Mechanism 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*

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The effects of a single molecularly cloned subspecies of human leukocyte interferon (IFN-αA) on vesicular stomatitis virus (VSV) macromolecular synthesis in human amnion U cells were examined. IFN-αA was found to uniformly inhibit VSV protein synthesis to an extent sufficient to account for the overall inhibition of viral infectivity. IFN-αA treatment also prevented the shutoff of cellular protein synthesis observed in untreated, VSV-infected U cells. By use of the VSV mutant tsG41, which is competent in RNA transcription but defective in RNA replication at 40 °C, it was shown that IFN did not significantly inhibit the accumulation of VSV primary transcripts, although the *in vivo* translation of primary viral transcripts was greatly impaired as a function of IFN treatment. Thus, the major, and possibly only, effect of IFN-αA on VSV replication was translation inhibition. Analysis of RNA, separated by agarose gel electrophoresis after denaturation with glyoxal, with cDNA probes to individual VSV mRNAs, did not reveal any detectable difference in the structural integrity of VSV mRNA isolated from IFN-treated as compared to untreated cells. Likewise, *in vitro* protein synthesis did not reveal any major difference in the functional integrity of VSV mRNA isolated from IFN-treated as compared to untreated U cells. Viral mRNA isolated from either wild type or tsG41-infected U cells treated with IFN was translated only slightly less efficiently *in vitro* than viral mRNA from untreated cells. Thus, the principal cause of the IFN-induced inhibition of viral protein synthesis observed *in vivo* appears to be an alteration of a component of the translational machinery other than the mRNA template.

The replication of VSV is acutely sensitive to the antiviral action of IFN in almost every type of cell which responds to this class of regulatory proteins. However, in spite of this extreme sensitivity, there is little agreement concerning the molecular basis of the IFN-induced inhibition. Using a molecularly cloned subspecies of human leukocyte IFN (IFN-αA), we have studied the effect of IFN action in human amnion U cells on each stage of the VSV multiplication cycle in order to determine the site(s) (or points) acted upon by the “antiviral state.” In the preceding paper (1), we examined the kinetics of induction of antiviral activity against VSV, and we showed that IFN treatment of U cells has no detectable effect on the adsorption, penetration, and uncoating of infecting VSV virions, nor does IFN treatment cause aberrant VSV assembly or reduce the specific infectivity of released virus. In this paper, we report the effect of IFN on VSV macromolecular synthesis.

EXPERIMENTAL PROCEDURES

Materials. Cloned cDNAs in the pUC and a derivatives of VSV (1, 2) were generously provided by Dr. J. A. Roth, Yale University, New Haven, Connecticut. [m-32P]dUTP (3000 Ci/mmol) was from Amersham; [125I]anti-VSV (3000 Ci/mmol) was from Inform. The sources of all other reagents and reagents were exactly as described in the preceding paper (1).

Cells. Human amnion U. The growth of human amnion U cells and the growth and properties of wild-type and cloned VSV (infectious viruses) are described previously (1). The mutant tsG41 of VSV (infectious viruses) was obtained from Dr. F. I. Korsm, University of Connecticut, Storrs, CT. A stock of VSV that was prepared from infection of a confluent monolayer of amnion U cells with plaque-purified virus at a multiplicity of 0.1 plaques/cell was harvested after 24 h. These wild-type VSV-infected amnion U cells were incubated at 30 °C for 20 h after infection with 0.01 plaque-forming unit/cell. The rate of growth of plaques was determined at 40 °C ± 0.5 °C. Human amnion U cells were generated by Dr. K. C. Pike, Bethesda, MD 20814. Human amnion U cells were examined. IFN-αA was obtained from Dr. J. K. Rose, Salk Institute, San Diego, California, 92121.

