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Inhibition of Herpes Simplex Virus Type 1 Immediate-Early Gene Expression by Alpha Interferon Is Not VP16 Specific

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Pretreatment of tissue culture cells with alpha interferon (IFN-α) inhibits the transcription of herpes simplex virus type 1 (HSV-1) immediate-early (IE) genes, an effect which has been attributed to reduced transactivation of IE promoters by the virion protein VP16. Our previous demonstration that IFN-α inhibited the replication of the HSV-1 mutant *in*1814, which has a mutated VP16 unable to activate IE transcription, appeared to be incompatible with IFN-α having an effect on VP16 action (D. R. S. Jamieson, L. H. Robinson, J. I. Daksis, M. J. Nicholl, and C. M. Preston, J. Gen. Virol. 76:1417–1431, 1995). To investigate this observation further, cells were infected with a derivative of *in*1814 containing the *lacZ* gene controlled by the human cytomegalovirus IE promoter. The accumulation of HSV-1 IE RNA species was inhibited by IFN-α in these cells to the same extent as in cells infected with a virus rescued at the VP16 locus, and production of *lacZ*-specific RNA was also reduced, demonstrating that IFN-α can inhibit expression from a heterologous promoter that is not responsive to VP16. To provide a means of investigating the activity of VP16 on IE promoters not located in the HSV-1 genome, cell lines containing the neomycin phosphotransferase gene controlled by the HSV-1 IE ICP0 promoter were constructed. Activation of the IE promoter by VP16 was not inhibited when the ICP0 promoter was resident in the cell, demonstrating that VP16 function was unaffected by pretreatment of cells with IFN-α. The results suggest that IFN-α prevents the onset of IE transcription from the HSV-1 genome through a general mechanism rather than by having an effect specific to HSV-1 IE promoters.

Alpha interferon (IFN-α) is a widely studied antiviral agent that exerts its effects on a variety of cellular processes important for virus replication. In the case of herpes simplex virus type 1 (HSV-1), treatment of cells with IFN-α before infection results in inhibition of immediate-early (IE) gene expression (7, 16–18). Since the production of IE proteins is essential for transcription of the viral early and late genes (reviewed in reference 9), this block is sufficient to arrest HSV-1 replication at an early stage.

Transcription of HSV-1 IE genes is controlled primarily by the virion protein VP16 (otherwise known as Vmw65 or α-TIF), which interacts with the cell proteins Oct-1 and HCF to form a multiprotein complex at DNA sites containing the sequence TAATGARAT (R is a purine nucleotide) within IE promoters (4, 15, 19, 21, 22, 26). Assembly of the complex enables the C-terminal activating region of VP16 to contact proteins involved in the initiation of transcription and thereby to stimulate IE gene expression (23, 25). Sequence specificity of VP16 action is mediated by the binding of Oct-1 to TAAT GARAT rather than the direct interaction of VP16 itself with DNA. An HSV-1 mutant, *in*1814, which encodes a mutated VP16 unable to form the multiprotein complex, has been isolated (1). This mutant replicates inefficiently when cells are infected at a low multiplicity of infection, demonstrating a crucial role for VP16 in the initiation of productive infection.

Studies on the mechanism of inhibition of IE gene expression by IFN-α showed that IE transcription is reduced in nuclei from IFN-α-pretreated cell cultures (16, 17). This result concurred with the observation that fewer cells detectably produced IE proteins and that IE RNA levels were lower in pretreated cultures (7, 16, 17). Further investigation revealed that uptake and uncoating of HSV-1 DNA were not altered in IFN-α-pretreated cells, leading to the suggestion that the action of VP16 may be affected (18). This idea was supported by transient transfection experiments in which the activities of IE promoters and their stimulation by VP16 were examined. In IFN-pretreated mouse L cells, the response to VP16 of the promoter for the IE gene ICP4 was reduced (13), and similarly in pretreated human fibroblasts, the IE ICP0 promoter in a transfected plasmid was not activated by infection of cells with HSV-1 (7). It was concluded that IFN-α specifically blocked transactivation by VP16 (7).

