Mechanism of Interferon Action

EXPRESSION OF VESICULAR STOMATITIS VIRUS G GENE IN TRANSFECTED COS CELLS IS INHIBITED BY INTERFERON AT THE LEVEL OF PROTEIN SYNTHESIS*

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The effect of interferon on the expression of the vesicular stomatitis virus glycoprotein G gene was examined in simian COS cells transfected with the expression vector pSVGL containing the G gene under the control of the SV40 late promoter. When COS cells were treated with interferon 24 h after transfection, the synthesis of vesicular stomatitis virus G protein was inhibited by about 80% as compared to that in untreated controls. By contrast, under the same conditions, neither the plasmid copy number nor the G gene mRNA levels were detectably affected by interferon treatment. Likewise, the synthesis of simian virus 40 large T-antigen was not inhibited by interferon treatment of transfected COS cells even though the synthesis of vesicular stomatitis virus G protein was markedly inhibited. The residual G protein synthesized in transfected, interferon-treated COS cells appeared to be normally glycosylated.

Interferons are a family of regulatory proteins that profoundly affect a number of biologic processes in animal cells including their ability to support the replication of a wide range of RNA and DNA animal viruses (1–3). In many animal virus-host cell systems, the principal step of viral replication inhibited by IFN[1] is the synthesis of virus-specific macromolecules. Previous studies by this laboratory using highly purified, molecularly cloned human IFN-α and employing vesicular stomatitis virus (VSV) as the challenge virus have shown that the major and possibly only effect of IFN-α on VSV replication in human amnion U cells is translation inhibition (4–6). The translation of VSV primary transcripts within IFN-α-treated, VSV-infected U cells is selectively inhibited under conditions where overall cellular protein synthesis is unaffected by IFN-α treatment (5, 6). The inhibition of VSV primary protein synthesis by IFN-α does not involve a detectable quantitative or qualitative effect of IFN-α on VSV primary mRNA synthesis. Neither the structural nor the functional integrity of VSV mRNA is affected by IFN-α treatment as measured by Northern gel-blot hybridization or by in vitro translation of viral mRNA synthesized in vivo (5, 6).

In contrast to the specific IFN-mediated inhibition of the translation of VSV transcripts in vivo, the mode of action of IFN-α on SV40 replication in permissive cells appears to be mediated at a step other than the inhibition of viral mRNA translation. Various lines of evidence indicate that a very early step in SV40 replication, possibly the uncoating of SV40 virions, is inhibited by IFN-α treatment of simian cells prior to infection with virions (7–12). IFN treatment of simian cells after infection with intact SV40 virions does not reduce the synthesis of early or late RNA and polypeptides or yield of infectious SV40 progeny (8–10) even though the cells are IFN-sensitive, as measured by the inhibition of reovirus polypeptide synthesis in cells doubly infected with reovirus and SV40 (8, 9). Furthermore, the synthesis of SV40 early RNA, T-antigen, and DNA is not significantly inhibited in simian cells pretreated with IFN and then transfected with free SV40 DNA (7). In SV40-transformed mouse cells, neither T- nor t-antigen synthesis is inhibited by IFN (11, 12). These observations collectively indicate that the translation of SV40 mRNAs, like that of most cellular mRNAs, is not adversely affected by IFN treatment under conditions that lead to reduced VSV and reovirus mRNA translation.

A direct experimental approach toward understanding the structural basis of the IFN-induced inhibition of viral mRNA translation and the selective discrimination of the inhibition is to transfect animal cells with suitable recombinant DNA vectors containing individual viral genes and then to study the transient expression of these genes as a function of IFN treatment before and after the introduction of controlled alterations in their sequences. In view of the differential sensitivities of SV40 as compared to VSV and reovirus mRNAs toward the IFN-mediated inhibition of protein synthesis in simian cells (5, 9), the gene coding for the G protein of VSV was expressed in simian COS cells under the control of the SV40 late promoter, and the effect of human IFN-α on its expression was examined. In this study, we present evidence that the VSV G gene in an SV40 vector retains its sensitivity to IFN at the level of protein synthesis after transfection into an IFN-sensitive simian cell.

