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Herpes Simplex Virus Infection Selectively Stimulates Accumulation of Beta Interferon Reporter Gene mRNA by a Posttranscriptional Mechanism

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To study the mechanism of a novel herpes simplex virus (HSV) activity that stimulates expression of reporter genes containing beta interferon (IFN-β)-coding sequences, we have established permanent DNA-transfected cell lines that each contain two distinct hybrid genes encoding mRNA species with different half-lives. These reporter genes comprised either the human IFN-β- or bacterial chloramphenicol acetyltransferase (CAT)-coding and 3′ untranslated regions placed under the transcriptional control of the powerful major immediate-early promoter-enhancer region (IE94) from simian cytomegalovirus. Most of the dual-transfected cell lines yielded significant levels of steady-state IE94-CAT mRNA and abundant constitutive synthesis of CAT enzyme activity, whereas no accumulation of IE94-IFN mRNA could be detected. However, infection with HSV type 1 resulted in a 300-fold increase in IE94-IFN-specific mRNA transcripts, compared with no more than 3- to 5-fold stimulation of IE94-CAT-specific mRNA. In contrast, cycloheximide treatment increased stable mRNA levels and transcription initiation rates from both the IE94-IFN and IE94-CAT hybrid genes. Run-on transcription assays in isolated nuclei suggested that induction of IE94-IFN gene expression by HSV type 1 occurred predominantly at the posttranscriptional level. Enhancement of the unstable IFN mRNA species after HSV infection was also observed in cell lines containing a simian virus 40 enhancer-driven IFN gene (SV2-IFN). Similarly, in transient-transfection assays, both SV2-IFN and IE94-IFN gave only low basal mRNA synthesis, but superinfection with HSV again led to high-level accumulation of IFN mRNA. Finally, substitution of the SV2-IFN gene 3′ region with poly(A) and splicing signals from the SV2-CAT gene cassette led to stabilization of the IFN mRNA even in the absence of HSV. Therefore, we conclude that HSV infection leads to selective accumulation of IFN-β mRNA by a posttranscriptional mechanism that is reporter gene specific and promoter independent.

We are interested in the mechanisms that are used by herpesviruses to distinguish between host and viral mRNA species and to selectively express different classes of viral genes. Herpes simplex virus (HSV) has been shown to shut off host cell DNA, RNA, and protein synthesis and to destabilize the majority of host mRNAs (4, 6, 20, 23, 30), whereas complex transcriptional regulatory controls involving a virion factor and newly synthesized immediate-early (IE) effector gene products exert predominantly positive influences on viral gene expression (20–22, 29, 32). Many 5′ promoter regulatory sequences and *trans*-acting factors that are involved in HSV gene expression have been identified. However, posttranscriptional events, including stabilization or destabilization of mRNA species and alterations in mRNA transport or processing, are also thought to contribute significantly to overall gene expression in many systems. For example, conserved AU-rich sequences in the 3′ untranslated portions of c-*myc*, beta interferon (IFN-β), and other inducible cellular mRNAs with short half-lives have been shown to have important influences on mRNA stability (10, 31, 34), and the human immunodeficiency virus (HIV)-encoded Rev and possibly also Tat proteins carry out some of their functions at the posttranscriptional or postinitiation level.

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To study the posttranscriptional factors involved in regulation of viral mRNA accumulation during HSV infection, we have compared the properties of an intrinsically unstable mRNA encoding human IFN-β1 with an mRNA encoding the bacterial chloramphenicol acetyltransferase enzyme (CAT) that was demonstrated to have an intermediate half-life. The induction of endogenous human IFN-β mRNA expression by Newcastle disease virus infection or by poly(rI)-poly(rC) treatment is regulated at both the transcriptional and posttranscriptional levels. Treatment with inhibitors of protein synthesis, such as cycloheximide (CHX), greatly increases accumulation of endogenous IFN-β mRNA induction. Subsequent removal of the inhibitors results in reestablishment of transcriptional repression and a rapid degradation of the IFN mRNA with a half-life of 20 to 30 min (24, 25, 28, 33, 34). In contrast, reporter gene mRNA for the bacterial CAT enzyme has an intrinsically longer half-life (>4 h) in mammalian cells (17).