In our previous studies, we found that plaque formations by *in*1814 and by wild-type HSV-1 were equally sensitive to inhibition by IFN-α pretreatment of cells and noted that this result was not obviously compatible with the hypothesis that IFN-α affects VP16 action, since a virus lacking VP16 function would be expected to be resistant to inhibition by IFN-α (11). In the experiments reported here, we test further the possibility that the action of IFN-α on IE transcription is through having an effect on VP16 and conclude that this is not the case in the context of virus infection. Rather, the response of HSV-1 IE promoters to IFN-α is observed only when they are cloned into the HSV-1 genome, suggesting a more general effect on the onset of viral transcription.

MATERIALS AND METHODS

Plasmids. To clone the neomycin phosphotransferase (*npt*) gene, the starting point was plasmid pMJ102, the cloned HSV-1 *Bam*HI p fragment (containing the thymidine kinase [TK] gene) with an insert consisting of the ICP0 promoter upstream of the *Escherichia coli lacZ* gene (11). The *npt* gene from plasmid pMC1NeoPolyA (Stratagene; used as derivative pgDNeo, kindly provided by A. McGregor) was modified by conversion of the *Mlu*I site upstream of the *npt* coding sequences to a *Hin*dIII site by insertion of an oligonucleotide linker. The *npt* gene and polyadenylation sequences were excised by cleavage with *Hin*dIII and *Sal*I and cloned between the *Hin*dIII and *Xho*I sites of pMJ102, thereby replacing *lacZ* with *npt*, to yield pMJ90. The structures of pMJ102 and pMJ90 are shown diagrammatically in Fig. 1.

Cells and viruses. Human fetal lung (HFL) fibroblasts (Flow 2002) and Vero

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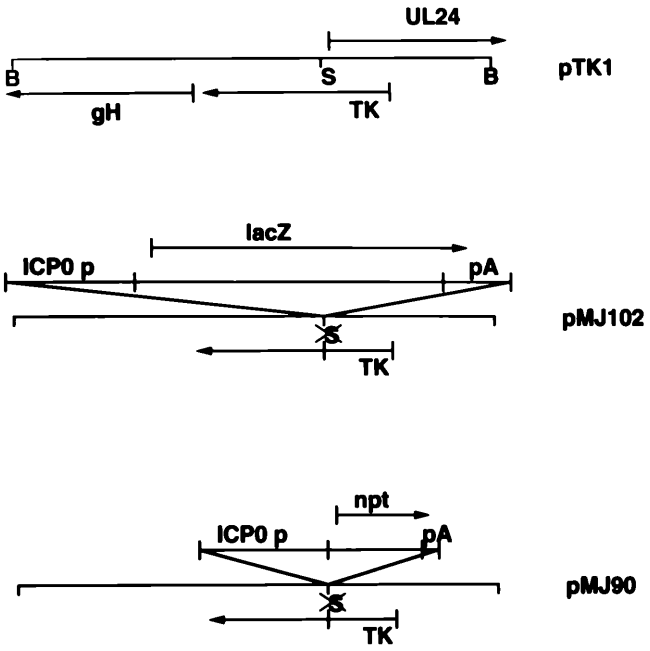

FIG. 1. Plasmid structures. pTK1 contains the HSV-1 *Bam*HI p fragment, which encodes TK and parts of the sequences for genes UL24 and glycoprotein H (gH). pMJ102 and pMJ90 contain insertions consisting of *lacZ* and *npt*, respectively, controlled by the ICP0 promoter (ICP0 p) and SV40 polyadenylation sequences (pA). Plasmid pMJ102 was used for the construction of *in*1854 and *in*1864. pMJ90 was used to construct *in*1321 and for transformation of Vero cells. Cleavage sites for *Bam*HI (B) and *Sst*I (S) are shown.