Labeling of In vivo Translated Viral Proteins. Proteins synthesized in VSV-infected U cells were isolated under conditions similar to those used to label the kinetics of induction of antiviral activity (1). Cells (4 × 10⁶) of untreated (2) and infected with 300 plaque-forming units of VSV at a multiplicity of 10 plaques/cell were incubated at 37 °C in the presence of [m-32P]dUTP (3000 Ci/mmol). For the purposes of quantitation, where indicated, NP40 extracts were prepared by addition to each sample of 0.5 ml of 10% sodium deoxycholate (SDC) on ice for 10 min with vortex agitation. The extracts were centrifuged at 10,000 x g at 4 °C for 10 min, and the supernatant fluids were used for the immunoprecipitation of viral antigens. ATP was measured in yeast extracts by the luciferase assay (16). Viral specific infectivity was determined by the method of Miles et al. (17).

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proteins. Samples were incubated at 0°C for 30 min, and then 100 μl of a 10%
(±)-suspension of heat-inactivated, formalin-fixed *Staphylococcus aureus*
(coated with 1 ml) was added to each T700 well. The reaction mixture was
incubated at 0°C for 5 min, before harvest and analyzed by NaDodSO4-polyacryla-
mide gel electrophoresis.

Following visual detection by autoradiography, the amount of radioactivity
in viral protein bands in non-immunoprecipitated and immunoprecipitated
samples was measured by directly excising the bands from dried NaDodSO4-
Polyacrylamide gels; background was measured by exciting the corresponding
areas of lanes of untreated, uninfected HeLa cells. Gel pieces were digested by
lyophilization in 0.1 ml of 100 μg/ml of protease K and the radiolabeled amino
acids were analyzed by quantitative amino acid analysis as described previously.

Preparation of RNA. RNA was isolated from cells which were treated
under conditions identical to those used to follow the induction of anti-
viral activity (1). Briefly, U cells monolayers (100 cm2 each), untreated or
infected with 300 units/ml IFN-αA for various times, were washed
with PBS, homogenized in 5 ml of 8 M urea, and separated by
DNAse treatment. The RNA was then precipitated with 0.5 M
amylose sulfate, 5 M NaCl, and 0.5 M ethylenediamine tetraacetic
acid (EDTA) at pH 7.2. The RNA was then resuspended in 0.5 M
glycine, 0.5 M NaCl, and 0.1 M EDTA pH 8.3, and single-stranded
RNA was isolated by agarose gel electrophoresis into a 1.0 M
formic acid solution followed by elution therefrom with 1.0 M
NaCl. The RNA was then precipitated with 0.5 M
ammonium acetate and 5 M ethanol.

The RNA preparations were hybridized with 0.25 μg of tritiated DNA, 1.5 μg of
5'-phosphate DNA, and 1.5 μg of unphosphorylated DNA, each
prepared by the methods described under "Experimental Procedures." All
samples were hybridized at 66°C and then incubated in a 20°C
water bath. Autoradiography of the blots was performed as described
under "Experimental Procedures." The RNA preparations were monitored in parallel by
Northern blot analysis, as described under "Experimental Procedures." By
Northern blot analysis, the highest IFN-αA doses were found to be best
suited for the detection of viral RNA. In IFN-treated, VSV-infected
samples, there was a concomitant increase in host cell protein synthesis, which,
at the highest IFN-αA doses (lanes g and h), approached the
level of protein synthesis seen in uninfected cells (lanes i and j). Larger aliquots of the samples shown in Fig. 1 were run on
NaDodSO4-polyacrylamide gels with wide sample wells, and viral protein bands were excised and counted for radioactivity (Fig. 2A); the same was done with sample aliquots that had been immunoprecipitated with anti-VSV antiserum in order to clearly resolve the N, G, and L viral proteins from protein bands of cellular origin (Fig. 2B). At both concentration doses of IFN-αA, IFN treatment was seen to cause a uniform inhibition of the synthesis of the five VSV polypeptides; no single polypeptide differed significantly from the others in its sensitivity to IFN-induced inhibition of synthesis. The extent of viral protein synthesis inhibition was, we believe, sufficient to account for the overall reduction in infectious virus yield measured in similar IFN-treated, VSV-infected samples harvested at the end of the standard 12-h single step growth cycle (Fig 2; see "Discussion").

