JOURNAL OF VIROLOGY, June 1990, p. 2588–2593
0022-538X/90/062588-06$02.00/0
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Vol. 64, No. 6

Specific Effect of Interferon on the Herpes Simplex Virus Type 1 Transactivation Event

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Received 6 December 1989/Accepted 21 February 1990

Human recombinant gamma interferon and to a lesser extent alpha interferon were shown to inhibit herpes simplex virus type 1 replication in the human cell lines WISH and HEp-2. Dot blot analysis of viral DNA synthesis and viral RNA transcription indicated that the inhibition occurred at an early step in infection. A study of the early events after herpes simplex virus type 1 infection indicated that adsorption, penetration, uncoating, and transport of viral DNA were not affected by interferon. Northern (RNA) blot analysis revealed that both immediate-early and delayed-early gene transcription was inhibited by interferon. Transactivation of the immediate-early responsive element linked to a reporter gene (CAT or *tk*) was specifically inhibited by both classes of interferon. Our data would indicate that either the transactivating protein VP16 or the complex formed between VP16 and a host protein(s) is attenuated by interferon.

The molecular basis of interferon (IFN) antiviral activity has been only partly elucidated. The double-stranded RNA-dependent 68-kilodalton protein kinase complex and the 2′-5′-oligoadenylate synthetase induced by IFN treatment of responsive cells have been directly correlated with the onset of the antiviral state for encephalomyocarditis and mengo virus (2). The mechanism whereby the IFN-induced genes discriminate between viral and cellular mRNAs has only been partially determined (7, 14). Both groups have proposed an intracellular localization of the 2′-5′-oligoadenylate synthetase and the protein kinase activities in the vicinity of viral double-stranded RNA molecules. Many DNA viruses are sensitive to IFN action by mechanisms still unknown (13).

Herpes simplex virus type 1 (HSV-1) replicates via the expression of at least three groups of genes regulated in a temporal cascade: immediate-early (IE), delayed-early (DE), and late (L) genes (6). Two IE genes, ICP0 and ICP4, are strictly required for the transcription of the DE genes (6, 22). No expression of DE and L genes occurs if IE genes are not properly expressed (since at least one of the DE genes is a transactivator of L genes). L genes are expressed only after the start of DNA synthesis, and HSV DNA is synthesized by a viral DNA polymerase that belongs to the DE gene group. All five IE genes share a common characteristic: an upstream enhancerlike regulatory sequence containing the motif TAATGARAT (R = purine) (18, 20, 26). This sequence is involved in the regulation of IE genes through a transactivation event mediated by the virion (tegument) protein VP16 (also called Vmw65 or *trans*-inducing factor). Preston et al. (20) and O'Hare et al. (17, 18) have shown by gel-shift assays that the IE gene transactivation by VP16 occurs via the formation of a protein complex (IE complex [IEC]) rather than by direct interaction of the protein with HSV-1 DNA sequences. This complex is made up of the cellular octamer binding factor, TAATGARAT recognition factor (5), or αH (8) and possibly other cellular factors. The cellular component(s) of this complex are responsible for the interaction with DNA, while VP16 is required for interaction with the RNA polymerase II (21, 23).

The model described for the IE transactivation suggests that VP16 is required for efficient infection with HSV-1. This concept has been confirmed by Triezenberg and co-workers (4), who demonstrated that cells expressing a truncated form of VP16 (lacking the activating C-terminal domain) are restricted in HSV-1 replication.

Obermann and Panet (15, 16) have studied the effect of alpha IFN (IFN-α) on the early stages of HSV replication. They report inhibition of IE gene transcription but no effect of IFN on any steps prior to transactivation. Viral uncoating appears to be normal in IFN-treated cells. These authors propose that the effect of IFN-α must be on some facet of the VP16-complex interaction (16).

In agreement with the above findings, we report an effect of IFN-γ on the early stages of HSV-1 replication. Viral DNA synthesis, as well as IE and DE gene transcription, is inhibited in IFN-treated cells. Similar results were found when chimeric plasmids were used, involving activation of IE genes by VP16. These results indicate that inhibition of HSV-1 replication by IFN involves a step in the interaction of VP16 with the IEC complex or action on VP16 itself.