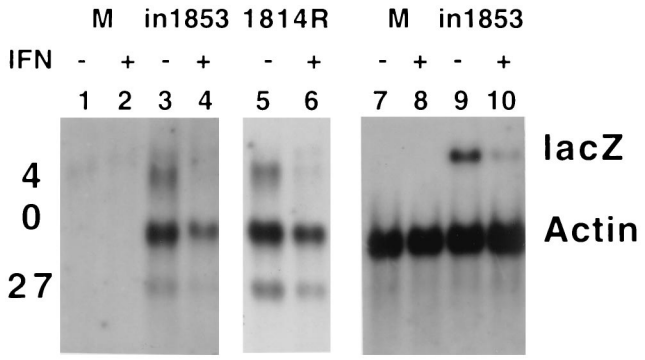

FIG. 2. Inhibition of IE RNA accumulation by IFN-α pretreatment of cells. HFL cell monolayers were pretreated with IFN-α (lanes 2, 4, 6, 8, and 10) or mock pretreated (lanes 1, 3, 5, 7, and 9) for 24 h prior to infection with 5 PFU of *in*1853 (lanes 1 to 4 and 7 to 10) or 1814R (lanes 5 and 6) per cell in the presence of cycloheximide. At 6 h postinfection polyadenylated RNA was prepared and analyzed by RNA blotting with mixtures of probes specific for IE RNAs encoding ICP4, ICP0, and ICP27 (lanes 1 to 6) or γ-actin and *lacZ* (lanes 7 to 10). Monolayers were infected with *in*1853 (lanes 3, 4, 9, and 10) or 1814R (lanes 5 and 6) or were mock infected (lanes 1, 2, 7, and 8). Lanes 1 to 6 were derived from the same autoradiograph, and lanes 1 to 4 were exposed for four times longer than lanes 5 and 6.

cells were used for experiments, whereas BHK cells were used for propagation of viruses. For construction of transformed cell lines, Vero cells were transfected with pMJ90 and propagated in the presence of geneticin (800 μg/ml). Two cell lines were maintained, one derived from a single colony and the other derived from a mixture of approximately 20 colonies. Once established, cell lines were propagated in 500 μg of geneticin per ml. The HSV-1 mutant *in*1814 and rescued revertant 1814R have been described previously (1). The derivative *in*1854 was constructed by insertion of pMJ102 into the genome of *in*1814, as described previously (8, 11), and *in*1864 was derived from *in*1854 by rescue of the VP16 mutation after cotransfection of BHK cells with *in*1854 DNA and plasmid pMC1 (4). Mutant *in*1853, a derivative of *in*1814 containing the *lacZ* gene controlled by the human cytomegalovirus (HCMV) major IE promoter, was described previously (8). Mutant *in*1321 was constructed by recombination of plasmid pMJ90 with DNA of HSV-1 mutant *in*1820K (a derivative of *in*1814) and selection for TK-deficient progeny. Mutant *in*1321 contains the VP16 mutation with a replacement of the ICP0 promoter by the Moloney murine leukemia virus long terminal repeat promoter (11) and a temperature-sensitive mutation in the coding sequences of ICP4. The additional mutations affecting ICP0 and ICP4 activities are not relevant to the studies reported here. The genome structures of all mutants were confirmed by Southern hybridization with long exposure times to ensure homogeneity of recombinant viruses.

RNA analysis. Where appropriate, cells were pretreated with 10^3 U of human lymphoblastoid IFN-α (Sigma) per ml for 24 h at 37°C. Monolayers consisting of 10^7 cells on 100-mm-diameter tissue culture plates were infected with 5 PFU of virus per cell and maintained at 38°C in the continuous presence of 50 μg of cycloheximide per ml for 6 h. Polyadenylated RNA was extracted and purified with Dynabead magnetic beads (Dynal). RNA was applied to formaldehyde-agarose gels, electrophoresed, and transferred to a GeneScreen Plus nylon membrane (DuPont, NEN). Hybridization was carried out with probes radiolabeled by random primer extension (10), as described by the manufacturers of GeneScreen Plus. Fragments specific for the IE genes encoding ICP0, ICP4, and ICP27 were prepared as described by Ace et al. (1), and the γ-actin probe was as described by Daksis and Preston (6). The β-galactosidase- and *npt*-specific probes were 500-bp *Kpn*I-*Hpa*I and 587-bp *Mlu*I-*Sph*I fragments, respectively, from the gene coding sequences.

