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Novel Induction by Herpes Simplex Virus of Hybrid Interferon Gene Transcripts Driven by the Strong Cytomegalovirus IE94 Promoter

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We have constructed stable DNA-transfected LTK$^+$ cell lines containing two different coselected hybrid interferon (IFN) genes driven by the usually strong and constitutive promoter from the immediate-early 94K protein (IE94) gene of simian cytomegalovirus. Surprisingly, and unlike hybrid IE94-chloramphenicol acetyltransferase gene constructs, both of the IE94-IFN genes (one with and one without the complex spliced intron region) produced relatively low basal titers of biologically active human IFN in the mouse cell lines. However, IFN expression could be stimulated up to 120-fold by superinfection with herpes simplex virus (HSV), although not with cytomegalovirus. To examine the mechanism of this unexpected HSV induction process, we measured the levels of both IE94-IFN mRNA and IFN protein produced under various infection protocols. Compared with similar previously characterized cell lines containing hybrid IFN genes under the control of HSV IE or delayed-early (DE) promoters, activation of IFN expression first occurred at an intermediate time. Both IE94-IFN cell lines also produced an unusual pattern of response to infection with the HSV IE regulation-deficient mutants *ts*K and *ts*B7: stimulation of IFN synthesis occurred in the absence of a functional HSV IE175 (or ICP4) gene product, but did not occur in the absence of uncoating of virus capsids. Cycloheximide treatment (without virus infection) also gave a rapid 30-fold increase in steady-state levels of correctly initiated mRNA from both types of IE94-IFN hybrid genes, but had no effect on cells containing the IE175-IFN construct. Therefore, we conclude that the use of the IE94-IFN constructs identifies a novel HSV regulatory response that requires a previously unrecognized function of HSV and does not involve either IE175 or the pre-IE "virion factor" *trans*-activators that are known to stimulate transcription of HSV IE and DE genes, respectively.

Herpesvirus-infected cells undergo complex regulatory controls in which virion factors and newly synthesized gene products exert both positive and negative influences on viral and cellular gene expression. Most of these control processes, especially at early times after infection, are believed to occur at the transcriptional level. In herpes simplex virus (HSV) infections, all five immediate-early (IE) genes appear to possess both medium-strength basal-level elements (18, 21, 24, 36, 49) and specific virion factor-dependent positive *cis*-acting signals (5, 25, 30, 34, 36). In contrast, expression of the HSV thymidine kinase (*tk*) and other delayed-early (DE) genes requires the presence of an active IE175 or ICP4 gene product (7, 23, 33, 35, 41, 42, 47). Furthermore, in short-term DNA cotransfection assays both the IE175 and the IE110 (or ICP0) proteins are capable of *trans*-activating HSV DE promoters (9, 12, 31, 37). Late HSV genes appear to possess negative elements in their promoter regions that have to be bypassed with the help of these or other virus-encoded transcription factors (7, 8, 11, 43) and in some cases may require an altered conformation related to DNA replication events. In addition, rapid transcriptional and translational shutoff of host gene expression (and possibly also of IE gene expression) by both virion factors and de novo viral gene products has been described in a number of studies (14, 29, 32, 39).

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The only viral mRNA expressed abundantly after simian cytomegalovirus (CMV) (Colburn) infection of permissive human fibroblast (HF) cells in the presence of cycloheximide is that encoding the IE94 protein, which is the only detectable simian CMV protein expressed immediately after cycloheximide reversal in HF cells or at any time in nonpermissive BALB/3T3 cells (15, 17; K.-T. Jeang, Ph.D. thesis, Johns Hopkins University, Baltimore, Md., 1984). Furthermore, in a previous study (K.-T. Jeang, J. Mosca, J. Shero, D. Gay, M. Pizzorno, and G. S. Hayward, submitted for publication) we found that (i) hybrid IE94-interferon (IFN) gene constructs give between 50- and 500-fold higher basal IFN titers after microinjection into *Xenopus* oocytes than the previously described HSV TK-IFN and IE175-IFN hybrid genes (28, 41), (ii) hybrid IE94-chloramphenicol acetyltransferase (CAT) gene constructs produce three- to six-fold more basal CAT activity in transient expression assays in transfected Vero or LTK$^-$ cell cultures than does the equivalent SV2-CAT construct containing the complete simian virus 40 (SV40) early promoter-enhancer region, and (iii) numerous independent permanent Neo^r Vero and LTK$^-$ cell lines containing cotransfected IE94-CAT hybrid genes give continuous basal expression of enzyme product at levels at least as high as those produced in parallel cell lines receiving SV2-CAT. These findings, together with the observation that a DNA-transfected mouse LTK$^+$ cell line containing the isolated intact IE94 gene continuously expresses large amounts of the phosphorylated IE94 protein product (16), indicate that the IE94 promoter has unusually strong constitutive properties. Structurally, the CMV IE94 upstream

promoter-regulatory region is very different from the HSV IE175 promoter-regulatory region and contains a complex arrangement of four sets of multiple, short repeat elements, including 11 interspersed copies of the 16-base-pair (bp) consensus palindromic sequence CCATTGACGTCAATGG (Jeang, thesis, 1984). Furthermore, an equivalent upstream region from the major IE gene of human CMV has been shown to have very strong "enhancer" properties (4). In contrast to the HSV IE175 gene, but similar to the human CMV IE68 gene (45), the simian CMV IE94 gene also contains multiple splice sites, with a 5′ noncoding exon and a large 900-bp intron (Jeang, thesis, 1984).

In earlier reports, we described both transient and long-term DNA transfection systems for studying herpesvirus gene regulation, using isolated intact IE genes and hybrid target gene constructs containing various HSV IE or DE promoters driving the transcription of IFN or CAT genes (26, 30–32, 41). In particular, we showed that in coselected mouse LTK$^+$ cell lines, IFN expression from resident DNA-transfected TK-IFN and IE175-IFN hybrid genes, acquired in a stably integrated and presumably chromatin-associated form, could be stimulated between 30- and 500-fold at both the RNA and protein levels by superinfection with HSV (28). Differences in response to infection with the HSV-1 *ts*B2 virus mutant, which is temperature sensitive for IE175 functions, indicates that the regulation of the foreign cloned genes introduced into these cells faithfully mimics that of IE or DE genes in the viral genome at equivalent stages of the infection process. In addition, in cells receiving the IE175-IFN hybrid gene construct, which contains the *ori*-S viral DNA replication origin, the copy number of the resident IFN DNA sequences could be amplified 10- to 30-fold within 20 h after HSV infection (28). Here we describe superinfection experiments showing that HSV also activates hybrid CMV IE94-IFN genes introduced into stable coselected LTK$^+$ cell lines, but that this stimulation occurs by a different regulatory mechanism than those involved in the *trans*-activation events acting on either the HSV IE or DE hybrid IFN genes.