The major IE gene (IE94, or MIE) of simian cytomegalovirus (SCMV) produces the most abundant viral mRNA synthesized in infected human fibroblasts in the presence of CHX and encodes the only viral mRNA and protein species expressed after SCMV infection of nonpermissive rodent cells (7). The MIE gene mRNA and at least one of its protein products (IE94, or IE1) is also expressed constitutively in stable DNA-transfected Ltk$^+$ cell lines (8). The strong expression from this promoter is believed to be primarily conferred by the IE94 upstream enhancer region, which consists of a complex arrangement of multiple interspersed

repeated elements. These include consensus NF-κB-like and serum response factor-binding sites, as well as nuclear factor I (NFI) binding sites and cyclic AMP response elements (3, 3a, 9). Consistent with these properties, IE94-CAT reporter genes show high constitutive basal expression that is not affected by HSV infection in transient-expression assays (9, 13a).

By contrast, when a human IFN-β cDNA reporter gene was placed under the transcriptional control of the SCMV IE94 promoter-enhancer region and integrated into stable Vero or Ltk$^-$ cell lines, no constitutive steady-state levels of IFN mRNA could be detected (16). Surprisingly, the apparently inactive IE94-IFN hybrid gene could be induced more than 200-fold (at both the mRNA and protein levels) by HSV superinfection of these cell lines, although SCMV had no effect. This induction proved to represent a novel HSV regulatory mechanism, distinct from those acting on reporter genes containing either HSV IE or delayed-early (DE) promoters in similar cell lines. For example, both IE175-IFN and IE175-CAT reporter genes respond specifically to the VP16 transcription factor (also called VF65, Vmw65, and α-TIF) that is introduced with the infecting virus particles, whereas stimulation of the expression of thymidine kinase (TK)-IFN and TK-CAT reporter genes requires de novo synthesis of the IE175 (or ICP4) transcriptional transactivator protein (18). In contrast, induction of IE94-IFN occurs independently of both the virion factor and the presence of an active IE175 protein, but does require de novo synthesis of other HSV IE proteins (16).

The observed low basal accumulation and HSV inducibility of IE94-IFN in permanent cell lines appeared to be a unique feature of the IFN reporter gene and may also have been dependent in same way on the target gene's being in an integrated state. For a more detailed investigation of this unusual HSV induction phenomenon, we considered it necessary to (i) demonstrate directly by microinjection into *Xenopus* oocytes that the IE94-IFN constructions do have the expected high basal transcription initiation properties; (ii) ask whether the induction is specific for the IE94 promoter-enhancer region or the particular IFN reporter gene used; (iii) measure the rates of transcription initiation and the half-life of the IFN mRNA produced before and after induction in our permanent DNA-transfected cell lines; and (iv) evaluate the induction in dual-transfected cell lines containing both IFN and CAT reporter genes controlled by identical IE94 promoter-enhancer regions. Our results indicate that HSV infection leads to an increased half-life and stable accumulation of human IFN-β mRNA but not of bacterial CAT mRNA in both permanent cell lines and transient-expression assay systems.

MATERIALS AND METHODS

Cell lines, viruses, and cycloheximide induction. Ltk$^-$ cells (thymidine kinase-deficient murine fibroblast line), Ly cells (murine fibroblast line), Vero cells (African green monkey kidney cell line), GM2504 cells (human fibroblast line with trisomic chromosome 21), and cloned G418-resistant transfected cell lines containing stably integrated plasmid DNA were grown in either Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum or OPTI-MEM medium supplemented with 2.5% fetal calf serum. HSV-1(MPcl-20) virus stock was prepared and titered on Vero cells. HSV-1 infection of G418-resistant cell lines and in transient-expression assays was usually carried out at a multiplicity of infection (MOI) of 5 and allowed to proceed for 8 h at 37°C before harvesting. Cycloheximide was used at 6 μg/ml for Ltk$^-$ cell lines and 50 μg/ml for Vero cell lines for 4 h; the treatment was reversed by washing with three changes of fresh warm medium and incubation for an additional 0 to 16 h. Actinomycin D was not used in the reversal steps because of previous evidence that it leads to alterations in the turnover rate of IFN-β mRNA. Control SCMV(Colburn)-infected cells (7, 8) were usually harvested at 48 h for S1 analysis or CAT assays.