RESULTS

Effect of IFN-α on IE RNA accumulation. In view of the observation that IFN-α pretreatment of HFL cells inhibits plaque formation by *in*1814 (11), the effect of IFN-α on the synthesis of IE RNAs was investigated. Mutant *in*1853, which has the *lacZ* gene controlled by the HCMV IE promoter inserted into the TK coding sequences of *in*1814, was used so that the activity of a promoter that is not responsive to VP16 could be monitored. HFL monolayers were pretreated with IFN-α for 24 h and infected with *in*1853 or 1814R in the presence of cycloheximide for 6 h. Polyadenylated RNA was extracted, and the production of IE and *lacZ* RNAs was determined by electrophoresis, blotting, and hybridization to specific probes. Accumulation of the three IE RNAs examined was inhibited by three- to fivefold in both *in*1853- and 1814R-infected cells, as determined by PhosphorImaging analysis (Fig. 2, lanes 3 to 6). In addition, synthesis of *lacZ*-specific RNA was reduced by approximately fivefold in *in*1853-infected cells (Fig. 2, lanes 9 and 10). This experiment confirms that the observed reduction in titer of *in*1814 on IFN-α pretreated cells was reflected in lower accumulated IE RNA levels and also demonstrates that expression from the HCMV enhancer, which does not contain functional TAATGARAT elements and is not responsive to VP16 (references 2 and 24 and unpublished observations), is also inhibited by IFN-α.

Responsiveness of the ICP0 promoter to VP16. The above data suggest that IFN-α pretreatment does not act through having an effect on VP16, although it is possible that the HCMV IE promoter contains other sequence elements that mediate the inhibition. To examine directly whether activation of IE transcription was affected by pretreatment of cells with IFN-α, cell lines containing an introduced gene (the *npt* gene) that was responsive to VP16 were constructed. HFL cells were unsuitable for this purpose because of their finite life span in culture; thus, Vero cells were chosen because human IFN-α also inhibits IE gene expression in this cell type (23a). As a control experiment, the responses of the *lacZ* and *npt* genes, controlled by the HSV-1 ICP0 upstream region and located at identical sites within the TK coding region, were investigated. Vero cells were pretreated with IFN-α and infected with a mixture of *in*1854 and *in*1321 in the presence of cycloheximide. Since both viruses contained the *in*1814 mutation, cells were coinfected with 1814R to provide VP16. RNA was prepared at 6 h postinfection, and levels of *lacZ* and *npt* RNAs were de-

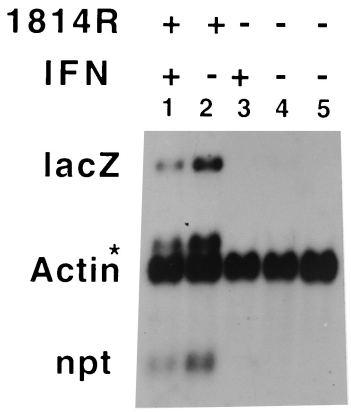

FIG. 3. Repression of *lacZ*- and *npt*-specific RNA accumulation by IFN-α pretreatment. Vero cells were pretreated with IFN-α (lanes 1 and 3) or mock pretreated (lanes 2, 4, and 5) and infected with a mixture of *in*1854 and *in*1321 (3 PFU of each virus per cell; lanes 1 to 4) or mock infected (lane 5) in the presence of cycloheximide. Polyadenylated RNA was extracted at 6 h postinfection and analyzed on RNA blots with a mixture of probes specific for γ-actin, *lacZ*, and *npt*. An *npt*-related transcript is marked with an asterisk.