RESULTS

Viral Protein Synthesis in Vivo—The effect of IFN-αA on the synthesis of VSV polypeptides was examined under conditions identical to those used to study the kinetics of induction of antiviral activity against VSV (1). Human amnion U cells, which had been untreated or treated with 300 or 10 units/ml of IFN-αA for various times from 2 to 8 h, were infected with VSV at a multiplicity of 10 pfu/cell in the presence of 0.5 μg/ml of actinomycin D. Cells were labeled with [3H]methionine from 4 to 4.5 h postinfection and harvested at 5.5 h postinfection and analyzed by NaDodSO4-polyacrylamide gel electrophoresis as described under "Experimental Procedures."
These results show that all or almost all inhibition of VSV replication during the first kinetic phase of IFN treatment (1) could be attributed to a block at or prior to viral protein synthesis. Such an effect could be due to an inhibition of either viral transcription or viral translation, or to a combination of these processes, since the overwhelming majority of VSV protein synthesis results from translation of mRNA synthesized on progeny nucleocapsids. In order to distinguish a possible direct effect by IFN on VSV transcription from a direct effect on the translation of VSV mRNA, it was necessary to examine only primary transcription and translation i.e. viral mRNA synthesis and translation occurring in the absence of amplification by parental genome replication. Investigators have typically examined VSV primary transcription by use of an appropriate VSV mutant strain (12) or by examining wild type VSV transcription in the presence of the protein synthesis inhibitor cycloheximide (13-15), since VSV protein synthesis results from translation of mRNA synthesized on progeny nucleocapsids. In order to distinguish such an effect could be due to an inhibition of host protein synthesis, nor did IFN treatment noticeably affect VSV cell protein synthesis.

Viral RNA Synthesis in Vivo—Whole cytoplasmic RNA was isolated at 4 h postinfection from IFN-treated, VSV tsG41-infected U cell cultures parallel to those in which primary protein synthesis had been labeled. Because we wanted to quantify primary viral mRNA levels, RNA samples were not selected for polyadenylated fractions since such selection often gives variable recovery yields; also, we could not preclude the possibility that viral message polyadenylation could be affected by IFN. Aliquots (10 μg) of RNA were reacted with glyoxal, separated by electrophoresis through agarose, and blotted onto nitrocellulose membranes which were probed with [α-32P]dTTTP-labeled cDNA to VSV N mRNA (Fig. 4) or to VSV M mRNA (Fig. 5). As can be seen in lanes f-j of Figs. 4 and 5, there was little or no decrease in the amount of primary VSV N or M message over the course of 0 to 6 h of treatment of U cells with 300 units/ml of IFN-αA. In order to demonstrate that the amount of hybridization to a band indeed reflects the concentration of hybridizing sequences present in the bands, dilutions of RNA from untreated cells infected with wild type VSV at 37°C were analyzed on the same blots to provide known standards for direct comparison (Figs. 4 and 5, lanes a-e). Clearly, the amount of hybridizing N or M primary message in all of the samples prepared from tsG41-infected cells, either untreated or IFN-treated, was slightly less than the amount in the 0.03-μg standards and considerably greater than that of the 0.01-μg standards.

The relative levels of total (primary plus secondary) N and M mRNA at 4 h postinfection in untreated and IFN-treated, wild type VSV-infected U cells were also measured by RNA blotting. Figs. 6 and 7 show hybridization of N and M gene cDNAs, respectively, to successive dilutions of RNA from untreated, VSV-infected cells (lanes a-e) compared with hy-