EXPERIMENTAL PROCEDURES

Materials—Plasmid vectors pSVGL and pGR125 (14) were generously provided by Dr. J. K. Rose, Salk Institute, San Diego, CA. DEAE-dextran (average M_r 500,000) was obtained from Pharmacia P-L Biochemicals; protein A-Sepharose, chloroquine diphosphate, and tunicamycin were purchased from Sigma. The sources of all other materials have been described (5, 14).

Cells, Virus, and IFN—COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Stocks of the Indiana serotype of VSV were obtained from confluent monolayers of mouse L929 cells as described (4). Sendai virus-induced human leukocyte IFN-α was generously provided by Dr. K. Cantell, Central Public Health Laboratory, Helsinki, Finland. The specific

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[1] The abbreviations used are: IFN, interferon; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; VSV, vesicular stomatitis virus; DMEM, Dulbecco's modified Eagle's medium.

activity was 1.9×10^6 units/mg of protein.

Measurement of the Antiviral State against VSV in COS Cells— The induction of antiviral activity against VSV as a function of IFN concentration or duration of IFN treatment was measured as the reduction of single cycle virus yield essentially as described earlier (4) except that monolayers of COS cells (LUX 21-cm² dishes) were used instead of human amnion U cells.

*Transfection of COS Cells—*Monolayer cultures (LUX 21-cm² dishes) of COS cells that were about 75% confluent were transfected with pSVGL using the DEAE-dextran technique (15). Medium was removed by aspiration, and 1 ml of serum-free DMEM containing 600 μg/ml DEAE-dextran and 20 μg/ml plasmid DNA was added. After 30 min at 37 °C, the transfection medium was removed and 3 ml of DMEM containing 5% fetal calf serum and 100 μM chloroquine diphosphate was added (16); the dishes were then incubated for 3 h at 37 °C. After this period, the chloroquine-containing medium was removed, and the monolayers were washed two times with cold phosphate-buffered saline. Cells were then briefly trypsinized, collected by scraping, resuspended in DMEM containing 5% serum, and washed twice by centrifugation in the same medium. Cells were reseeded at one-half their original density. For the mock transfections, the same procedure was also carried out on COS cells but with the omission of plasmid DNA during transfection. Twenty-four hours post-transfection, dishes received either 3 ml of fresh medium (untreated) or medium containing IFN-α at a concentration of 1000 units/ml if not otherwise stated (IFN-treated).

*Labeling and Analysis of Proteins Synthesized in Vivo in COS Cells—*Monolayer cultures of COS cells, either transfected or not and then left untreated or IFN-treated for 24 h as indicated, were washed with methionine-free DMEM and then incubated for 1 h at 37 °C in methionine-free DMEM containing 0.5% fetal calf serum. The medium was then removed, and the monolayers were washed with methionine-free DMEM. Cultures were then labeled with methionine-free DMEM containing [³⁵S]methionine (100 μCi/ml, 1 ml/60-mm dish) for 90 min. The labeling medium was removed, and the cultures were washed with cold phosphate-buffered saline and then lysed by the addition of 1 ml of a 10 mM Tris-HCl (pH 7.4) buffer containing 1% Nonidet P-40, 0.4% deoxycholate, 66 mM EDTA, and 50 μg/ml phenylmethylsulfonyl fluoride. Nuclei were removed by centrifugation (12,000 × g, 2 min), and 5 μl of anti-VSV antiserum (6) were added to the supernatant fraction. After incubation for 16 h at 0 °C, antigen-antibody complexes were precipitated with protein A-Sepharose, washed three times with cold STN buffer (9, 17), and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography as previously described (5). Levels of T-antigen were similarly measured in extracts prepared from parallel [³⁵S]methionine-labeled IFN-treated and -untreated dishes using hamster anti-SV40 T-antigen serum (9).

[³⁵S]Methionine-labeled VSV polypeptides, used as markers during electrophoresis, were prepared by pulse labeling COS cells 4–4.5 h post-infection, essentially as described (5). Labeled VSV polypeptides were also prepared from tunicamycin-treated, VSV-infected cells. In this case, tunicamycin treatment (0.5 μg/ml) was initiated 1 h post-infection and was continued throughout the subsequent incubation.