MATERIALS AND METHODS

Cell lines and viruses. Cell lines and viruses were obtained from the American Type Culture Collection (ATCC, Rockville, Md.). African green monkey kidney (Vero), human larynx carcinoma (HEp-2), amniotic (WISH), and murine L-929 cells were grown in Dulbecco minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 100 μg of streptomycin per ml, 100 U of penicillin per ml, 2 mM glutamine, and 5% fetal bovine serum (Hyclone, Logan, Utah).

HSV-1 (strain MP, provided by B. Roizman, Chicago, Ill.) was plaque purified and was grown and titered on Vero cells. Aliquots of the original stock obtained after plaque purification were stored at −80°C, and each vial was used only once. Encephalomyocarditis virus was purchased from ATCC (strain VR 129-B), plaque purified, and grown and titered on L-929 or WISH cells.

Viral infections. Viral infections were performed in 60- or 100-mm plastic tissue culture dishes (Corning Glass Works, Corning, N.Y.). Immediately prior to infection, the medium was removed and the monolayer was rinsed twice with phosphate-buffered saline. One of the cultures was

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trypsinized to calculate the total cell number per dish. A 0.2- to 0.4-ml portion of viral lysate (diluted to the appropriate concentration of PFU per milliliter) was added to the monolayer at a multiplicity of infection (MOI) of 1. Virus was allowed to adsorb at 37°C for 60 min, after which 3 or 10 ml of medium was added. Viral incubation was terminated at appropriate times, and cells were harvested either by using a cell scraper or by trypsinization.

IFN pretreatment of cells. WISH, HEp-2, or Vero cells were treated 14 to 16 h prior to infection with human recombinant IFN-α or -γ (Genentech Inc., San Francisco, Calif.) at the concentration shown in each experiment. Following the IFN treatment, virus was added as described above. Fresh IFN was added to the medium during viral incubation in some experiments. All IFN preparations were assayed routinely on WISH or HEp-2 cells by using encephalomyocarditis virus, and they were titered with National Institutes of Health standard IFN preparations.

Plaque assays. HSV-1 lysates were titered on Vero cells. Plaque assays were performed on monolayers of Vero cells overlaid with 1.3% methylcellulose and 2% calf serum. The monolayers were stained with crystal violet for plaque counting 36 to 48 h postinfection.

Purification and labeling of herpes viral particles. HSV-1 was grown on Vero or WISH cells as described above. Viral particles were purified by the method of Spear and Roizman (25), with some modifications. After clarification of the viral supernatants, the virus was pelleted by centrifugation in a rotor (model SW27; Beckman Instruments, Inc., Fullerton, Calif.) at 15,000 rpm for 2 h at 4°C. The viral pellet was then suspended in 50 mM Tris hydrochloride (pH 7.5)–10 mM EDTA and layered onto a continuous 5 to 40% sucrose gradient made in the same buffer. The virus was centrifuged through the sucrose gradient in a rotor (model SW27; Beckman) at 15,000 rpm for 2 h at 4°C. Purified virions were collected as a light-scattering band about halfway through the gradient by piercing the tube with an 18-gauge needle. Labeled virions were obtained by adding 5 μCi of [^{3}H]thymidine per ml to the incubation medium after viral adsorption.

RNA isolation. Total RNA from infected, transfected, and uninfected cells was isolated by the single-step method (1). RNA concentrations were determined by optical density readings at 260 and 280 nm.

Plasmids and molecular probes. HSV-1 recombinant plasmids pRB201 (containing IE genes on *Hin*dIII restriction fragments H and M in pBR322) and pRB123 (containing DE and L genes on *Bam*HI restriction fragment J in pBR322 [24]) were kindly provided by B. Roizman, Chicago, Ill.

Plasmid pIE110CAT (5) containing the IE regulatory sequence linked to a bacterial CAT gene in pUC9 was provided by P. O'Hare, Oxted, United Kingdom, and plasmid pS12TKU containing the IE regulatory sequence linked to the HSV *tk* gene was provided by C. M. Preston, Glasgow, United Kingdom. Plasmid pSAM-I containing a mouse *aprt* gene was provided by J. Tischfield (Indianapolis, Ind.).