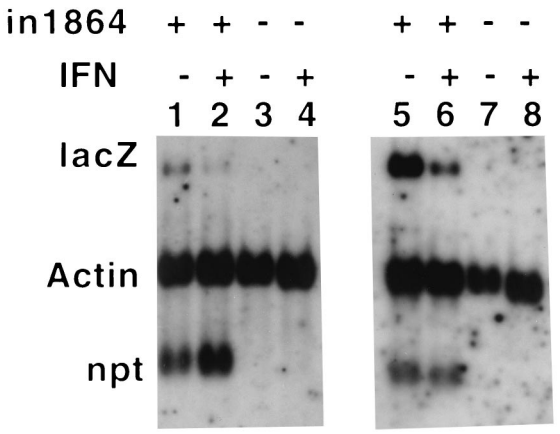

FIG. 4. Effect of IFN-α in *npt*-transformed Vero cells. Transformed cell lines derived from a mixture of colonies (lanes 1 to 4) or a single colony (lanes 5 to 8) were pretreated with IFN-α (lanes 2, 4, 6, and 8) or mock pretreated (lanes 1, 3, 5, and 7). After 24 h, monolayers were infected with 5 PFU of *in*1864 per cell (lanes 1, 2, 5, and 6) or were mock infected (lanes 3, 4, 7, and 8) in the presence of cycloheximide and polyadenylated RNA was extracted at 6 h postinfection. RNA was analyzed by blotting with a mixture of probes specific for γ-actin, *lacZ*, and *npt.*

termined by hybridization to RNA blots (Fig. 3). The accumulation of both RNAs, when VP16 was present, was reduced to the same extent (approximately threefold) (Fig. 3, lanes 1 and 2), demonstrating that there was no specificity of inhibition toward the coding sequences of the two genes. An RNA species migrating slightly more slowly than actin mRNA was detected when cells were infected with *in*1321 (Fig. 3). This transcript was not observed in cells transformed with pMJ90, and its size is consistent with that expected of an RNA produced by readthrough of the polyadenylation signal in pMJ90 and by utilization of the proximal site for the HSV-1 UL24 transcript (5, 14).

Vero cell lines in which the *npt* gene was controlled by the ICP0 promoter were established. The transfected plasmid pMJ90 contained the *npt* gene with ICP0 promoter and TK flanking sequences identical to those present in the viruses *in*1854, *in*1864 (which have *lacZ* controlled by the ICP0 promoter), and *in*1321 (which has *npt* controlled by the same ICP0 upstream region). The resultant cell lines thus contained the ICP0-*npt* cassette flanked by sequences identical to those in the ICP0-*lacZ* cassette within virus mutants *in*1854 and *in*1864. Two lines were propagated, one being a single colony isolate and the other being a mixture of approximately 20 colonies. It was established that the efficiency of plaque formation by HSV in both lines was reduced to the same extent (5- to 10-fold) as in the parental Vero cells (results not shown). In addition, *npt* activity was present in extracts of transformed cells, although mRNA was not detected by RNA blots since presumably only low enzyme levels are required to maintain G418 resistance of Vero cells.

The cell lines were pretreated with IFN-α (or mock treated) and infected with *in*1864 in the presence of cycloheximide, and RNA was analyzed by hybridization to blots (Fig. 4). In both lines, expression of *lacZ*-specific RNA was inhibited by approximately fivefold, whereas the accumulation of *npt*-specific RNA was unaffected. Activation of the ICP0 promoter upstream of *npt* by VP16, present in *in*1864 virions, was therefore unaffected by pretreatment of cells with IFN-α. To confirm this conclusion, the mixed cell line was pretreated with IFN-α and infected with *in*1854 in the presence of cycloheximide, with or without coinfection with 1814R. In this case, functional VP16 provided by 1814R was available to act in *trans* on the ICP0 promoter either in the viral genome (controlling *lacZ*) or in the cellular genome (controlling *npt*). It was found that the accumulation of *lacZ*-specific RNA was inhibited but that there was no effect on *npt*-specific RNA (Fig. 5, lanes 1 and 2), reemphasizing that the location of the ICP0 promoter in the *in*1814 genome, as opposed to the cell genome, was the crucial factor determining its responsiveness to inhibition by IFN-α.

DISCUSSION

Previous studies suggested an effect of IFN-α on the transactivator protein VP16 by IFN-α (7, 13). Since VP16 acts in a sequence-specific manner, this proposed mechanism of inhibition predicts that promoters lacking TAATGARAT elements are resistant to inhibition by IFN-α. It is difficult to test this prediction, however, because most promoters cloned into the HSV genome behave with early or late kinetics and are thus inactive in the presence of cycloheximide (20). Expression lev-