MATERIALS AND METHODS

Cells and viruses. Murine LTK$^-$ cells, diploid HF cells, and cloned LTK$^+$ cell lines containing transfected plasmid DNA were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. Maintenance medium for LTK$^+$ cell lines contained in addition selective HAT medium (15 μg of hypoxanthine, 1 μg of aminopterin, and 5 μg of thymidine per ml). HSV-1 (MPc1-20), HSV-1 (KOS) *ts*B2, HSV-1 (HFEM-STH2) *ts*B7, HSV-1 (17) *ts*K, and HSV-2 (333cl-6) virus stock were prepared and titers were determined as described previously (26, 41). Human CMV (Towne) and simian CMV (Colburn) were prepared and used as described previously (15, 19). Induction of HF cells with Newcastle disease virus was carried out with the NJ-LaSota strain at 10 PFU/cell.

DNA transfection. The procedures used for *tk* coselection were similar to those described previously (28, 40) except that no carrier DNA was used. LTK$^-$ cell cultures were seeded at 3×10^5 cells per 60-mm dish and transfected 24 h later with 2 μg of supercoiled pTJ204 or pTJ211 plasmid DNA plus 100 ng of the pGR18 plasmid containing an intact HSV-2 *tk* gene. The calcium-DNA precipitate was removed after 4 h, and the cells were washed with phosphate-buffered saline and then treated with 10% glycerol for 2 min. The cell cultures were rinsed twice with phosphate-buffered saline and overlaid with DMEM supplemented with 20% fetal calf serum and 5 mM sodium butyrate. The cultures were placed in fresh DMEM after 16 h and in HAT selective medium after 48 h. After 2 weeks, individual colonies were isolated from the original dish and grown into mass culture for biochemical analysis.

Assay for IFN expression before and after superinfection. Cells were grown in 100-mm plates for simultaneous measurement of IFN in the culture medium and RNA isolation from the cell monolayers. Superinfection and procedures for detection of IFN production were carried out essentially as described previously (28, 41). Confluent cultures at 8×10^6 cells per 100-mm plate either received a fresh medium change for basal expression assays or were infected with HSV-1, HSV-2, or CMV, usually at a multiplicity of infection of 3 to 5 PFU/cell. After 90 min at 37°C in medium without fetal calf serum, the virus was removed and the cells were overlaid with 4 ml of DMEM supplemented with 3% fetal calf serum per 100-mm plate and incubated further at 37°C. At the times indicated (hours after addition of virus) the medium was collected and the IFN titer was determined on Vero cells, an African green monkey kidney cell line deficient in IFN production (27).

Preparation of cellular DNA and RNA. High-molecular-weight DNA for dot blot hybridization analysis was prepared from detergent-salt lysates of cells followed by treatment with proteinase K, RNase, phenol, and chloroform, and ethanol precipitation. Total cellular RNA for RNA dot blot hybridization analysis was prepared by the CsCl-guanidine thiocyanate procedure from lysates of cell monolayers as described previously (6, 28). Cellular DNA remained at the interface, and the RNA which pelleted through the CsCl cushion was suspended and precipitated with ethanol. Poly(A)-containing [poly(A)$^+$] RNA was isolated by binding and recovery from two successive oligo(dT)-cellulose columns.

DNA and RNA dot blot hybridization. For dot blot analysis, twofold dilutions of either DNA or RNA were applied to nitrocellulose sheets with a 96-well filtration manifold (Bethesda Research Laboratories, Inc.). Cell DNA was treated with 0.5 M NaOH for 10 min on ice, neutralized with an equal volume of 4 M ammonium acetate, and applied to nitrocellulose in 10× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.7). Ethanol-precipitated RNA was evaporated to dryness and suspended in 10 μl of formamide containing 8% formaldehyde, and 20 mM MOPS (morpholinepropanesulfonic acid, pH 7.0), 5 mM sodium acetate, and 1 mM EDTA. The RNA sample was heated at 65°C for 15 min, diluted with 200 μl of 10× SSC containing 3% formaldehyde, and applied to the nitrocellulose.

RNA analysis by Northern blotting. For Northern blot analysis, 1 μg of poly(A)$^+$ RNA was evaporated to dryness and suspended in 10 μl of formamide containing 8% formaldehyde, 20 mM MOPS (pH 7.0), 5 mM sodium acetate, and 1 mM EDTA. The suspended RNA was heated at 65°C for 5 min, cooled on ice, and electrophoresed at 30 V for 12 h through a 1.5% agarose gel containing 1% formaldehyde. The gel running buffer was 40 mM MOPS (pH 7.0)–5 mM sodium acetate–1 mM EDTA. After electrophoresis, the gel was soaked in 10× SSC for 45 min and transferred to nitrocellulose paper overnight. Filters were rinsed in 2× SSC and baked for 3 h at 80°C in a vacuum oven. Prehybridization and hydridization were performed at 60°C as described by Mosca et al. (28), except that a riboprobe (Promega Biotec system) was used in blot analysis. The plasmid pSP64-β-IFN, used as the template for synthesis of