When plasmids were used as probes, they were radiolabeled by using the oligonucleotide random-priming method (3), with some modifications. The DNA (0.2 μg) was denatured by boiling and was labeled in a reaction buffer containing 50 mM Tris hydrochloride (pH 7.2); 10 mM $MgSO_4$; 0.1 mM dithiothreitol; 50 μg of bovine serum albumin per ml; 1 nM (each) dCTP, dGTP, dTTP; 100 pM [α-^{32}P]dATP (Amersham Corp., Arlington Heights, Ill.); 1 μg of hexaoligonucleotide random primer; and 2 U of *Escherichia coli* DNA polymerase I Klenow fragment (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in a total volume of 30 μl. The reaction was incubated at 10°C for 12 h, after which the labeled DNA was purified by ethanol precipitation. This method routinely yields probes with specific activities of 1×10^8 cpm/μg. The probe was denatured by boiling before use.

Molecular hybridizations. The hybridization of DNA filters was by the method of Maniatis et al. (11). Some modifications were introduced for RNA filters. The nitrocellulose (NC) membranes were first wetted in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and underwent a prehybridization step for 2 h at 65°C in 3× SSC–35% formamide–1× Denhardt solution–0.5% sodium dodecyl sulfate–100 μg of sonicated salmon sperm DNA per ml. The prehybridization buffer was then removed, and the filter was hybridized for 12 h with the denatured probe in the prehybridization buffer adjusted to 10 mM EDTA. The filter was washed sequentially at room temperature with 2× SSC–0.5% sodium dodecyl sulfate for 15 min, 1× SSC–0.5% sodium dodecyl sulfate for 15 min, and 0.1× SSC–0.5% sodium dodecyl sulfate at 55°C until the radioactive background was low. Autoradiography was done overnight at −70°C with an intensifying screen. Dot blot assays for DNA and RNA were performed as described previously (19).

DNA transfections. DNA was transfected into cells by the DEAE-dextran (M_w 500,000; Pharmacia, Uppsala, Sweden) method (10), with some modifications. The DNA-DEAE-dextran mixture was prepared in prewarmed TBS buffer (25 mM Tris hydrochloride [pH 7.4], 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$) at 37°C. The mixture was then mixed with an equal volume of prewarmed 2× Dulbecco modified Eagle medium and layered onto washed monolayers. The final concentration of DEAE-dextran applied to the cells was 500 μg/ml, and the final concentration of DNA was 1 μg/ml. The volume used was 0.3 ml on 35-mm dishes, 0.8 ml on 60-mm dishes, and 2 ml on 100-mm dishes. Cells were incubated for 4 h, after which the medium was removed and 10% dimethyl sulfoxide in phosphate-buffered saline was added for 2 to 5 min at room temperature. Cells were then washed twice with phosphate-buffered saline and incubated in growth medium. After overnight incubation, the medium was replaced and IFN was added as needed. Samples were incubated a further 48 h and harvested.

RESULTS

Quantitation of HSV-1 viral yield in the presence of IFNs in different cell lines. In order to determine the in vivo effect of IFNs on HSV-1 replication, three different cell lines, HEp-2 (human epithelial fibroblasts), Vero (African green monkey kidney), and WISH (human amnion fibroblasts), were pretreated for 16 to 18 h with different amounts of human recombinant IFN-α or -γ and infected with HSV-1 at a calculated MOI of 0.1. The viral yield was determined by plaque assay (in duplicate samples) of the lysates 48 h later. The viral yield was compared with that of untreated controls (Table 1). (Previous experiments had shown that at a higher MOI, protection by 100 U of IFN (α or γ) per ml was not complete.) The virus titer decreased over 10,000-fold when WISH and HEp-2 cells were treated with at least 100 U of IFN-γ per ml. In Vero cells, however, large amounts of infectious virus were scored in the presence of IFNs, indicating that Vero cells are refractile to inhibition of HSV-1 by human IFN at this MOI. At lower MOIs, there was some inhibition. IFN-α appears to be considerably less effective than IFN-γ in inhibiting HSV-1 replication in these cell lines.

TABLE 1. Inhibition of HSV-1 replication by interferons[a]

IFN and cells used	Yield in untreated controls	Yield with following concn of IFN		
		10^2 U/ml	10^3 U/ml	10^4 U/ml
IFN-α				
Vero	6×10^7	5×10^7	2×10^7	1.2×10^7
WISH	4×10^5	2×10^5	5×10^4	4×10^4
HEp-2	2×10^5	1.3×10^4	3×10^3	2×10^3
IFN-γ				
Vero	6×10^7	4.9×10^7	1.9×10^7	1.1×10^7
WISH	4×10^5	$<10^2$	$<10^2$	$<10^2$
HEp-2	2×10^5	$<10^2$	$<10^2$	$<10^2$

[a] Effect of human IFN-α and IFN-γ on HSV-1 yield in Vero, WISH, and HEp-2 cells. Infection was at a MOI of 0.1. Values were determined by plaque assay of the viral lysates produced in the presence or absence of IFN. Plaque assays were performed on Vero cells. The results are expressed in PFU per milliliter.