Plasmid DNA, reporter genes, and riboprobes. Construction of the human IFN-β cDNA reporter genes HSV-1 TK-IFN (in plasmid pGR192), HSV-1 IE175-IFN (plasmid pGR238), SCMV IE94(−3500/+1100)-IFN (plasmid pTJ204), and SCMV IE94(−3500/+30)-IFN (plasmid pTJ211) was described by Mosca et al. (16). The IE94(−990/+30)-CAT gene in plasmid pTJ278 and the IE94(−300/+30)-CAT gene in plasmid pTJ280 together with pSV2-CAT were those used by Jeang et al. (9). The target plasmids pTJ211 and pJM50 were created by inserting either IFN or CAT coding sequences downstream from the SCMV IE94 promoter-enhancer region and upstream from the IE94 exon 4 polyadenylation signal (Fig. 1A). The starting plasmid for these constructions (pTJ148) encompasses an intact 2.3-kb spliced transcript encoding the major 94-kDa SCMV IE1 acidic nuclear protein. Cleavage of pTJ148 with *Sac*I removed 3.1 kb from inside the IE1 gene, permitting insertion of heterologous coding sequences 30 bp downstream from the IE1 mRNA start site under the transcriptional control of the IE94 promoter and poly(A) signal. A 760-bp *Hin*cII fragment from the genomic clone pIFR (17), encompassing the entire coding sequence for the human IFN-β gene including 3 bp upstream from the IFN-β initiation codon and 200 bp downstream from the protein termination codon, was inserted with the addition of *Sac*I linkers into the deleted pTJ148 to yield an IE94-IFN reporter gene in plasmid pTJ211. Similarly, a 1,500-bp *Hin*cII fragment from pCATB′, encompassing the entire CAT gene coding sequence starting 50 bp upstream from the initiation codon and including the small t-antigen splice signal (but not the associated simian virus 40 [SV40] early region poly[A] signal) was inserted into the *Sac*I-deleted pTJ148 to make another version of the IE94-CAT reporter gene in plasmid pJM50. A mutated variant of the pTJ211 plasmid containing a nonfunctional IFN coding region with a 10-bp *Cla*I linker inserted at the *Pvu*II site is referred to as pTJ211mod (see Table 2).

The SV2-IFN genes in plasmids pJM40 and pJM41 both contain human IFN-β reporter genes that are under the transcriptional control of the SV40 promoter-enhancer region (−340 to +58) from pSV2-CAT, but they differ in their 3′ untranslated regions. SV2-IFN(40) contains an 810-bp *Taq*I fragment from the human genomic IFN-β gene in pIFR bounded by *Hin*dIII and *Bam*HI linkers and thus retains part of the IFN-β 3′ untranslated region, including the natural poly(A) signal. In contrast, in SV2-IFN(41) the 220-bp 3′ noncoding region beyond the *Bgl*II site was replaced with an 850-bp *Xho*II fragment from SV2-CAT, which contains the SV40 early-region splice signals and poly(A) site cassette.

The general structure of the four template plasmids used for riboprobe synthesis is summarized in Fig. 1B. For detection of IE94-IFN mRNA, the appropriate 1,800-bp *Ava*I-*Bgl*II fragment from pTJ211 was inserted between the *Ava*I and *Bam*HI sites in pGEM1 to form pJM142. For detection of IE94-CAT mRNA, a 1,250-bp *Sal*I-*Eco*RI fragment from pTJ278 was inserted into pGEM2 to form pJM158. For detection of SV2-IFN mRNA, an IFN-β ribo-