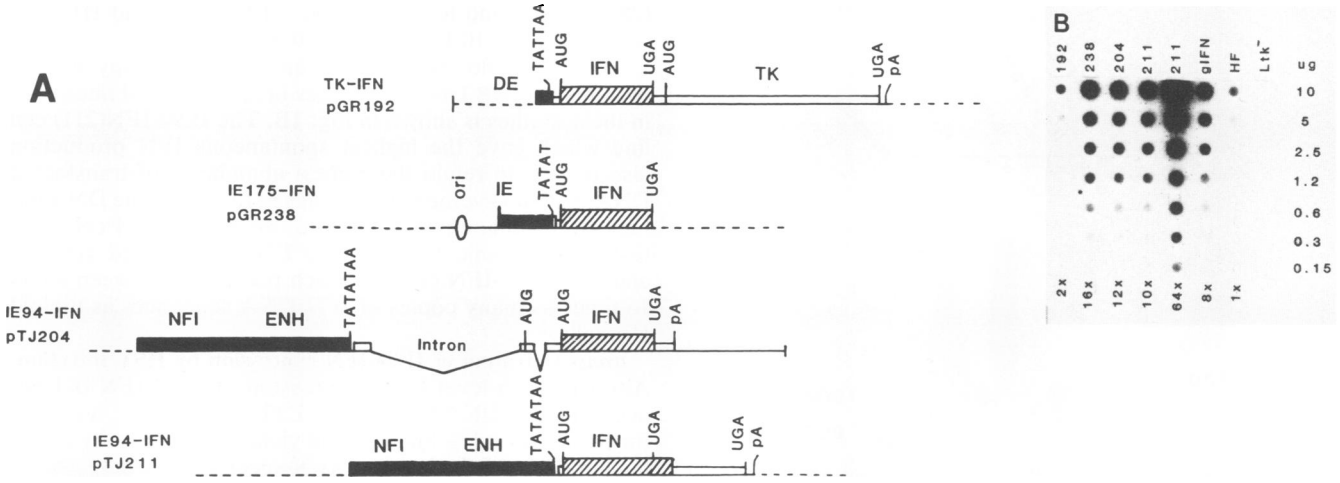

FIG. 1. (A) Structure of the four hybrid IFN genes containing herpesvirus promoter-regulatory regions. Hatched bars represent human IFN gene sequences. Details of the construction of plasmid pGR192 (TK-IFN) containing a 560-bp human IFN-β cDNA inserted into the 5′ leader sequence of the HSV-1 *tk* gene were given in Reyes et al. (41). The pGR192 construct would be expected to produce a DE polycistronic hybrid mRNA of 1,800 bp by utilizing the poly(A) signal (pA) from the HSV-1 *tk* gene (open bar). The first AUG and intact reading frame are those coding for the complete and unaltered human IFN protein, including the signal peptide. Approximately 800 bp of upstream HSV DNA sequences are present at the 5′ end, including the proximal and distal elements of the HSV DE class *tk* gene promoter (solid bar) and 50 nucleotides from the 5′ leader sequence of *tk* mRNA. Construction of plasmid pGR238 (IE175-IFN) containing the same 560-bp IFN-β cDNA under the transcriptional control of the HSV-1 IE175 promoter-regulator region was described by Mosca et al. (28). The initial AUG and first open reading frame in pGR238 encode the complete human IFN-β polypeptide and produce hybrid mRNA molecules containing 30 bp from the 5′ leader sequence for IE175 mRNA. However, this construct lacks a defined poly(A) addition signal. The complex IE175 promoter-regulatory region (solid bar) and the *ori*-S replication origin are included in the 1,900 bp of 5′ upstream viral sequences present. The construction and some properties of plasmids pTJ204 [IE94-IFN(204)] and pTJ211 [IE94-IFN(211)] containing CMV IE94-IFN genes that either retain or lack the large 895-bp 5′ intron sequence of IE94 have been described by Jeang et al. (submitted for publication). In pTJ204, the same 560-bp IFN-β cDNA fragment was inserted in phase at the *Bam*HI site in the IE94 coding region, creating a fusion protein with 98 amino acids from the CMV IE protein at the NH_2-terminus in front of the IFN signal peptide. A 3′ poly(A) site is provided from the late capsid gene of SV40. This construct also contains the SV40 2 × 72-bp enhancer region derived from pSV2gpt, although that lies more than 4,000 bp away from the IE94 RNA start site. In pTJ211, a slightly larger 820-bp fragment from the genomic IFN-β clone pIFR (50) was inserted at the *Sac*I site 30 bp downstream from the IE94 mRNA start site in pTJ148. Therefore, the construct encodes an intact unaltered IFN-β protein, and the entire complex IE94 intron region together with most of the IE94 5′ leader sequence have been deleted. At the 3′ end, the construct retains the COOH-terminal portion of the IE94 coding region and the IE94 polyA site. (B) Determination of the copy number of human IFN-β DNA sequences in the IE94-IFN and control cell lines. The autoradiograph shows the results of DNA dot blot hybridization with an isolated 560-bp ^{32}P-labeled human IFN-β cDNA probe. Each vertical filter series contains twofold dilutions from a initial 10 μg of total DNA preparation extracted from uninfected cell cultures. Cell lines tested (left to right): LH_2p192-8 (TK-IFN), LH_2p238-38 (IE175-IFN), LH_2p204-32 (IE94-IFN), LH_2p211-10 (IE94-IFN), LH_2p211-6 (IE94-IFN), LH_2pIFR-8 (containing genomic IFN from plasmid pIFR), diploid HF cells, and LTK^- (parent mouse) cells. Estimations of the total copy number of IFN cDNA fragments per haploid cell genome for each cell line (relative to single-copy HF DNA) are given below each lane. Confirmation that equal amounts of DNA were applied to each dilution series was obtained by UV visualization of ethidium bromide-stained DNA after transfer.

the complementary-strand RNA probe, was constructed by insertion of an *Eco*RI-*Hin*dIII fragment from position −282 to +1350 in the human β-IFN gene derived from plasmid pIFR (50) into the pSP64 vector DNA between the *Hin*dIII and *Eco*RI sites. The pSP64-β-IFN DNA was linearized with *Eco*RI, incubated with SP6 RNA polymerase, extracted with phenol-chloroform, passed through a G25 Sephadex column to remove unincorporated [α-^{32}P]rNTPs and ethanol precipitated before use in RNA hybridization reactions. Hybridized filters were washed in 50 mM NaCl–20 mM sodium phosphate (pH 6.5)–1 mM EDTA–0.1% sodium dodecyl sulfate at 60 to 65°C, with four washes of 20 min each.

S1 nuclease mapping of mRNA 5′ termini. Plasmid pTJ211 DNA was 5′-end labeled with [α-^{32}P]ATP and T4 nucleotide kinase at the *Bgl*II site 560 bp inside the IFN coding sequence. Cleavage with *Ava*I generated an asymmetrically labeled fragment probe that was isolated, denatured, and mixed with 10 μg of total RNA from each culture sample. Hybridization was performed in 80% formamide buffer for 15 h at 50°C (2, 48). The 5′ hybrids were treated with 300 μg of S1 nuclease per ml at 37°C for 30 min, denatured, and electrophoresed through a 5% polyacrylamide gel containing 7 M urea.

RESULTS

Basal expression and DNA copy number in IE94-IFN cell lines compared with other hybrid IFN cell lines. The structures of the four types of hybrid herpes virus promoter-IFN genes that were used in these studies are shown in Fig. 1A. Permanent cell lines containing the HSV TK-IFN and IE175-IFN constructs were used here for comparison of their basal and induced expression with that of the IE94-IFN hybrid genes. Establishment of an LTK^+ cell line (LH_2p192-8) retaining the HSV-1 TK-IFN hybrid gene from plasmid pGR192 in a form inducible by HSV superinfection was described by Reyes et al. (41). Similarly, the specificity of HSV induction of the LH_2p238-38 cell line containing the HSV IE175-IFN hybrid gene from plasmid pGR238 was described previously by Mosca et al. (28). Construction and comparison of the two IE94-IFN plasmids, pTJ204 and pTJ211, has been reported elsewhere (K.-T. Jeang and J. D.

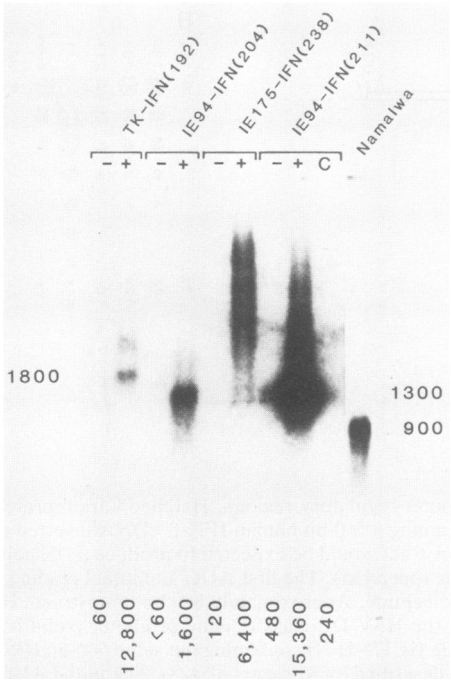

FIG. 2. Northern RNA blot hybridization analysis illustrating the size and abundance of IFN-specific mRNA induced in various cell lines after HSV-1 superinfection. Each track contains the selected poly(A)$^+$ RNA fraction from 10 μg of total extracted cell RNA fractionated by electrophoresis through a formaldehyde-agarose gel. Lanes: −, basal expression samples obtained after mock infection; +, parallel samples obtained 12 h after infection with HSV-1 (MP) at 10 PFU/cell. The cell lines used were LH_2p192-8 (TK-IFN), LH_2p204-32 (IE94-IFN), LH_2p238-38 (IE175-IFN), and LH_2p211-6 (IE94-IFN). Lane C, Sample infected with simian CMV (Colburn). The final lane contains poly(A)$^+$ RNA from an equal number of human lymphoblasts (Namalwa cells) induced by Sendai virus infection and used as a size marker [900 bp plus poly(A)]. Sizes are indicated to left and right (in base pairs). Numbers below each lane indicate IFN-β titers (microliters per milliliter) obtained from the culture supernatants.