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**TABLE I**

Quantitation of Primary Viral Protein Synthesis in IFN-treated Ammonium U cells infected with VSV tsG41.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>M RNA polymerase synthesis</th>
<th>L</th>
<th>N</th>
<th>NS</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>No IFN</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>300 units IFN-αA</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2 h</td>
<td>0.80</td>
<td>0.61</td>
<td>0.57</td>
<td>0.88</td>
<td>0.22</td>
</tr>
<tr>
<td>3 1/3 h</td>
<td>0.37</td>
<td>0.28</td>
<td>0.23</td>
<td>0.54</td>
<td>&lt;0.12</td>
</tr>
<tr>
<td>4 2/3 h</td>
<td>0.23</td>
<td>0.40</td>
<td>0.14</td>
<td>0.32</td>
<td>&lt;0.09</td>
</tr>
<tr>
<td>6 h</td>
<td>0.05</td>
<td>0.13</td>
<td>0.03</td>
<td>0.17</td>
<td>&lt;0.09</td>
</tr>
</tbody>
</table>

Fig. 3. Primary viral protein synthesis in IFN-treated ammonium U cells infected with VSV tsG41. U cell monolayers were untreated (a, a', f, f') or treated with 300 units/ml of IFN-αA for 2 h (b, b', 3/4 h (c, c'), 4 1/2 h (d, d'), or 6 h (e, e') at 37°C. Monolayers were then shifted to 40°C and were left uninfected (f, f') or were infected with VSV tsG41 (a-e, a'-e') at a multiplicity of 10 pfu/cell in the presence of 0.5 μg/ml of actinomycin D. Cells were labeled with [35S]methionine from 3.5 to 4 h postinfection. Viral proteins were then immunoprecipitated with anti-VSV antiserum and analyzed by NaDodSO4-polyacrylamide gel electrophoresis (Fig. 3, lanes a-f). Primary viral protein synthesis was seen to decrease as a function of duration of IFN treatment, although the magnitude of this decrease, which was quantitated by densitometry (Table I), was not as great as the overall decrease of secondary viral protein synthesis (Figs. 1 and 2). An IFN-αA treatment of 300 units/ml for 6 h produced an average 10-fold reduction in the rate of in vivo translation of VSV primary message. Primary synthesis of L protein, and perhaps also NS protein, appeared more sensitive to IFN-induced inhibition than did synthesis of the other VSV proteins; we do not know if this difference was significant.

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Interferon Inhibition of Vesicular Stomatitis Virus

concentration  

RNA (pg)

N mRNA-

M mRNA-

42 S-

FIG. 4. Primary N gene mRNA synthesis in IFN-treated amnion U cells infected with VSV tsG41. U cell monolayers (parallel cultures to those used in Fig. 3) were untreated (f, h) or treated with 300 units/ml of IFN-αA for 2 h (g), 3½ h (h), 4½ h (i), or 6 h (j) at 37 °C. Monolayers were then shifted to 40 °C and were left uninfected (k) or were infected with VSV tsG41 (f-j) at a multiplicity of 10 pfu/cell in the presence of 0.5 μg/ml of actinomycin D. At 4 h postinfection, RNA was purified from cells and 10-μg samples were glyoxylated, electrophoresed on a 1.2% agarose gel, blotted onto nitrocellulose, and probed with [α-32P]dTTP-labeled cDNA to VSV N message as detailed under “Experimental Procedures.” For comparative purposes, dilutions of RNA from (wild type) VSV-infected U cells were run on the same gel: 0.1 μg (a), 0.03 μg (b), 0.01 μg (c), 0.003 μg (d), and 0.001 μg (e).

Fig. 5. Primary M gene mRNA synthesis in IFN-treated amnion U cells infected with VSV tsG41. Lane assignments and experimental conditions are identical to those in Fig. 4, except that the probe used was [α-32P]dTTP-labeled cDNA to VSV M message.

Fig. 6. N gene mRNA synthesis in IFN-treated, VSV-infected amnion U cells. U cell monolayers were untreated (a-e, k) or treated with 300 units/ml of IFN-αA for 6 h (f-j). Monolayers were left uninfected (k) or were infected with (wild type) VSV (a-j) at a multiplicity of 10 pfu/cell in the presence of 0.5 μg/ml of actinomycin D. At 4 h postinfection, RNA was purified from cells, and samples of 1 μg (a, f, k), 0.3 μg (b, g), 0.1 μg (c, h), 0.03 μg (d, i), and 0.01 μg (e, j) were glyoxylated, electrophoresed on an agarose gel, blotted onto nitrocellulose, and probed with [α-32P]dTTP-labeled cDNA to VSV N message as detailed under “Experimental Procedures.”