In order to study the IFN effect under conditions of approximately total inhibition of virus replication, WISH cells treated with 1,000 U of IFN-γ and infected at a MOI of 1 were used in subsequent experiments. In some experiments, IFN-α was also used.

IFN-induced inhibition of HSV-1 DNA synthesis. A time course of the effect of 1,000 U of IFN-γ per ml on HSV-1-infected WISH cells was performed. Infected cells were harvested at different times after infection and immediately applied onto NC membranes. The samples were processed for DNA analysis as described previously (19). The results (Fig. 1) indicated a marked inhibition (and delay) of HSV-1 DNA synthesis in the presence of IFN-γ at an early time after infection (6 to 10 h). There was a recovery of viral DNA synthesis at 18 to 24 h postinfection, probably reflecting escape synthesis. That this delayed synthesis might be due to decay of the IFN effect was ruled out by adding high concentrations of IFN-γ to the incubation medium after viral infection. Since HSV DNA synthesis requires a specific DNA polymerase, a viral DE gene product, we examined transcription of IE and DE genes.

IFN-induced inhibition of HSV-1 transcription. (i) RNA dot-blots. WISH cells were treated with IFN-γ and infected

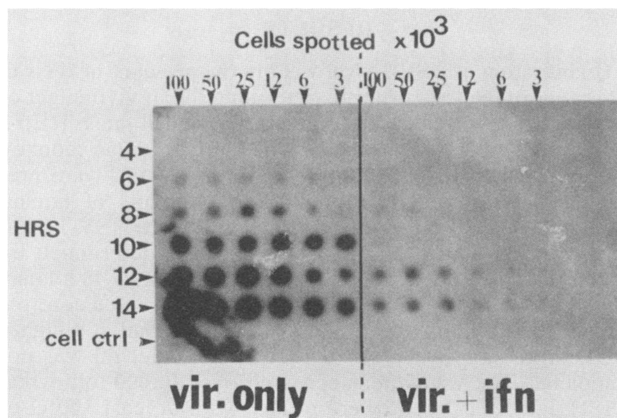

FIG. 1. HSV DNA replication in IFN-γ-treated WISH cells. Cells pretreated for 12 h with IFN-γ were infected with HSV-1 at a MOI of 1 in the presence of 10^3 U of IFN-γ per ml. Samples were collected at 2-h intervals. Twofold serial dilutions were blotted onto NC membranes, denatured, and immobilized for DNA hybridization as described previously (19). Plasmid pRB201 was used as a probe.

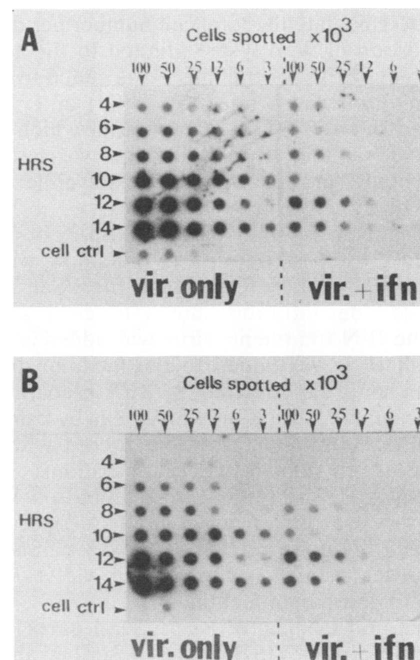

FIG. 2. Detection of HSV-1 RNA transcripts in IFN-γ-treated WISH cells. Cells were treated with IFN-γ and infected, and cells were harvested as described in the legend to Fig. 1. NC membranes were fixed in 1% glutaraldehyde and probed as described previously (19). (A) IE gene transcription (pRB201); (B) DE gene transcription (pRB123).

with HSV-1 as described in Materials and Methods. Cell samples were harvested and counted, and twofold dilutions were blotted onto NC paper (19). After fixation and proteolytic digestion, the filter paper was probed with labeled plasmids containing different HSV-1 DNA coding sequences. Figure 2 shows that the inhibition of the two types of viral messenger RNAs tested followed the pattern observed for DNA synthesis (cf. Fig. 1 and 2); HSV-1 IE gene transcripts appeared in the untreated control 4 h after infection (Fig. 2A), while glycoprotein D transcripts followed at 6 h (Fig. 2B). DNA synthesis was first detected at about 8 h. Thus, all steps of this cascade are inhibited by IFN-γ treatment. In order to confirm that specific mRNAs were inhibited by IFN-γ and that the results were not due to degradation of viral mRNA, the newly synthesized RNA was analyzed by Northern (RNA) blots.