Mosca, submitted for publication). One of these CMV IE hybrids (pTJ204) produces a fused protein and retains the complete 5′ leader sequence plus most of exons 2 and 3 in the complex NH_2-terminal spliced region, whereas the other (pTJ211) lacks the entire IE94 gene intron structure and much of the 5′ leader sequence. As reported in those earlier studies, basal expression of biologically active IFN from all four constructs after microinjection into *Xenopus* oocytes varies by several orders of magnitude in the following approximate ratios: TK-IFN, 1; IE175-IFN, 15; IE94-IFN(204), 100; and IE94-IFN(211), 800.

To ask whether the same strong and constitutive properties of IE94-IFN would also be displayed after long-term DNA transfection, we established LTK$^+$ cell lines receiving pTJ204 or pTJ211 DNA by unlinked TK coselection of mouse LTK$^-$ cells. A number of LTK$^+$ colonies surviving in HAT medium were grown to mass culture to measure basal levels of human IFN-β expression. Surprisingly, none of over 30 clones of IE94-IFN cell lines tested gave the expected high-level basal human IFN-β titers. The highest yields were 120 U/ml over 20 h for LH_2p204-32 cells and 960 U/ml for LH_2p211-6 cells, compared with 120 U/ml for our standard TK-IFN cells and 240 U/ml for IE175-IFN cells (28). Background levels in LTK$^-$, LH_2p18-1, and HF cells were all below 10 U/ml over a 20-h collection.

A DNA dot blot hybridization analysis of the copy number of human IFN-β DNA sequences in all of the cell lines used in these studies is shown in Fig. 1B. The IE94-IFN(211) cell line which gave the highest spontaneous IFN production also proved to retain the highest abundance of transfected DNA, with an average of 64 copies of the IFN gene DNA per haploid genome equivalent. Two other IE94-IFN cell lines tested, a genomic IFN plasmid DNA-transfected cell line and the IE175-IFN cell line, each possessed between 8 and 16 times as many copies of IFN DNA sequences as diploid HF cells.

***trans*-Activation of IE94-IFN expression by HSV infection.** Although high-level basal expression of IE94-IFN did not occur in the DNA-transfected LTK$^+$ cell lines, we were further surprised to find that the yields of biologically active human IFN and the levels of IFN-specific RNA detected by dot blot analysis were inducible by superinfection with HSV. The average IFN titer produced by 10 of 24 LTK$^+$ cell lines receiving IE94-IFN(204) DNA increased four- to eightfold by 20 h after infection, and three of eight IE94-IFN(211) cell lines tested gave increases of at least 30-fold. In comparison, both the TK-IFN and IE175-IFN control cell lines gave at least 50-fold induction. The stimulation of IFN-specific RNA levels measured by initial RNA dot blot hybridization analyses was 4-fold in two IE94-IFN(204) cell lines tested, 4-fold in the TK-IFN cells, 8- to 16-fold in the three positive IE94-IFN(211) cell lines, and 16- to 20-fold in the IE175-IFN cells (not shown). The LH_2p211-6 line, which gave the highest constitutive IFN levels (960 U/ml over 20 h), produced between 30,000 and 120,000 U/ml over 20 h after HSV infection in at least six separate experiments. Parallel infections with human or simian CMV had no significant effect on basal IFN production at either the RNA or protein level in any of the hybrid IFN cell lines (not shown). HSV-1 infection failed to induce the endogenous single-copy IFN-β gene in HF cells to any detectable degree (less than 10 U/ml over 20 h), although strong induction was obtained in the same culture by Newcastle disease virus infection or poly(rI:rC) addition. Similarly, no induction was obtained after HSV infection in three cell lines receiving genomic IFN DNA that were all inducible with poly(rI:rC).

An initial experiment with the HSV-1 *ts*B2 virus for superinfection induction of IE94-IFN(204) cells revealed that IFN RNA stimulation was equally efficient at the permissive and nonpermissive temperatures (not shown). This result was similar to previous observations with the IE175-IFN cells (28) and implied that the *trans*-activation may not be dependent on a functionally active HSV IE175 gene product. Therefore, to further examine the nature of the novel HSV *trans*-activation of IE94-IFN cells, we made a detailed comparison of their responses to superinfection relative to those of IE175-IFN and TK-IFN cells. The highly inducible LH_2p211-6 cell line was chosen as representative of the IE94-IFN(211) cell lines for use in most of these studies, although comparisons with an IE94-IFN(204) cell line were also done when appropriate.

Size analysis of induced mRNA in IE94-IFN cell lines. Northern RNA blot analysis of poly(A)-selected mRNA after fractionation from infected and uninfected samples by formaldehyde-agarose gel electrophoresis is shown in Fig. 2, together with the yields of biologically active IFN released into the supernatant medium from the same cultures. In this experiment, IFN-specific RNA was not detectable in the uninfected TK-IFN, IE175-IFN, and IE94-IFN(204) cell

lines, although a low-abundance basal-level mRNA species of 1,300 bp was present in IE94-IFN(211) cells. At 20 h after HSV-1 infection, greatly increased levels of poly(A)$^+$ IFN mRNA were present in both IE94-IFN cell lines as well as in the control cell lines. Both IE94-IFN cell lines gave major induced IFN mRNA species of approximately 1,300 bp, which corresponded closely to the expected sizes of the hybrid mRNA produced in each construct. In contrast, infection of the IE94-IFN(211) cells with simian CMV again gave no increase in the amount of the 1,300-bp mRNA over basal levels in uninfected cells. The infected TK-IFN cells contained a major species of 1,800 bp, corresponding to the expected size for the hybrid TK-IFN mRNA. These results suggested that the *trans*-activated IFN mRNA species present in both IE94-IFN cell lines and in the TK-IFN cell line probably all initiated correctly at the virus promoters present in the hybrid gene constructs. Infected IE175-IFN cells produced a range of relatively high-molecular-weight RNA species, which was consistent with the lack of any defined poly(A) addition site signals in the parent pGR238 construct. We showed previously that the poly(A)$^+$ IFN RNA species represents only a small fraction of the total IFN-specific RNA produced in the IE175-IFN cells (28). However, S1 nuclease analysis of 5′ ends in DNA-RNA hybrids formed with IFN-specific mRNA in the HSV-infected IE175-IFN cells confirmed that they too initiated close to the expected viral mRNA start site (not shown).