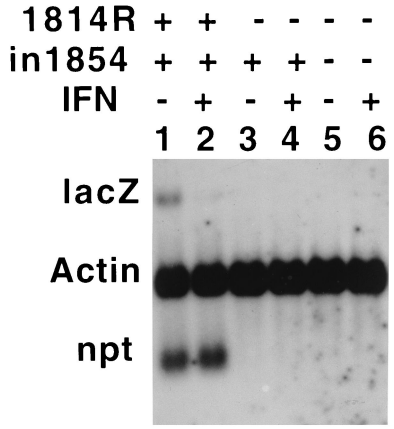

FIG. 5. Effect of IFN-α in *npt*-transformed cells. Transformed cells derived from a mixture of colonies were pretreated with IFN-α (lanes 2, 4, and 6) or mock pretreated (lanes 1, 3, and 5). After 24 h, cultures were infected with 5 PFU of *in*1854 per cell (lanes 1 to 4) or mock infected (lanes 5 and 6) in the presence of cycloheximide and additionally coinfected with 5 PFU of 1814R (lanes 1 and 2). At 6 h postinfection, polyadenylated RNA was extracted and analyzed by RNA blotting with probes specific for γ-actin, *lacZ*, and *npt.*

els of early and late promoters, and of the HCMV IE promoter, are reduced in IFN-pretreated cells when infection is carried out without cycloheximide (12, 16, 17). In these cases, however, it is not possible to distinguish between a direct effect on the promoter concerned and an indirect effect due to the inhibition of IE protein synthesis. The HCMV major IE promoter, however, is active in the presence of cycloheximide when cloned into the HSV-1 genome, although even in this case much greater expression is observed at early times in infection once IE proteins have been produced (21a).

The HCMV IE promoter does not contain TAATGARAT elements and is not responsive to VP16 (references 2 and 24 and unpublished observations); nonetheless, its expression in cycloheximide-treated cells was reduced by pretreatment of cells with IFN-α, suggesting that the effect of IFN-α is not specific for the HSV-1 IE promoters. The construction of cell lines provided a means of assaying simultaneously the activities of VP16 on the same promoter element in the viral and cellular genomes, and the results demonstrated that when the promoter resident in the transformed cells was used to monitor the activity of VP16, IFN-α pretreatment had no detectable effect on activation of the ICP0 promoter. Our results were therefore inconsistent with the previous reports that stated IFN-α inhibits the activity of VP16. It is likely, on the basis of previous findings (16, 17), that the inhibitory effect of IFN-α is at the level of transcription, although this has not been formally demonstrated with HFL or Vero cells, and other effects, e.g., on mRNA stability or export from the nucleus, are possible. The precise mechanism of action of IFN-α must be viewed in light of the fact that genes located within the HSV-1 genome are preferentially inhibited.

The reason for the discrepancy between our results and the previous findings is not clear, but it may reside in the use of transfection for the study of gene expression. In the report of LaMarco and McKnight (13), plasmids containing the HSV-1 TK gene controlled by the simian virus 40 (SV40) early promoter or the HSV-1 IE ICP4 promoter were transfected into cells together with a plasmid expressing VP16. Pretreatment of cells with IFN-α reduced the level of expression from the IE-TK construct, but not from the SV40-TK construct, in the presence of the VP16-expressing plasmid. A reduction in the basal expression level of the IE-TK plasmid was also observed, however. The study by De Stasio and Taylor showed that chloramphenicol acetyltransferase-specific RNA from a transfected plasmid (with chloramphenicol acetyltransferase controlled by the ICP0 promoter) was detectable when cells were infected with HSV-1 but not when transfected cells were pretreated with IFN-α prior to infection (7). The effect of IFN-α on the levels of chloramphenicol acetyltransferase-specific RNA in uninfected cells was not reported; thus, it is possible that basal expression was inhibited in their study.

As in other examples in which IFN blocks transcription by DNA-containing viruses, the basis for the effect on HSV IE gene expression is unclear. Our results suggest that the gross structure of the viral genome shortly after infection is a crucial factor, but unfortunately little is known on this subject. The most obvious analogy is with the study of Brennan and Stark (3), who showed that IFN-α pretreatment of cells prevented the initiation of SV40 early transcription but had no effect once the process had commenced. Despite the many differences between the two viruses, it is possible that IFN-α blocks the initial transcription of SV40 and HSV-1 by a common mechanism.

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