*RNA and DNA Dot-blot Hybridizations—*Plasmid DNA and G-specific mRNA levels in IFN-treated, pSVGL-transfected cells were compared to those in untreated, transfected cells by dot-blot hybridization. For this purpose, dishes parallel to those used for quantitation of VSV G protein and SV40 T-antigen were analyzed. For measurement of DNA copy number, cellular extracts were prepared by the procedure of Hirt (18) and, in order to hydrolyze RNA, made 0.5 N in NaOH and then incubated for 12 h at 37 °C (19). Extracts were then heated at 100 °C for 5 min to fragment plasmid DNA (20), chilled, neutralized with concentrated HCl, and then denatured with formaldehyde directly as in the case of cytoplasmic RNA extracts as described below. For comparison of G gene-specific RNA levels, the direct cytoplasmic dot hybridization procedure of White and Bancroft (21) was employed. Dot blots were also examined with purified total cytoplasmic RNA (22) prepared from IFN-treated and -untreated COS cells. For all dot-blot hybridization analyses, serial 2-fold dilutions of equal quantities of crude extracts or purified total RNA were made in 20 × SSC and transferred directly to nitrocellulose using a Bethesda Research Laboratories Hybri-Dot manifold (28).

Filters were probed with plasmid pGR125 containing cDNA to the VSV G gene (13) labeled with [α-³²P]dTTP by nick translation (23) to a specific activity of 8×10^7 cpm/μg DNA. Hybridization and subsequent washing of filters were done under stringent conditions, described in detail earlier (5, 6). Under these conditions, DNA and

RNA blots of extracts prepared from mock-transfected cells or RNA blots of extracts prepared from transfected cells but which had been alkali-treated (0.5 N NaOH, 12 h, 37 °C) and then neutralized prior to blotting did not show any detectable hybridization with the labeled G gene probe.

RESULTS

*Induction of the Antiviral State in COS Cells—*The effect of different concentration doses of human leukocyte IFN (IFN-α) on the replication of VSV was examined in simian COS cells. As shown in Fig. 1A, a progressive inhibition of VSV replication in COS cells occurred as a function of treatment for 24 h with increasing concentrations of IFN-α. At an IFN dose of 300 units/ml, the yield of infectious VSV progeny was reduced by more than 99% relative to that of untreated controls. The kinetics of induction of the antiviral state in COS cells was also examined (Fig. 1B). The observed kinetic curve exhibited a short lag period (1–2 h) followed by a rapid but biphasic decline during which the major extent of viral inhibition occurred. These observations regarding the IFN dose dependence and the biphasic kinetics of induction of the antiviral state, as well as studies on the IFN-mediated inhibition of VSV polypeptide synthesis (data not shown), are similar to the results previously obtained during a detailed analysis of the inhibition of VSV replication in human amnion U cells by IFN-α (4–6).

*Effect of IFN-α on Protein Synthesis in pSVGL-transfected COS Cells—*In order to study the effect of IFN-α on the expression of a single viral gene in the absence of virion infection, we chose the expression vector pJC119 (24) and the VSV Indiana G gene (13). The expression vector construct pSVGL contains the complete coding sequence for VSV G protein under the control of the SV40 late promoter in pJC119 (14). The vector can be propagated in simian COS-1 cells which support its replication by supplying SV40 large T-antigen (25). The G protein encoded by pSVGL and expressed in COS cells is of the correct size, is glycosylated, and is functionally normal (14).
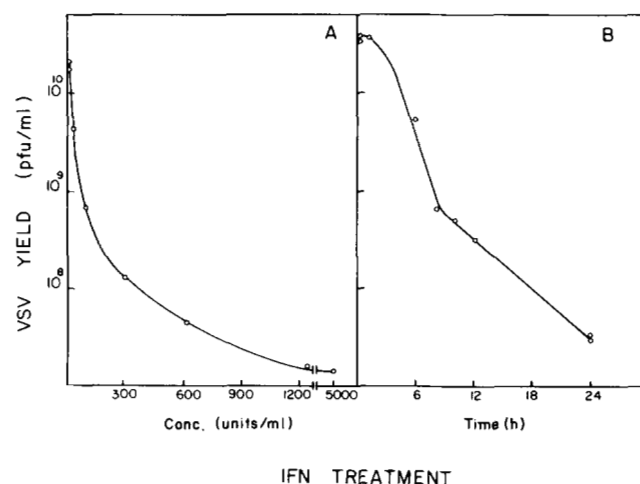