Kinetics of induction of IE94-IFN mRNA and its biologically active protein product. Comparisons of both the steady-state levels of human IFN mRNA and the titers of accumulated mature IFN protein produced at different times after HSV-1 infection of the DNA-transfected cell lines are presented in Fig. 3. The diagram shows the relative amounts of IFN-specific RNA measured by dot blot hybridization analysis (solid symbols), together with the yields of human IFN-β activity obtained from supernatants of the same culture samples for all three hybrid cell types at early times after HSV-1 (MP) superinfection. Basal levels accumulated over 20 h in parallel cultures of infected and mock-infected cells are also shown. The major purpose of this experiment was to examine whether initial accumulation of IE94-IFN RNA occurred concurrently with IE, DE, or late viral gene activation. Synthesis of biologically active IFN lagged several hours behind IFN-RNA induction and accumulation was maximal at late times (20 h) after infection, whereas steady-state RNA levels increased rapidly at early times and decreased substantially in all three cell types at later times. Induced IFN RNA first appeared within 1 h after virus adsorption in the IE175-IFN cells, but not until 2 to 3 h in IE94-IFN(211) cells and between 4 to 6 h in TK-IFN cells. Therefore, the *trans*-activation of IFN expression from the simian CMV IE promoter appeared to be more consistent with either a DE form of induction or of some intermediate category rather than activation at the IE level.

Relative to the amounts of IFN-specific RNA produced, the translation and processing of biologically active IFN product in the TK-IFN cell lines occurred at an efficiency similar to that after induction of the endogenous IFN-β gene in HF cells by Newcastle disease virus infection (not shown). In comparison, the ratio of biological activity to RNA produced was 2- to 3-fold lower for IE94-IFN(211) cells and at least 20-fold lower in both the IE175-IFN and IE94-IFN(204) cell lines. The low ratio of mature protein product to induced RNA levels in IE175-IFN cells relative to TK-IFN cells was noted previously (28), and in the case of the IE94-IFN(204) construct, we presume that transport and

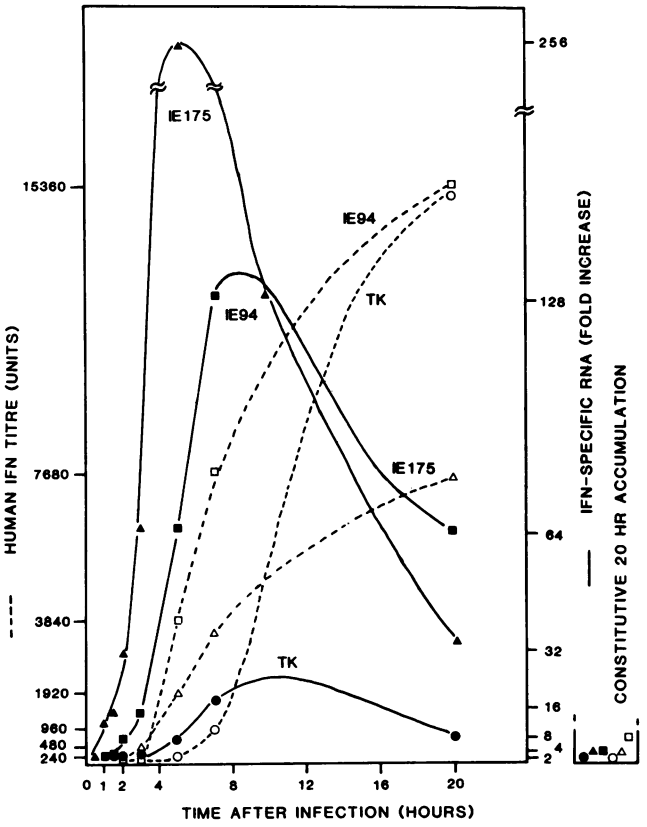

FIG. 3. Time course of IFN RNA and protein induction after HSV-1 superinfection. Parallel cultures of LH_2p192-8(TK-IFN), LH_2p238-38 (IE175-IFN), and LH_2p211-6 (IE94-IFN) cells were infected with HSV-1 (MP) and harvested at the times indicated for IFN-specific RNA analysis by RNA dot blot assay (solid line). Supernatants from the same cultures were assayed for human IFN-β biological activity in the vesicular stomatitis virus cytopathic reduction test on Vero cells (dashed lines). Basal or constitutive expression titers that accumulated after 20 h in parallel mock-infected cultures are given in the inset to the right of the figure. Solid symbols, RNA; open symbols, titer; ●,○, TK-IFN; ▲,△, IE175-IFN; ■,□, IE94-IFN.

removal of the signal peptide from the fusion protein was much less efficient in HSV-infected LTK$^+$ cells than in microinjected oocytes.

Induction of hybrid IFN in the presence of cycloheximide. The classical definition of whether a herpesvirus early gene has the properties of an IE or DE class gene depends on whether its mRNA is expressed after infection in the presence of a protein synthesis inhibitor such as cycloheximide. The results of an experiment designed to examine this point in all four cell types are shown in Fig. 4. RNA samples were assayed by dot blot analysis with an IFN DNA probe at 3 to 8 h after addition of cycloheximide alone, after infection with HSV-1 alone, or after infection with HSV-1 in the presence of cycloheximide. As expected, the TK-IFN cells gave no detectable RNA induction until 8-h after infection, and the activation at 8 h was abolished in the presence of cycloheximide (Fig. 4A). On the other hand, increased IFN RNA levels occurred within 3 h after infection in IE175-IFN cells (Fig. 4C), and the stimulation either remained equally strong or was enhanced slightly in the presence of cycloheximide. In both IE94-IFN cell lines (Fig. 4B and D), considerable induction of IFN RNA occurred by 8 h after infection,

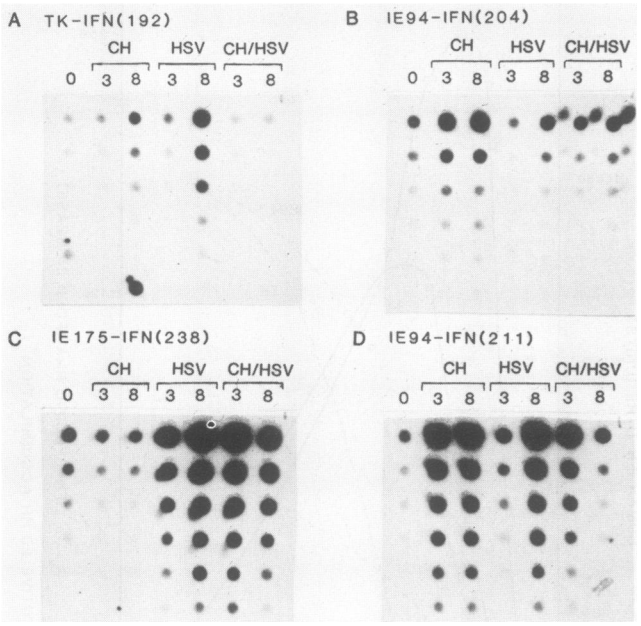

FIG. 4. Assay for IFN-specific RNA induced in the presence of cycloheximide. The autoradiograph shows RNA dot blot hybridization assays of total RNA from (A) TK-IFN (LH_2p192-8), (C) IE175-IFN (LH_2p238-38), (B) IE94-IFN (LH_2p204-32), and (D) IE94-IFN (LH_2p211-6) cells at 3 and 8 h after treatment with cycloheximide alone (CH), infection with HSV-1 (HSV), or infection in the presence of cycloheximide (CH/HSV). Each vertical column contains serial twofold dilutions starting with 10 μg of total cell RNA. The hybridization probe used was the isolated 560-bp IFN cDNA fragment labeled with [α-^{32}P]CTP by nick translation.

although not at 3 h (i.e., DE-type kinetics), and the induction was not abolished by cycloheximide but was clearly enhanced at 3 h after infection in the presence of cycloheximide. Unexpectedly, in both IE94-IFN cell lines, the control samples with cycloheximide but without virus infection were also induced to levels at least as high as those obtained after infection. The enhancing effect of cycloheximide was readily apparent even within only 3 h after addition of the drug. Most significantly, cycloheximide gave no enhancement over basal RNA levels in the IE175-IFN cell lines. Some enhancement of TK-IFN RNA may also have occurred after cycloheximide addition; however, if reproducible, the effect is much slower and weaker than that observed in either type of IE94-IFN cells.