Fig. 7. M gene mRNA synthesis in IFN-treated, VSV-infected amnion U cells. Lane assignments and experimental conditions are identical to those in Fig. 6, except that the probe used was [α-32P]dTTP-labeled cDNA to VSV M message.

of message and genome length (42 S) material. These were clearly of viral origin, since they did not appear in the lanes of RNA from uninfected control cells (lane k); in addition, they were not due to fortuitous hybridization of pBR322 sequences because viral cDNA probes were excised from their parent vector and also because [α-32P]dTTP-labeled pBR322 did not hybridize at all to other blots of the same RNA samples (data not shown). The mobility of glyoxylated RNA in our agarose gels was calibrated with the following samples of known molecular weight: the N mRNA, M mRNA, and 42 S mRNA standards cell RNA (10 pg).
RNA of VSV, the 18 S and 28 S rRNAs of U cells, and the l class, m class, and s2 mRNAs of reovirus. The relative mobilities of these species were linear with the logarithms of their molecular weights, allowing an estimation of the sizes of the intermediate RNAs on blots of the same gel (data not shown). The intermediate hybridizing species were found to correspond to tandem multimers of VSV mRNAs encoded by adjacent genes. The more strongly N-hybridizing bands, denoted in Fig. 6, were N-NS, N-NS-M, and N-NS-M-G RNAs, in order of increasing size. The more strongly M-hybridizing bands, denoted in Fig. 7, were NS-M, M-G, NS-M, and NS-M-G RNAs, in order of increasing size. These molecules are probably the polycistronic mRNAs that have previously been detected in vitro by heteroduplex analysis by Herman et al. (20, 21) and have been shown to contain tracts of polyadenylate connecting adjacent messages. As is obvious from Fig. 7 and as was also noted by these investigators, the N-M tandem message was by far the most numerous of the polycistronic mRNAs. In this context, it is of interest that all of the VSV Indiana intergenic junctions (including the region between the L gene and the 5' nontranscribed portion of the genome) consist of an identical 18-base nucleotide sequence, except for the NS-M junction, which differs from the consensus sequence by a single base (22).

Translation in Vitro of Viral RNA Synthesized in Vivo—RNA from untreated and IFN-treated U cells infected with wild type VSV was used to program in vitro protein synthesis in wheat germ cell-free extracts in order to determine what portion of the VSV mRNA from IFN-treated cells, detected structurally by RNA blot hybridization, was functionally active. Because blot hybridization had shown that a 6-h treatment of U cells with 300 units/ml of IFN-aA caused approximately a 10-fold reduction in viral mRNA (Figs. 6 and 7), the RNA isolated from untreated, infected cells was diluted 10-fold with RNA isolated from untreated, uninfected cells in order that the protein synthesis reactions would be carried out with nearly comparable amounts of both viral and cellular mRNA. In order to test the possibility that viral message from IFN-aA-treated cells was deficient in cap methylation (23), protein synthesis reactions were performed in the presence of either 1 mM AdoHcy or 20 mM AdoMet. In the presence of AdoHcy, the wheat germ system shows a greatly reduced capacity to translate unmethylated VSV or reovirus mRNAs; in the presence of AdoMet, unmethylated viral messages are translated with almost the same efficiency as methylated messages, presumably due to an endogenous methylating activity in wheat germ extracts (11, 24). Protein synthesis reactions were also done at low (75 mM) and high (125 mM) concentrations of potassium ion because a number of effects, including cap methylation, which cause differences in mRNA translation efficiency, show more pronounced differences as potassium ion concentration increases (25, 26). As is shown in Fig. 8, there were small but consistent differences in the abilities of VSV RNAs from IFN-treated as compared to untreated U cells to program the synthesis in vitro of the four smaller VSV polypeptides, M, N, NS, and G. The largest VSV protein, L, was not efficiently synthesized in vitro. The amount of radioactivity in excised VSV polypeptide bands was determined for the gel in Fig. 8 and for a second set of in vitro translations in which the relative effects of 1 mM AdoHcy or 20 mM AdoMet were tested. For all of these reaction conditions, VSV message from untreated cells appeared on the average about 1.6-fold more efficient than an equivalent amount of VSV message from IFN-treated cells in reactions which measured both the rate (30 min) and extent (90 min) of protein synthesis (data not shown). At least in part, this small apparent difference probably reflected an overestima-