IFN TREATMENT

FIG. 1. **Induction of the antiviral state against VSV in simian COS cells treated with IFN-α.** *A*, concentration dose dependence of the IFN-α-induced antiviral activity against VSV in COS cells. Monolayers of COS cells were treated with varying concentrations of IFN-α for 24 h and then infected with VSV at a multiplicity of 10 plaque-forming units (*pfu*)/cell in the presence of actinomycin D. After 12 h of incubation, viral yields were determined. *B*, kinetics of induction of the antiviral state against VSV in COS cells treated with IFN-α. COS cell monolayers were treated with a saturating concentration dose (1000 units/ml) for various lengths of time and then infected with VSV in the presence of actinomycin D. The virus yields after 12 h were then determined.

As shown in Fig. 2 (*lanes 5* and *6*), G protein encoded by pSVGL was readily detected in untreated COS-1 cells at 48 h after transfection by pulse labeling with [^{35}S]methionine and immunoprecipitation. However, if at 24 h after transfection the COS-1 cells were treated with a saturating concentration dose of IFN for 24 h and then pulse-labeled with [^{35}S]methionine, the amount of G protein synthesized was significantly reduced as compared to untreated cells (Fig. 2, *lanes 5–8*). In contrast to the inhibitory effect of IFN-α on VSV G protein synthesis in transfected COS-1 cells (Fig. 2, *lanes 5–8*), IFN-α treatment with a saturating concentration dose (1000 units of IFN/ml) did not affect SV40 T-antigen synthesis in the transfected COS-1 cells (Fig. 2, *lanes 9–12*).

The extent of IFN-induced inhibition of G protein synthesis in pSVGL-transfected cells was dependent upon the IFN concentration used to treat the COS-1 cells (Fig. 3). The dose dependence for inhibition of G protein synthesis in pSVGL-transfected COS-1 cells (Fig. 3) was similar to that observed for inhibition of VSV replication in virion-infected COS-1 cells (Fig. 1). Likewise, the kinetics of the inhibition of G protein synthesis in IFN-treated transfected COS cells were similar to the kinetics observed in the case of VSV replication in virion-infected COS cells (data not shown). The extent of inhibition of IFN treatment of G protein synthesis in transfected cells was independent of the extract concentration analyzed by immunoprecipitation and densitometric scanning (Fig. 4), further establishing the quantitative nature of the assay. Treatment with 1000 units/ml IFN routinely caused about a 5-fold reduction in G protein synthesis in pulse-labeled IFN-treated as compared to untreated transfected COS-1 cells, as shown for example by the results in Fig. 4.
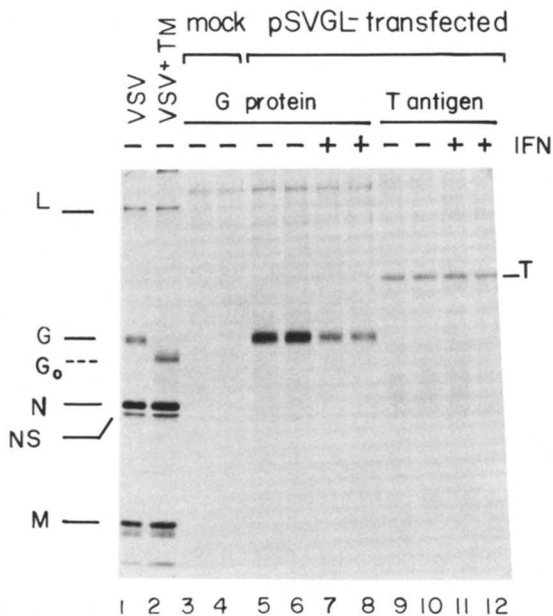

FIG. 3. **Concentration dose dependence of the inhibition of G protein synthesis by IFN-α in transfected COS cells.** Monolayers of pSVGL-transfected COS cells were either left untreated or treated 24 h post-transfection with the indicated concentration doses of IFN-α. After 24 h, cells were pulse-labeled with [^{35}S]methionine. Extracts were then analyzed (as in Fig. 2) by SDS-PAGE and fluorography after immunoprecipitation with anti-VSV antiserum as described under "Experimental Procedures." G protein synthesis in transfected COS cells was quantitated by densitometric scanning of the fluorograph (*inset*) using an LKB Ultroscan XL Laser densitometer. The data were then plotted as a function of increasing concentration of IFN-α.