Therefore, the results with IE175-IFN and TK-IFN cells were essentially those expected for correct and typical IE and DE activation mechanisms, respectively. In contrast, the IE94-IFN cell lines behaved differently from either of the control cell lines, and the results in both the IE94-IFN(204) and IE94-IFN(211) cells were qualitatively identical, although magnified in the latter. These findings gave the first unambiguous indications that the mechanism of HSV *trans*-activation of the CMV IE94-IFN hybrid genes differed from those acting on either the HSV IE-IFN or DE-IFN constructs and that it may represent a previously unrecognized HSV regulatory process.

IE94-IFN *trans*-activation does not require an active IE175 gene product. The results described above suggested, on the one hand, that IE94-IFN activation occurs with DE-type kinetics, but also, on the other hand, that de novo viral gene expression may not be involved, because the virus-induced activation was not abolished by cycloheximide. However, the ability of cycloheximide alone to increase IE94-IFN RNA levels in these cells obviously greatly complicated the interpretation of the latter experiment. As an alternative approach to unraveling the mechanism of IE94-IFN induction, we compared the induction patterns in the IE94-IFN(211) cells and both HSV cell types after infection with HSV conditional mutants blocked at various levels at early stages in the infection process. In the first set of experiments we examined the results of infection with HSV-1 (KOS) *ts*B2 and the supposedly less "leaky" mutant HSV-1 (17) *ts*K viruses, which both contain mutations within the IE175 gene and fail to transcribe DE genes after infection at 39°C (35). Table 1 shows the levels of IFN activity accumulated by 20 h after infection with *ts*B2 and *ts*K under both permissive and nonpermissive conditions and lists the ratios of product at 39 and 33°C in each of several experiments. As expected, in TK-IFN cells, neither mutant was able to stimulate IFN synthesis at 39°C. In contrast, IE175-IFN cells were hardly affected, presumably because the stimulation in this case required only the incoming virion factor without de novo viral gene expression. Induction in IE94-IFN(211) cells was also relatively unaffected by nonpermissive conditions after *ts*B2 and *ts*K infection. Examination of IFN RNA levels by dot blot analysis at both 5 and 20 h from one of these same sets of experiments (Fig. 5) gave essentially concordant results. Induction of IFN RNA levels with *ts*K was temperature sensitive in the TK-IFN cell line but not in IE175-IFN or IE194-IFN(211) cells. Considering that IFN RNA induction in IE94-IFN(204) cells was also equally effective at either 33 or 39°C with *ts*B2 virus, we conclude that a fully active IE175K gene product is not required for *trans*-activation of the CMV IE promoter in either type of IE94-IFN cell line.

IE94-IFN *trans*-activation does not occur in the absence of viral gene expression. Others have shown that the mutant virus HSV-1 (HFEM) *ts*B7, which fails to release input viral DNA into the nucleus at the nonpermissive temperature, is nevertheless capable of activating expression of HSV IE175-

TABLE 1. Comparison of IFN induction in different cell lines in response to infection with mutants deficient in HSV IE gene functions

Cell line	Superinfecting virus	IFN (U/ml)		Ratio (39°C/33°C)
		33°C	39°C	
TK-IFN(192)	None	30	30	
	*ts*B2	3,840	60	<0.015
	*ts*B2	61,440	480	0.008
	*ts*B2	16,000	125	0.008
	*ts*K	7,680	30	<0.004
	*ts*B7	8,000	1,000	0.125
IE175-IFN(238)	None	120	120	
	*ts*B2	3,840	1,920	0.5
	*ts*B2	7,680	7,680	1.0
	*ts*B2	8,000	4,000	0.5
	*ts*K	3,840	7,680	2.0
	*ts*B7	3,840	1,920	0.5
	*ts*B7	8,000	2,000	0.25
	*ts*B7	3,600	2,800	0.7
IE94-IFN(211)	None	500	1,000	
	*ts*B2	50,000	30,000	0.6
	*ts*B2	7,680	7,680	1.0
	*ts*K	30,700	15,360	0.5
	*ts*B7	64,000	2,000	<0.03
	*ts*B7	16,000	1,000	<0.06

TK and IE175-CAT constructs, presumably because positive regulation at this level is mediated by the pre-IE factor released from incoming virions (1, 28, 36). Therefore, in the series of comparative experiments with the three hybrid IFN cell lines, we also examined the results of infection at 33 and 39°C with this "uncoating" mutant. Infection with *ts*B7 gave induced levels of IFN product under nonpermissive conditions only in IE175-IFN cells, whereas induction at 39°C for 20 h in TK-IFN cells and IE94-IFN cells was relatively temperature sensitive (Table 1). In our hands, *ts*B7 consistently gave some breakthrough of DE expression at later stages of infection, but failed to give any significant increase in IFN activity above basal levels at the nonpermissive temperature in IE94-IFN(211) cells. Again, examination of the RNA levels at both 5 and 20 h reinforced these conclusions (Fig. 5). IFN RNA was not stimulated in TK-IFN cells with either *ts*K or *ts*B7 at 39°C, whereas it was still activated strongly in IE175-IFN cells with both *ts*K and *ts*B7 at 39°C. In contrast, IE194-IFN(211) cells infected with *ts*K gave increased IFN RNA levels at 39°C, but the cultures infected with *ts*B7 did not. We conclude from these observations that, unlike the results with the HSV IE promoter in IE175-IFN cells, the HSV pre-IE virion factor is not responsible (or at least not sufficient) for *trans*-activation of the CMV IE promoter in either type of IE94-IFN cell line.

Correct initiation of IE94-IFN mRNA after HSV or cycloheximide induction. An S1 RNA-DNA hybrid protection experiment with cycloheximide-induced IE94-IFN RNA was carried out to examine whether the RNA produced after protein synthesis inhibition represented authentic IE94-IFN hybrid mRNA. Most of the IFN-specific mRNA synthesized in IE94-IFN(211) cells after either cycloheximide treatment or HSV-1 infection was indeed initiated at a single location 590 bp upstream from the *Bgl*II site in the IE94-IFN hybrid gene (Fig. 6). This site corresponds to the authentic IE94 start site in the virus promoter at position +30 upstream from the *Sac*I site (Jeang et al., submitted for publication). Furthermore, up to 16,000 U of biologically active IFN-β per ml was released into the culture fluid within 3 h after removal of the cycloheximide. The amount of IFN-specific S1-protected RNA also increased at the 2- and 3-h time points after cycloheximide release but decreased thereafter (not shown). Therefore, whatever the mechanism of the cycloheximide induction in the IE94-IFN(211) cells, it also gave rise to functional IFN mRNA driven by the input CMV IE94 promoter-regulatory region.