(ii) Northern blot analysis. The steady-state level of transcription of IE and DE genes was determined by Northern blot analysis following IFN-γ treatment of WISH cells. Infected cell samples (treated and untreated) were harvested at 10 h postinfection, and RNA was resolved by denaturing agarose gel electrophoresis. The blots were hybridized with different HSV-1 probes, and the results obtained are shown in Fig. 3. A remarkable decrease in both the IE and DE signals indicates that IFN-γ specifically inhibited IE mRNA expression. DE gene transcription is probably inhibited as a consequence of the inhibition of IE expression, although we cannot eliminate a multitarget effect of IFN. Additionally, overexposure of the autoradiograms (Fig. 3B and D) showed that the same pattern of transcription found in the untreated control could be detected in the IFN-treated sample. This result suggests that the IFN-γ action was due to inhibition (or delay of the onset) of transcription rather than degrada-

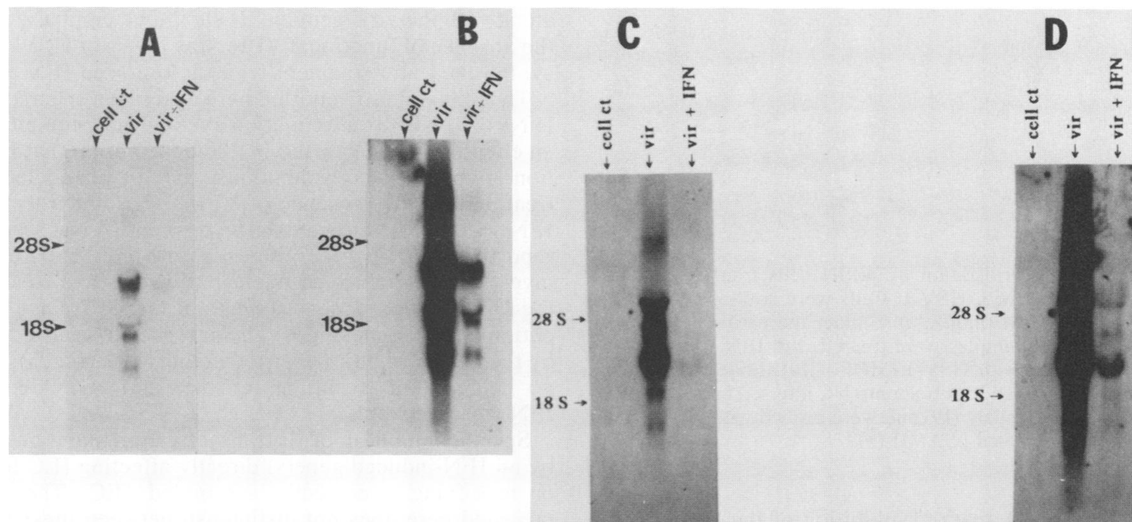

FIG. 3. Northern blot analysis of HSV-1 RNA transcripts. Total RNA was extracted from 10^7 WISH cells, either uninfected (cell ct) or pretreated with IFN at 6 h postinfection (vir + IFN). A 10-μg portion of each sample was run on a 1% agarose denaturing gel. The samples were transferred to NC and hybridized to labeled HSV-1 DE gene sequences (A and B) or IE gene sequences (C and D). Two exposures of each blot are presented to reveal intact messages in the IFN-treated samples. (A) 1-h exposure; (B) 20-h exposure; (C) 3-h exposure; (D) 13-h exposure.

tion of preexisting HSV IE messages. Degradation should result in a smear on the autoradiogram rather than intact bands. These results support the idea that IFN acts at (or prior to) the transcription of IE genes.

