the absence of detectable protein kinase. Arch. Virol. **72**: 137–142.
10. **Knipe, D., H. F. Lodish, and D. Baltimore.** 1977. Analysis of the defects of temperature-sensitive mutants of vescular stomatitis virus: intracellular degradation of specific viral proteins. J. Virol. **21**:1140–1148.
11. **Krug, R. M., M. Shaw, B. Broni, G. Shapiro, and O. Haller.** 1985. Inhibition of influenza viral mRNA synthesis in cells expressing the interferon-induced Mx gene product. J. Virol. **56**:201–206.
12. **Kusari, J., and G. C. Sen.** 1986. Regulation of synthesis and turnover of an interferon-inducible mRNA. Mol. Cell. Biol. **6**: 2062–2067.
13. **Lengyel, P.** 1982. Biochemistry of interferons and their actions. Annu. Rev. Biochem. **51**:251–282.
14. **Maheshwari, R. K., A. E. Demsey, S. B. Mohanty, and R. M. Friedman.** 1980. Interferon-treated cells release vesicular stomatitis virus particles lacking glycoprotein spikes: correlation with biochemical data. Proc. Natl. Acad. Sci. USA **77**:2284–2287.

15. **Manders, E. K., J. G. Tilles, and A. S. Huang.** 1972. Interferon-mediated inhibition of virion-directed transcription. Virology **49**:573–581.
16. **Marcus, P. I., D. L. Englehardt, J. M. Hunt, and M. J. Sekellick.** 1971. Interferon action: inhibition of vesicular stomatitis virus RNA synthesis induced by virion-bound polymerase. Science **174**:593–598.
17. **Marcus, P. I., and M. J. Sekellick.** 1978. Interferon action. III. The rate of primary transcription of vesicular stomatitis virus is inhibited by interferon action. J. Gen. Virol. **38**:391–408.
18. **Masters, P. S., and C. E. Samuel.** 1983. Mechanism of interferon action: inhibition of vesicular stomatitis virus replication in human amnion U cells by cloned human leukocyte interferon. I. Effect on early and late stages of the viral multiplication cycle. J. Biol. Chem. **258**:12019–12025.
19. **Masters, P. S., and C. E. Samuel.** 1983. Mechanisms of interferon action: inhibition of vesicular stomatitis virus replication in human amnion U cells by cloned human leukocyte interferon. II. Effect on viral macromolecular synthesis. J. Biol. Chem. **258**:12026–12033.
20. **Masters, P. S., and C. E. Samuel.** 1984. Mechanism of interferon action. Inhibition of vesicular stomatitis virus in human amnion U cells by cloned human leukocytic interferon. Biochem. Biophys. Res. Commun. **119**:326–334.
21. **McClure, M. A., and J. Perrault.** 1986. RNA virus genomes hybridize to cellular rRNAs and to each other. J. Virol. **57**: 917–921.
22. **Moyer, S. A., S. C. Baker, and J. L. Lessard.** 1986. Tubulin: a factor necessary for the synthesis of both Sendai virus and vesicular stomatitis virus RNAs. Proc. Natl. Acad. Sci. USA **83**:5405–5409.
23. **Pringle, C. R.** 1970. Genetic characteristics of conditional lethal mutants of vesicular stomatitis virus induced by 5-fluorouracil, 5-azacytidine, and ethyl methanesulfonate. J. Virol. **5**:559–567.
24. **Repik, P., A. Flamand, and D. H. L. Bishop.** 1974. Effects of interferon upon the primary and secondary transcription of vesicular stomatitis and influenza viruses. J. Virol. **14**:1169–1178.
25. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
26. **Rose, J. K., and C. J. Gallione.** 1981. Nucleotide sequences of the mRNAs encoding the vesicular stomatitis virus G and M proteins determined from cDNA clones containing the complete coding regions. J. Virol. **39**:519–528.
27. **Schubert, M., G. G. Harmison, and E. Meier.** 1984. Primary structure of the vesicular stomatitis virus polymerase (L) gene: evidence for a high frequency of mutations. J. Virol. **51**:505–514.
28. **Sen, G. C.** 1984. Biochemical pathways in interferon-action. Pharmacol. Ther. **24**:235–257.
29. **Sen., G. C., and R. E. Herz.** 1983. Differential antiviral effects of interferon in three murine cell lines. J. Virol. **45**:1017–1027.
30. **Sen, G. C., R. E. Herz, V. Davatelis, and S. Pestka.** 1984. Antiviral and protein-inducing activities of recombinant human leukocyte interferons and their hybrids. J. Virol. **50**:445–450.
31. **Sen, G. C., R. E. Herz, and B. Y. Rubin.** 1983. Antiviral, anticellular and enzyme-inducing activities of interferons in RD-114 cells. J. Gen. Virol. **64**:2213–2220.
32. **Simili, M., F. DeFerra, and C. Baglioni.** 1980. Inhibition of RNA and protein synthesis in interferon-treated HeLa cells infected with vesicular stomatitis virus. J. Gen. Virol. **47**:373–386.
33. **Staeheli, P., O. Haller, W. Boll, J. Lindenmann, and C. Weissmann.** 1986. Mx protein: constitutive expression in 3T3 cells transformed with cloned Mx cDNA confers selective resistance to influenza virus. Cell **44**:147–158.
34. **Thacore, H. R.** 1978. Effect of interferon on transcription and translation of vesicular stomatitis virus in human and simian cell cultures. J. Gen. Virol. **41**:421–426.
35. **Ulker, N., and C. E. Samuel.** 1985. Mechanism of interferon action: inhibition of vesicular stomatitis virus replication in human amnion U cells by cloned human γ-interferon. I. Effect on early and late stages of the viral multiplication cycle. J. Biol. Chem. **260**:4319–4323.
36. **Ulker, N., and C. E. Samuel.** 1985. Mechanism of interferon action: inhibition of vesicular stomatitis virus replication in human amnion U cells by cloned human γ-interferon. II. Effect on viral macromolecular synthesis. J. Biol. Chem. **260**:4324–4330.
37. **Unger, J. T., and M. E. Reichmann.** 1973. RNA synthesis in temperature-sensitive mutants of vesicular stomatitis virus. J. Virol. **12**:570–578.
38. **Whitaker-Dowling, P. A., D. K. Wilcox, C. C. Widnell, and J. S. Youngner.** 1983. Interferon-mediated inhibition of virus penetration. Proc. Natl. Acad. Sci. USA **80**:1083–1086.