Integrated IE94-IFN does not respond to the HSV-induced DNA amplification mechanism. Finally, we asked whether the DNA amplification events shown previously to occur after HSV infection of IE175-IFN cells, but not in TK-IFN cells (28), might also contribute to IE94-IFN induction. The results of both DNA and RNA dot blot analyses with IFN-specific probes showed that HSV infection of IE94-IFN(204) cells did not induce amplification of the transfected IFN DNA sequences under conditions in which six- to eightfold stimulation in IFN RNA synthesis occurred (not shown). In contrast, in parallel experiments the IFN DNA sequences in IE175-IFN cells were amplified at least eightfold by 20 h after infection, and these replication events required the viral DNA polymerase, as shown by sensitivity to phosphonoacetic acid treatment.

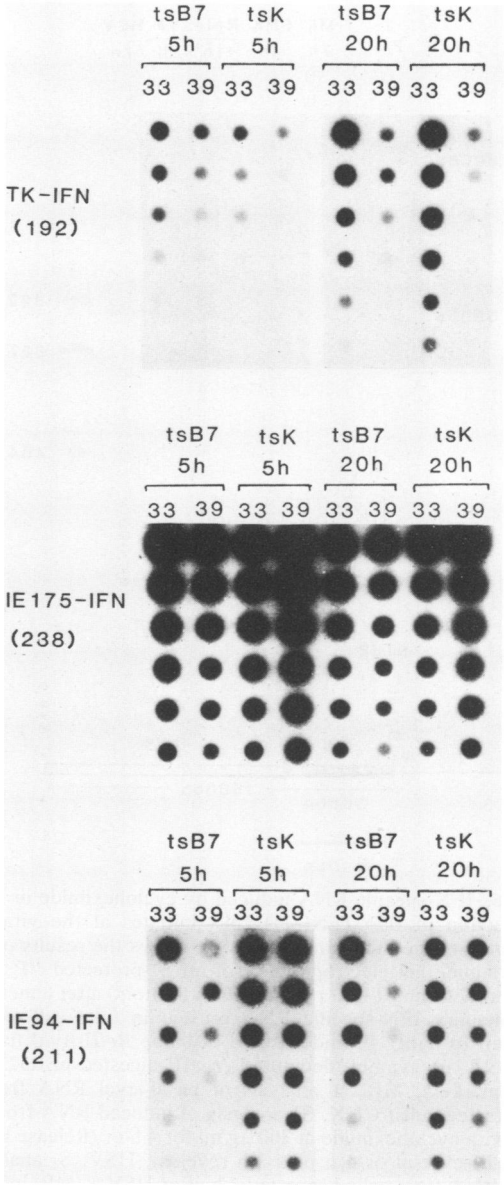

FIG. 5. Superinfection induction of IE94-IFN(211) RNA requires viral gene expression but is also independent of a functional IE175 gene product. The autoradiograph shows an RNA dot blot hybridization analysis of IFN-specific RNA present in TK-IFN (LH_2p192-8), IE175-IFN (LH_2p238-38), and IE94-IFN (LH_2p211-6) cells at 5 and 20 h after infection with either *ts*B7 or *ts*K virus under permissive (33.5°C) and nonpermissive (39.5°C) conditions.

DISCUSSION

The experiments described here were undertaken initially for two reasons: first, to further demonstrate the strong constitutive character of the IE94 promoter (4, 16; Jeang, thesis, 1984), and second, to ask whether the HSV virion factor mechanism for activation of the IE promoters through the specific TAATGARAT signals (5, 18, 21, 36) might cross-react with the many TAATGA and CAATGG elements present in the 5′ upstream regions of the IE94 promoter-regulatory region (Jeang, thesis, 1984; Jeang et al, submitted for publication). Therefore, the lack of constitutive expression of IE94-IFN in the coselected LTK^+ cell lines and our evidence for HSV *trans*-activation by some other mechanism were both major surprises. In all other situations in which we have introduced IE94 promoter-

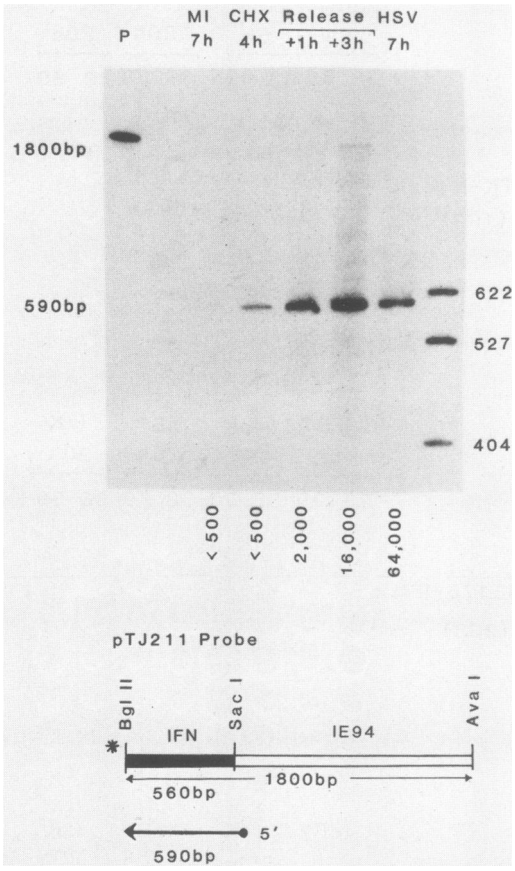

FIG. 6. IFN-specific RNA induced by cycloheximide or HSV-1 superinfection in IE94-IFN(211) cells initiates at the viral IE94 promoter start site. The autoradiograph shows the results of urea-polyacrylamide gel electrophoresis of an S1-protected ^{32}P 5′-end-labeled probe from pTJ211 plasmid DNA (bottom) after annealing to complementary IFN-specific RNA present in total cell extracts. Lanes (left to right): P, undenatured 1,800-bp *Bgl*II-*Ava*I fragment probe DNA; no symbol, denatured *Hae*III-digested pBR322 DNA as size markers; MI, S1 analysis of basal-level RNA from 7-h mock-infected cells; CHX, S1 analysis of induced RNA from cells treated with cycloheximide at 200 μg/ml for 4 h or (Release lanes) 4 h plus 1-h reversal or 4 h plus 3-h reversal; HSV, S_1 analysis of induced RNA from cells harvested 7 h after HSV-1 (MP) infection; no symbol, additional single-stranded pBR322 *Hae*III DNA size markers. IFN titers for supernatants of the same cultures are listed below each lane.

containing plasmids into eucaryotic cells we have observed strong constitutive expression: (i) the IE94-IFN(204) and IE94-IFN(211) constructs give several hundred-fold greater yields of biologically active IFN in microinjected oocytes than do IE175-IFN and TK-IFN constructs (Jeang et al., submitted for publication); (ii) IE94-CAT constructs with 5′ upstream sequences identical to those in IE94-IFN(211) give equal or greater yields of CAT product than those produced by the complete SV40 early promoter-enhancer region (pSV2-CAT) in both transient expression assays and long-term unlinked Neo^r coselection systems in LTK$^-$ and Vero cells (Jeang et al., submitted for publication); and (iii) the intact IE94 gene itself, when transfected into LTK$^-$ cells by linked TK coselection, yields cell lines constitutively expressing sufficient amounts of the viral IE94 protein to be detectable directly as both a [^{35}S]methionine-labeled polypeptide and as the major ^{32}P-labeled polypeptide in the culture extracts (16).