FIG. 2. **Effect of IFN-α treatment on the synthesis of VSV G protein and SV40 T-antigen in transfected COS cells.** COS cells were transfected with pSVGL (*lanes 5–12*) or mock-transfected (*lanes 3* and *4*). After 24 h, cells were either left untreated (*lanes 3–6, 9,* and *10*) or treated with 1000 units/ml IFN-α (*lanes 7, 8, 11,* and *12*). Following incubation for 24 h, cells were labeled with [^{35}S]methionine. Extracts were prepared, immunoprecipitated with either anti-VSV antiserum (*lanes 3–8*) or SV40 anti-T antiserum (*lanes 9–12*), and analyzed by SDS-PAGE as described under "Experimental Procedures." *Lanes 1* and *2* contained nonimmunoprecipitated extracts made from [^{35}S]methionine-labeled, VSV virion-infected COS cells either left untreated (*lane 1*) or treated with 0.5 μg/ml tunicamycin (*TM*) following infection. The identities of the various VSV-encoded polypeptides are indicated on the left margin.
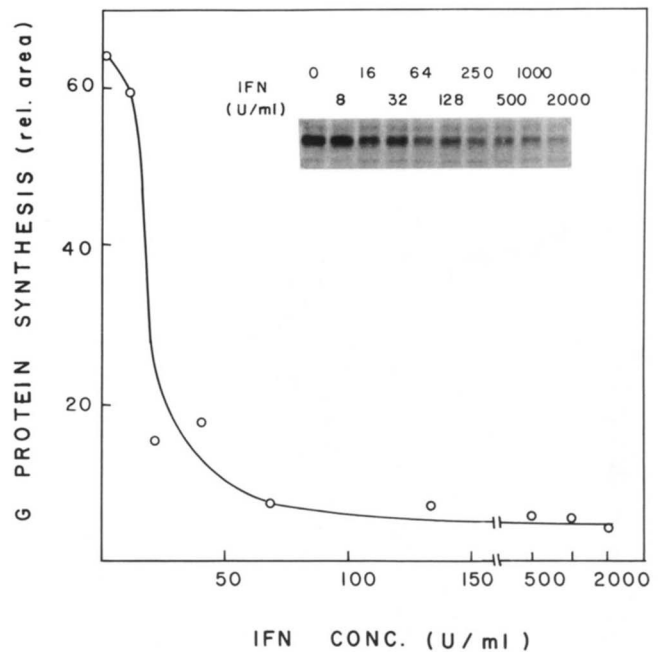
G protein synthesized in pSVGL-transfected COS-1 cells was indistinguishable from authentic G protein synthesized in virion-infected COS-1 cells as measured by mobility on SDS-PAGE (Fig. 2, *lanes 1, 5,* and *6*). However, the mobility of underglycosylated G protein (G_0) produced in virion-infected COS-1 cells as the result of treatment with the glycosylation inhibitor tunicamycin (26, 27) was readily distinguishable from normally glycosylated G protein (Fig. 2, *lanes 1* and *2*). Although IFN-α treatment caused about an 80% reduction in the amount of G protein synthesized in the experiment shown in Fig. 2, the mobility of the residual G protein synthesized in IFN-treated cells was typical of normally glycosylated G rather than underglycosylated G_0 (Fig. 2, *lanes 1, 2,* and *5–8*).