IFN effect on the HSV-1 attachment and penetration. Even though the above data indicate that IE transcription is the primary step inhibited, steps in viral infection that occur before transactivation could still cooperate in the general inhibitory effect that is observed in vivo. To test this, we measured (i) virus adsorption and penetration and (ii) viral uncoating and migration of viral DNA to the nucleus. The first stages of the HSV-1 infectious cycle involve the interaction of the virus with the host cell surface receptor (attachment), fusion of the viral membrane with the host cell membrane, and penetration of the viral particle into the host cell. These events were studied by adsorbing ^{3}H-labeled viral particles to permissive cells and samples harvested at various times after viral addition (the unadsorbed virus being washed off the cells with phosphate-buffered saline). The acid-precipitable radioactivity in the cells was then determined. The effect of IFN was studied by comparing the adsorption-penetration curve of untreated cells with the curves obtained with cells pretreated with 1,000 U of IFN-γ per ml overnight. No difference in the adsorption-penetration patterns of HSV-1 was found in the presence of IFN compared with an untreated control.

Dot-blot of DNA extracted from isolated nuclei. The transport of viral DNA to the nucleus in the presence of IFNs was studied by harvesting total DNA from isolated nuclei 4 h postinfection and then quantitating the amount of HSV-1 DNA contained in the nuclei by molecular hybridization (19). At this time, HSV-1 DNA has not yet replicated in WISH cells. These experiments showed no difference between the treated and untreated samples, indicating that IFNs do not inhibit transport of HSV-1 DNA to the nucleus and that uncoating and transport of viral DNA occur normally in both cases (data not shown).

IFN-induced inhibition of HSV-1 IE gene transactivation. To create a model system in which the transactivation event was isolated from other viral functions, a chimeric plasmid containing a reporter gene (CAT) under the control of the IE regulatory sequence was transfected into WISH cells. Transactivation was performed by adding intact HSV-1 at 60 h posttransfection for 6 h in the presence of cycloheximide. Figure 4 shows a Northern blot analysis of the total RNAs extracted from transfected cells and probed with a ^{32}P-

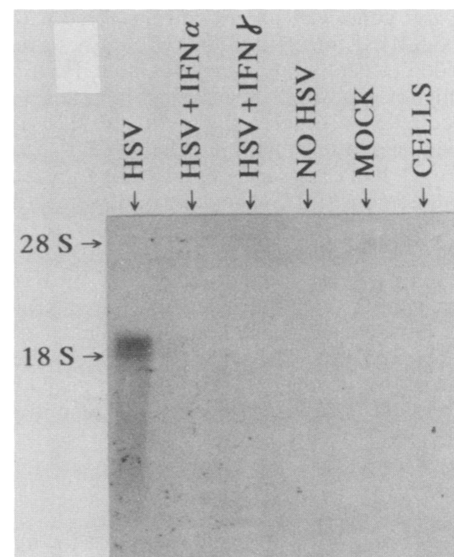

FIG. 4. Northern blot analysis of total RNA extracted from cells transfected with pIE110CAT and superinfected with HSV-1. Cells were transfected with 2 μg/100-mm plate with pIE100CAT DNA. Twelve hours later, IFN-α or -γ was added at 1,000 U/ml, and it was maintained on the cells throughout the experiment. At 60 h posttransfection, HSV-1 and cycloheximide (50 μg/ml) were added for 6 h and cells were harvested for RNA extraction. No HSV, The nontransactivated control; mock, transfection with pBR322; cells, no transfection.

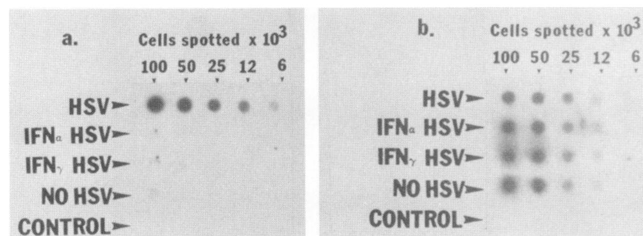

FIG. 5. Dot-blot hybridization of WISH cells transfected with pIECAT and activated with HSV-1. Cells were cotransfected with pIE110CAT and pSAM-I (mouse *aprt* under the native promoter). After 24 h, two of the samples were treated with IFN. At 68 h, all samples were infected with HSV-1 (MOI = 10). Four hours later, samples were harvested and spotted onto NC filters. The filters were fixed as described previously (19) and were probed with P^{32}-labeled pIECAT (a) and P^{32}-labeled pSAM-I (b).