Mouse LTK$^-$ cells are nonpermissive for human and simian CMV, and in contrast to BALB/3T3 cultures, although viral DNA enters the nucleus, the infection process is blocked before synthesis of the major IE94 or IE68 proteins occurs (20; R. L. LaFemina and G. S. Hayward, submitted for publication). However, it is clear from the above observations that L cells do not lack appropriate factors for transcription from the IE94 promoter. Therefore, we surmise that the absence of strong basal expression of integrated IE94-IFN in mouse LTK$^+$ cell lines (but not in oocytes) involves either (i) intrinsic instability of the IFN mRNA sequences (38) compared with both CAT mRNA and intact IE94 mRNA in mouse L cells, (ii) selection against constitutive expression of the biologically active IFN protein product, or (iii) specific repression because of some unknown feature of the IE94-IFN constructs. Considering first that two quite different IE94-IFN constructs were used (one with and one without the major IE94 intron region) and second that 5′ and 3′ flanking sequences identical to those in IE94-IFN(211) are present in the intact viral IE94 gene and in the IE94-CAT constructs being expressed abundantly in the LH_2p198-3, VNp50-1, and LNp278-1 cell lines (16, Jeang et al., submitted for publication), we conclude that the unique unstable or repressible feature of these constructs probably resides within the IFN mRNA sequences. It should be emphasized that both low basal expression and HSV induction were observed in many independent cell clones receiving IE94-IFN plasmids and that these properties are therefore unlikely to reflect features of a specific unusual integration site.

The possibility that HSV and CMV possessed cross-reacting virion factor activation mechanisms was suggested by our earlier experiments with transient assays in Vero cells in which CMV (Colburn) superinfection activated expression from an IE175-CAT construct by 100-fold (30). However, we have subsequently found that this phenomenon represents a nonspecific CMV *trans*-activation mechanism which does not require the 5′ upstream TAATGARAT signals in the IE175 promoter (P. O'Hare, L. Sha, and G. S. Hayward, unpublished data). Similarly, the experiment described here with HSV-1 *ts*B7 infection, in which the integrated IE175-IFN gene but not IE94-IFN was activated, clearly rules out any cross-reacting "pre-IE" virion factor mechanism. Others have recently described evidence for a weak CMV virion factor mechanism for activation of the very similar human CMV IE68 promoter (44, 46), but we have not observed any significant effect of CMV (Colburn) superinfection on the IE94-IFN constructs in LTK$^+$ cells as described here or on cotransfected IE94-CAT constructs in fully permissive Neo^r Vero cell lines (Jeang et al., submitted for publication). The IE94-IFN cell lines also differ greatly from those carrying IE175-IFN by being strongly inducible by treatment with cycloheximide alone without HSV superinfection. We have shown that this induction leads to correctly initiate IE94-IFN mRNA and gives biologically active IFN protein product after reversal of the protein synthesis block. Boom et al. (3) have reported recently that expression of the isolated intact human IE68 gene in cotransfected Rat-1 cell lines can also be induced by cycloheximide treatment.

The mechanism of IE94-IFN induction by HSV superinfection in these cotransfected cell lines also differs from that of the standard *trans*-activation of both intact DE genes in the input infecting virus genome and of DE hybrid promoter

targets. For example, in our hands, the TK-IFN construct in the LH_2p192-8 cell line and TK-CAT or 38K-CAT constructs in both transient and long-term assay systems all respond to HSV infection in an IE175-dependent manner (28, 30, 31, 41). However, both IFN-specific RNA analysis and biological activity measurements indicated that induction of IE94-IFN was equally efficient at permissive and nonpermissive temperatures after infection with the HSV-1 *ts*K or *ts*B2 mutant viruses, which produce an inactive form of the IE175 regulatory gene product at 39°C (35). Since the response occurs at least as rapidly as the standard DE induction of TK-IFN, we conclude that either an IE gene or another virion factor, one that is not released by infection with HSV-1 *ts*B7 under nonpermissive conditions, must be responsible. Assuming that an HSV IE gene product is involved, there are four other IE genes (IE110, IE65, IE63, and IE12) that could be candidates and whose functions currently remain unclear. We also cannot rigorously exclude the possibility of a function involving IE175 that is not affected by the *ts*B2 or *tsK* lesion. At this stage, the IE110 protein might be the most obvious candidate, because it exhibits nonspecific *trans*-activation of a variety of heterologous target constructs in some transient expression assays and appears to act synergistically with IE175 in other systems (9, 12, 31, 32, 37). However, the mechanism of IE110 action (either direct transcriptional, posttranscriptional processing, or mRNA stabilization) has not yet been defined. Recent studies have shown that HSV superinfection also activates both cellular heat shock proteins (22) and integrated cotransfected globin genes (10), but in both cases these phenomena appear to be IE175 dependent.

In assessing the relative significance of the various different *trans*-activation effects described here, one should also take into account the gene copy number of the cell lines involved. Assuming that a significant proportion of the 128 copies of the hybrid gene DNA in the IE94-IFN(211) clone 6 cell line are functionally active and since the TK-IFN cell line with only 4 copies of the TK-IFN hybrid gene gave a nearly equal overall induction response, it is not unreasonable to suggest that the magnitude of the novel IE94-IFN response in relation to the number of functional genes present may be as much as an order of magnitude lower than that of the standard DE *trans*-activation mechanism acting on TK-IFN. Nevertheless, because the TK-IFN response observed was often as high as 500-fold at the protein level, the IE94-IFN effect is still one of considerable magnitude.

Finally, it is worth noting that HSV infection usually results in rapid shutoff and degradation of many cellular mRNA species. Therefore, whatever the mechanism of this novel *trans*-activation process, we believe that it must represent part of the normal process by which HSV either discriminates between viral and cellular mRNAs or selectively activates or stabilizes a small number of viral and cellular mRNAs (13). The evidence suggests that this particular mechanism will only be observed with an unstable mRNA species under the control of a naturally strong but perhaps "repressed" promoter-regulatory region. Whereas we cannot yet evaluate whether this novel HSV induction phenomenon might play any role in IFN-mediated antiviral responses, it is of interest that transcription of the endogenous IFN-β gene in cultured diploid HF cells can also be induced by HSV infection, but in this case only in the presence of cycloheximide and not by either agent alone (J. Mosca, P. Pitha, and G. S. Hayward, unpublished data). Details of the mechanisms of these two processes are currently under investigation.

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