Effect of IFN-α on Nucleic Acid Synthesis in pSVGL-transfected COS Cells—The IFN-induced inhibition in the synthesis of G protein observed in pSVGL-transfected COS cells (Fig. 2) could potentially be caused by a block either at the level of plasmid DNA replication or G mRNA transcription or, as demonstrated in the case of VSV-infected human amnion cells (4–6), at the level of viral mRNA translation. In order to distinguish between these possibilities, we measured by dot-blot hybridization the levels of plasmid DNA and G protein-encoding mRNA in untreated as compared to IFN-α-treated COS cells transfected with pSVGL. Quantitation of G-specific DNA and RNA in transfected cultures (Fig. 5) was carried out with cultures in parallel to those in which G protein synthesis was measured (Fig. 2). As shown in Fig. 5, the relative levels of G-specific DNA and RNA in untreated and IFN-treated transfected cells were comparable. Furthermore, Northern blot analysis of whole cytoplasmic RNA also
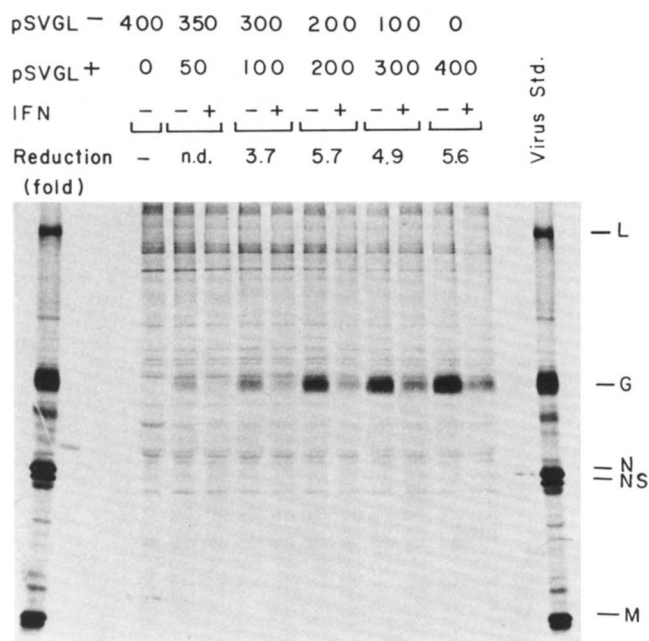
FIG. 4. **Quantitation of the IFN-induced inhibition of G protein synthesis in pSVGL-transfected COS cells.** Monolayers of COS cells were either mock-transfected or transfected with pSVGL. After 24 h, transfected cells were either left untreated or treated with a saturating concentration dose (1000 units/ml) of IFN-α. Following incubation for 24 h, cells were pulse-labeled with [^{35}S]methionine, and extracts were prepared as described under "Experimental Procedures." Varying amounts (0, 50, 100, 200, 300, and 400 μl) of extract prepared from either IFN-treated or -untreated pSVGL-transfected COS cells were then mixed with varying amounts of extract prepared from mock-transfected COS cells (total volume of mixture = 400 μl) and reacted with a constant amount (2.5 μl) of anti-VSV antiserum. Immunoprecipitates were then analyzed by SDS-PAGE and fluorography. G protein synthesis in the respective samples was quantitated by densitometric scanning. The fold inhibition of G protein synthesis in IFN-treated cells as compared to untreated cells using differing amounts of cell extract is indicated. *n.d.*, not determined.

FIG. 5. **Dot-blot analysis of VSV G gene-specific DNA and mRNA synthesized in IFN-α-treated COS cells transfected with pSVGL.** COS cell monolayer cultures parallel to those analyzed for protein synthesis (Fig. 2) were either mock-transfected (*lanes 1, 4,* and *7*) or transfected with pSVGL (*lanes 2, 3, 5, 6, 8,* and *9*). After 24 h, transfected cells were either left untreated (−) or IFN-treated (+) with a dose of 1000 units/ml IFN-α for 24 h. Monolayers were then processed, as described under "Experimental Procedures," either for the analysis of total G-specific DNA (*left panel*) or RNA (*center panel*) present in crude cell-free extracts or for the analysis of purified cytoplasmic G-specific RNA isolated from crude extracts (*right panel*). Reciprocal 2-fold dilutions (top to bottom in each row) of each sample were transferred to nitrocellulose filters and then probed with [α-^{32}P]dTTP-labeled cDNA to the VSV G gene mRNA.

did not reveal any qualitative or quantitative difference between G gene transcripts formed in IFN-treated as compared to untreated pSVGL-transfected COS cells (data not shown).