labeled CAT probe. A marked inhibition of the IE transactivation is shown in lanes HSV + IFN-α and HSV + IFN-γ when compared with the positive control HSV. The controls, NO HSV (corresponding to a sample that was not superinfected with HSV-1), MOCK (corresponding to cells transfected with 2 μg of pBR322), and CELLS (corresponding to untreated WISH cells), did not show any hybridization to the probe. The positive signal observed for the HSV sample versus little or no signal in the IFN lanes indicates inhibition of IE transactivation. Similar results were obtained by using a plasmid containing the HSV *tk* gene linked to IE sequences. VP16 is not synthesized de novo under these conditions but is brought in by the virus, as would be the case during normal infection.

In order to confirm that the IFN effect was not due to an effect on transfection efficiency or a general transcription inhibition, similar transfection experiments were done with the mouse *aprt* gene. Cells were cotransfected with pIE110 CAT and pSAMI. After 24 h, 1,000 U of IFN (α and γ) per ml were added to each plate, and 48 h later, HSV at a MOI of 1 was added. Cells were harvested 4 h later and were fixed in glutaraldehyde as described previously (19). Dot blots were probed separately with labeled pCAT and pSAM probes. After 20 h of exposure, control cells transfected with pCAT and infected with HSV gave a strong signal; however, cells treated with IFN gave no signal whatsoever, indicating inhibition of CAT transcription (Fig. 5a). At later times of exposure, some signal could be detected in IFN-treated cells. When a replica of the same filter was probed with pSAM, the mouse *aprt* gene, no signal could be detected until 48 h of exposure. This probably reflects the weaker promoter strength of *aprt*, a housekeeping gene. However IFN had no effect on transcription of this gene in the same transfected cells (Fig. 5b).

DISCUSSION

The evidence presented in Results supports the hypothesis originally proposed by Oberman and Panet (15, 16) that HSV-1 is inhibited by IFN-α at an early step in the virus replication cycle involving transactivation of IE genes by VP16. Transactivation of IE genes occurs via the formation of a protein complex defined as IEC (20). This complex results from protein-protein interaction between one or more host proteins (probably the octameric binding factor) and the viral transactivator VP16. The latter is imported into the host cell by the infecting virus and transported to the nucleus along with the viral genome. It should be emphasized that in the absence of functional VP16, low levels of HSV-1 are still synthesized, in agreement with the reported IFN effects.

The experiments reported here describe a specific effect of IFNs on the IE transactivation event. The specificity of such an effect is also supported by the observation that transcription of an unrelated gene (mouse *aprt*) is unaffected by IFN treatment in the presence of VP16. The inhibitory effect of IFN seems to be directed at the onset of IE transcription, as reported also by other investigators (12, 15, 16). These investigators performed nuclear runoff assays to show that the IE inhibition observed was not due to RNase L degradation of the IE messages. The latter result is also confirmed by the Northern blots reported in this work (Fig. 3), in which no degradation (resulting in smearing) is evident in the IFN-treated sample.

Specific inhibition of IE transactivation might be achieved by an IFN-induced gene(s) directly affecting IEC formation or modifying a correctly assembled IEC. The evidence provided here does not distinguish between these possibilities.

Another possibility is an effect on VP16 transport to the nuclei by IFNs. This possibility becomes important if one considers that IEC transactivating ability lies in the VP16 acidic domain. If, in the presence of IFN, VP16 is incorrectly transported to the host nuclei, then IEC formation would be impaired. Indirect evidence from other laboratories does not support this idea. HSV-1 induced early shutoff of host protein synthesis was not affected by IFN pretreatment of the cells (15). Early shutoff in HSV-1 infection is induced by the HSV protein imported into the cells by the infecting virions (9). This evidence suggests that IFNs do not affect the very early stages of HSV-1 infection, namely, penetration and uncoating. VP16 is similar to the shutoff protein in that it is both a structural and functional viral protein. If the shutoff protein is correctly imported into the cells in the presence of IFNs, so presumably is VP16.

If transport of VP16 to the nuclei is shown to be unaffected by IFNs, modification (or lack of modification) of IEC (one or more of its components) is the most likely possibility left to explain the failure of the complex to stimulate transcription.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant AI21898 from the National Institutes of Health and by the United Cancer Council (Indianapolis, Ind.).

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