FIG. 8. In vitro protein synthesis with RNA from IFN-treated, VSV-infected amnion U cells. RNA purified from VSV-infected (a, b, d, e, g, h, j, k) or untreated (c, f, i, l) U cells which had been untreated (a, e, d, g, i, j, l) or treated for 6 h with 300 units/ml of IFN-aA (b, e, h, k) was used to program in vitro protein synthesis in wheat germ cell-free extracts as detailed under "Experimental Procedures." The RNA isolated from untreated cells was diluted 10-fold with RNA isolated from untreated, uninfected cells so that protein synthesis reactions were all programmed with comparable amounts of both viral and cellular mRNA. Reactions were performed for 30 min (a-c, g-i) or 90 min (d-f, j-m), in the presence of 75 mM (a-f) or 125 mM (g-m) potassium ion and in the presence of 1 mM AdoHcy. Reactions were analyzed by NaDodSO4-polyacrylamide gel electrophoresis. Lane m is endogenous translation in wheat germ extract with no added RNA. Lane n is a standard of VSV-infected U cells labeled with 35S)methionine. Go denotes preprocessed VSV G protein.

DISCUSSION

We have examined in detail the effect of a molecularly cloned subspecies of human leukocyte IFN (IFN-aA) on VSV macromolecular synthesis in human amnion U cells. We focused our study on the first kinetic phase of the IFN-induced inhibition of VSV replication because this phase accounted
for the major part of the total reduction in infectious yield and because it exhibited first order kinetics with respect to duration of IFN treatment at all IFN-αA concentrations tested (1).

Treatment of U cells with either a saturating or a low dose of IFN-αA produced, as a function of duration of treatment, a continuous decrease in the rate of viral protein synthesis (Figs. 1 and 2). Treatment of cells was carried out with a low dose of IFN (10 units/ml) as well as a saturating dose (300 units/ml) to test the possibility that certain effects of IFN treatment which are manifested at low doses may become masked at higher doses (27). We found no evidence to support this notion. At each concentration of IFN-αA, synthesis of each of the five VSV proteins was inhibited to the same extent. In addition, our data presented in Fig. 1 is at variance with the assertion that IFN treatment causes an impairment of the glycosylation of the viral envelope protein G (28). If this were the case, then we would have expected to see G protein shift to an apparent lower molecular weight as the duration of IFN treatment increased, since unglycosylated G has a higher mobility than glycosylated G in NaDodSO₄-polyacrylamide gels (29).

The extent of the IFN-induced inhibition of VSV protein synthesis was not as great as the resultant decrease in viral infectious units for each temporal dose of IFN-αA at either a saturating or low concentration dose of IFN-αA (Fig. 2). We also found that the amount of viral protein which was incorporated into assembled nucleocapsids was not as rapidly reduced by IFN treatment as was viral infectivity (1). In both cases, these disproportionate reductions reflect the fact that VSV assembly is a net multimolecular reaction, and IFN inhibition reduces the concentrations of all of the participating reactants, the genome and the five viral polypeptides. Thus, for any given dose of IFN, the total effect on infectious units would be expected to exceed the decrease in synthesis of any single viral protein. We do not think it is necessary to invoke a further IFN-induced effect on any process subsequent to VSV protein synthesis.