DISCUSSION

The observations reported in this paper strongly indicate that the IFN-induced inhibition of the expression of the VSV G gene under the control of the SV40 late promoter in pSVGL-transfected COS cells is brought about at a step subsequent to plasmid DNA replication and transcription of the G gene sequence. The inhibition of G protein expression in transfected cells is possibly exerted at the level of translation.

The extent of the inhibition of G protein synthesis in transfected cells treated with a saturating concentration of IFN-α was typically between 5-fold, as was obtained in most experiments (*e.g.* as shown in Figs. 2 and 4), and about 7-fold, as was obtained in some experiments (*e.g.* as shown in Fig. 3). The inhibition of G protein synthesis in pSVGL-transfected COS cells was not as great as that observed for the inhibition of G protein synthesis in wild-type VSV virion-infected cells. In wild-type virion-infected cells, the inhibition of viral polypeptide synthesis by a saturating dose of IFN-α is nearly complete (5, 6). There are two possible explanations for this apparent difference. First, primary plus secondary expression is affected in wild-type VSV-infected cells, whereas conceptually only primary expression is affected in trans-
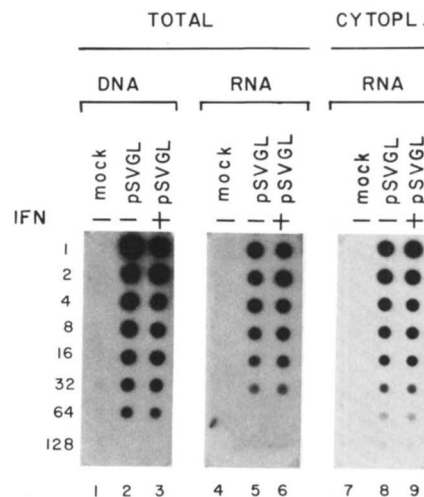
fected COS cells. Indeed, in previous studies from our laboratory (4–6), wherein primary translation was examined directly by use of the VSV mutant *ts* G41 which is temperature-sensitive in RNA replication, the IFN-induced inhibition in the translation of VSV primary transcripts was determined to be about 10-fold as compared to untreated controls. Second, the hybrid mRNA encoding the G protein in transfected cells possesses significant 5'-untranslated SV40 sequences preceding the 5'-untranslated VSV G mRNA nucleotides and has only 7 of the 29 5'-untranslated VSV G mRNA nucleotides. Nevertheless, in view of the fact that in this simplified model system the effect of IFN-α qualitatively approximated that observed in the case of infectious VSV virions, mutational analysis of the VSV G gene may permit the identification of regions in the VSV G mRNA, if indeed discrete regions exist, that are responsible for its reduced translation after IFN treatment.

It has been previously reported that IFN inhibits the oncogenic transformation and stable (*i.e.* long-term) expression of genes of both viral and cellular origin after their transfection into rodent cells by the CaPO$_4$ technique (31, 32). This inhibition seemed to be exerted at the level of stabilization and/or integration of exogenous DNA sequences into the cellular genome. In contrast, IFN was found not to prevent transient gene expression when the exogenous plasmid DNA was present in unintegrated form (32). Our results concerning the effect of IFN-α on the transient expression of the VSV G gene are consistent with these earlier studies by Chany and co-workers (32). Under conditions where the synthesis of G protein was markedly reduced by IFN treatment, we found that the levels of plasmid DNA and G-encoding mRNA were essentially unaltered, thereby suggesting that the inhibition was brought about at a step subsequent to plasmid DNA replication and G mRNA transcription. The inhibition of G protein expression in transfected cells was thus possibly ex-

erted at the level of translation.

Finally, the observation that the reduced amount of G protein synthesized in IFN-treated, transfected simian COS cells was apparently normally glycosylated is consistent with prior studies from our laboratory on the effect of IFN-α on VSV replication in human U cells (4-6). By contrast, Maheshwari *et al.* (29, 30) have reported that IFN treatment causes the underglycosylation of VSV G protein to an extent comparable with that observed in tunicamycin-treated VSV-infected cells.

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