A secondary consequence of IFN action on VSV in U cells is immediately evident in Fig. 1 i.e. as viral protein synthesis was progressively inhibited by increasing IFN treatments, there was an accompanying relief of the virally induced shutoff of host protein synthesis. Most, if not all, cellular protein bands which were absent or severely reduced in untreated VSV-infected cells increased in intensity as a function of IFN treatment, and at the higher doses of IFN they were restored to the levels of synthesis observed in uninfected cells. We have obtained the same result in IFN-treated, VSV-infected COS-1 (monkey) cells, and similar observations have been made by Baxt et al. (30) with both human amnion U cells and a monkey kidney cell line, LLC-MK2. In contrast, in murine S49 cells, primary rabbit kidney cells (31), and HeLa cells (32), IFN treatment did not reverse VSV-induced inhibition of host protein synthesis. It may be significant that, with one exception (31), IFN prevented the VSV shutoff of host protein synthesis when cells were grown in static monolayers, whereas, in all cases where cells were grown in suspension culture, IFN treatment failed to prevent shutoff. The mechanism of VSV inhibition of host cell protein synthesis possibly depends on this same variable (33).

In order to determine whether the effect of IFN-αA on total (primary plus secondary) VSV protein synthesis was due to an inhibition of viral transcription, viral mRNA translation, or both of these functions, we examined the synthesis and translation of primary viral transcripts in IFN-treated U cells infected with the VSV mutant tsG41 at the nonpermissive temperature (Figs. 3-5, Table 1). An IFN dose of 300 units/ml for 6 h was seen to produce 9- and 11-fold decreases, respectively, in the rates of primary synthesis of N and M mRNA levels observed with tsG41. This does not affect our conclusions about the relative effects of IFN treatment on viral transcription and translation.

As a result of differential rates of amplification from primary to secondary message, there were 10-fold lower levels of N and M mRNA in wild type VSV-infected U cells which had been treated with IFN-αA for 6 h as compared with untreated controls (Figs. 6 and 7). These RNA blot hybridizations also showed that in the IFN-treated sample, the ratio of N or M mRNA to 42 S viral RNA was at least as great as the same ratio in the untreated sample. This suggests that, unless IFN treatment grossly altered the relative amounts of 42 S+ and 42 S− viral RNA, at least as much N and M mRNA was synthesized per template in IFN-treated cells as in untreated cells, which supports our previous conclusion that IFN treatment does not directly affect viral transcription. It is also apparent from Figs. 6 and 7 and from blot hybridizations of much larger concentrations of the same samples that IFN treatment did not detectably increase the amounts of defective interfering particles or polycistronic mRNA species relative to the amounts of mRNAs of normal size. Thus, in IFN-treated cells, there was no marked increase in the frequency of aberrant viral transcription.

A comparison of our mRNA synthesis and protein synthesis data shows that an IFN-αA treatment of 300 units/ml for 6 h caused amounts of primary VSV mRNA approximately equal to those in untreated cells to be translated, on the average, into 10-fold less viral protein. At the level of secondary macromolecular synthesis, 10-fold lower amounts of viral

3 P. S. Masters and C. E. Samuel, unpublished results.
4 N. Ulker and C. E. Samuel, unpublished results.
message in IFN-treated cells were translated, on the average, into 23-fold lower amounts of viral protein. Thus, it would appear that IFN-induced translation inhibition was roughly four times as effective at the level of primary protein synthesis than at the level of secondary protein synthesis. A possible explanation for this could be that the IFN-induced translation inhibiting activity was well in excess of viral mRNA at the primary level but became saturated with viral mRNA at the secondary level. However, an alternative interpretation is suggested by the recent finding that in VSV-infected cells, by 4 h postinfection a substantial fraction of viral message becomes sequestered by VSV N protein into translationally inactive particles (36). In IFN-treated cells, since there was a reduction in the concentration of both N protein and secondary viral message, it would be expected that proportionately less sequestering of message would occur. If this is correct, then the actual quantity of free, translatable secondary viral mRNA in IFN-treated, VSV-infected U cells may have been considerably more than one-tenth of that in untreated cells. Consequently, the degree of IFN-caused translation inhibition at the secondary level may have been greater than we originally estimated.

In order to determine whether viral mRNA from IFN-treated cells was functionally altered, RNA from untreated and IFN-treated U cells infected with wild type VSV was translated in wheat germ cell-free extracts. RNA samples were diluted appropriately so that protein synthesis reactions were programmed with roughly the same amount of viral and cellular RNA. Viral message from IFN-treated cells, under all conditions tested, was somewhat less efficiently translated than viral message from untreated cells, but this small difference in translatability was insensitive to potassium ion concentration in vitro nor to the presence or absence of AdoHcy, and it did not diminish in the presence of AdoMet. Our data, then, is at variance with the recent report that an inhibition of mRNA cap methylation is brought about by IFN treatment of HeLa cells (23). Such an effect on cap methylation would have been distinguished by the various reaction conditions which we used in the wheat germ system in vitro translations (11, 24). Ball and White (37) likewise did not detect an IFN-mediated alteration in methylation of VSV mRNA synthesized in vitro by purified VSV added to extracts of untreated or IFN-treated chick cells.

The overall inhibition of VSV protein synthesis observed in IFN-treated U cells cannot be accounted for by a difference in either the structural or functional integrity of the VSV mRNA. This implies that in IFN-treated cells, some component of the translational machinery is altered in such a way that it recognizes and discriminates against VSV message. A comparison of Fig. 1 with Fig. 8 supports this interpretation. Treatment of U cells with 300 units/ml of IFN-αA for 6 h reduced viral protein synthesis in vivo to levels just barely detectable with respect to cellular protein bands (Fig. 1, lane g). However, when RNA was purified from VSV-infected cells which had been pretreated with the same IFN dose, it programmed the in vitro synthesis of viral proteins well in excess of the most prominent cellular protein bands (Fig. 8, lanes b, e, h, or k).

In this and the previous paper (1), we have shown that during the major kinetic phase of its action, IFN-αA inhibits VSV replication in human amnion U cells primarily, and probably solely, at the level of viral protein synthesis. This conclusion supports some prior in vivo (15, 30, 32) and in vitro (37) investigations but is at variance with other studies which reported an effect by IFN on primary viral transcription (12, 13). Our differences with these latter authors may derive from the reagents and methods of analysis used. In the work reported here, we have used cloned human IFN preparations free of any other eukaryotic proteins; we have also tried, wherever possible, to directly examine individual viral molecular species to assess the effects of IFN inhibition. While our results do not rule out an effect on VSV transcription at later times of IFN treatment (12–24 h), the apparent inhibition of primary transcription observed by others (12, 13) may have derived from a small IFN-induced difference in the uptake and uncoating of infecting virions (38).

Numerous studies of IFN action have focused on two translation-inhibiting enzymic activities induced by IFN in many animal cell lines, the P<sub>e</sub> or P<sub>e</sub>P<sub>2</sub>ε protein kinase (39) and the 2'5'-oligoadenylate synthetase (40), both of which are dependent upon double-stranded RNA. The 2'5'-oligoadenylate synthetase, when activated, polymerizes ATP into molecules of the series pppA<sub>2</sub>p<sub>5</sub>A<sub>n</sub> which, in turn, activate a constitutive endoribonuclease (41). Our finding that there is no significant reduction in the levels of primary VSV mRNA as a function of IFN treatment precludes a role for the latter enzyme system in the mechanism of IFN action against VSV in human cells. The data presented in this paper are compatible with an involvement of the IFN-induced protein kinase in the inhibition of VSV replication. However, other recent studies (42–44) make it unlikely that this enzyme system is necessary or sufficient to inhibit VSV in human cells. We are presently trying to learn the nature of the IFN-induced translation-inhibiting activity that operates against VSV.

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