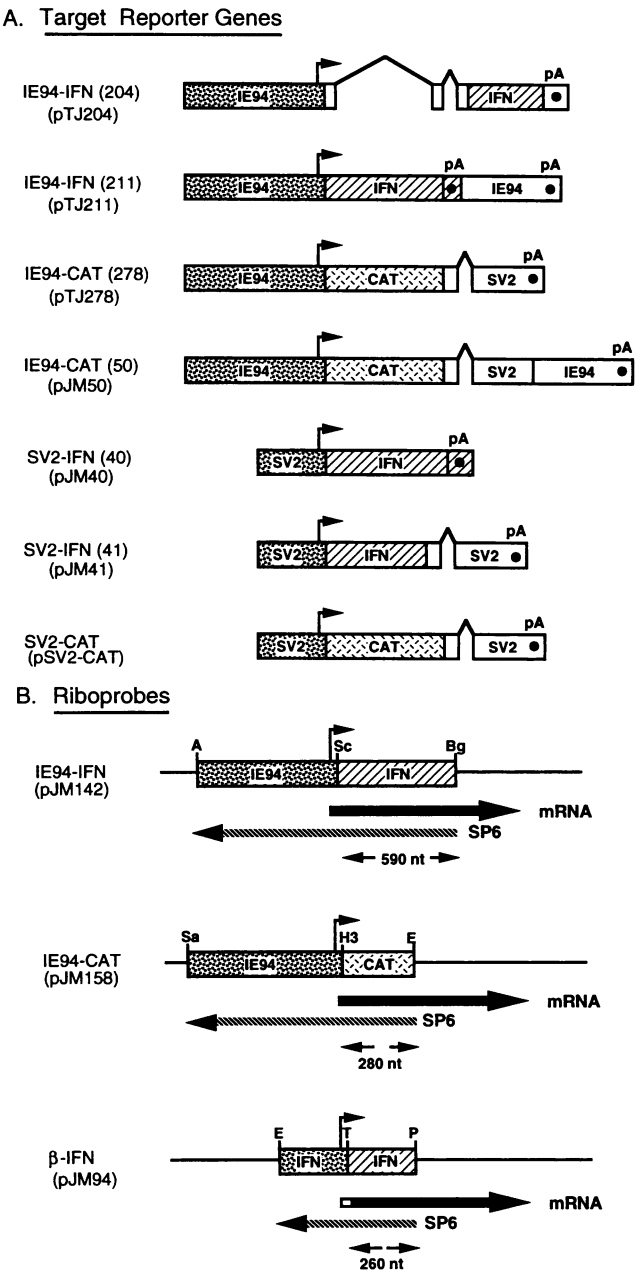

FIG. 1. Structures of relevant hybrid IFN and CAT reporter genes and riboprobes. (A) Diagramatic representations of the seven different versions of IFN and CAT target reporter gene expression vectors used in these studies. The CAT gene coding sequences and all cDNA sequences, coding regions, and genomic sequences derived from human IFN-β are designated by two different types of hatched bars. The SCMV major IE (IE94) and SV40 early (SV2) upstream promoter-enhancer domains are denoted by shaded bars, and downstream SV40- or SCMV-derived 3′ nontranslated domains are indicated by open bars. The mRNA start sites are designated by arrows, polyadenylation signals (pA) are shown by solid circles, and spliced-out intron regions are shown by inverted V symbols. (B) Diagramatic representations of the four different pGEM constructions used in SP6 polymerase synthesis of uniform ^{32}P-labeled riboprobes for S1 nuclease protection assays. The lengths of expected 5′ hybrid domains for the predominant correctly initiated mRNA species are given in nt. A, *Ava*I; Bg, *Bgl*II; E, *Eco*RI; H3, *Hin*dIII; P, *Pst*I; Sa, *Sal*I; Sc, *Sac*I; T, *Taq*I.

probe (pJM94) containing a 560-bp *Eco*RI-*Pst*I fragment from pIFR in pSP64 was used.

Establishment of permanent cell lines. Permanent Vero and Ltk$^-$ cell lines were generated by transfection with specific target plasmid DNA (10 μg per 100-mm dish) that had been coprecipitated with pSV2-NEO DNA (1 μg/100-mm dish). In the case of the dual-transfected stable cell lines, the DNA transfection mixture consisted of two target plasmid DNAs (10 μg each) and pSV2-NEO DNA. The calcium phosphate-DNA coprecipitate was prepared by mixing equal volumes of plasmid DNA in 0.25 M $CaCl_2$ solution with 2× HEPES-buffered saline (0.28 M NaCl, 0.01 M KCl, 0.0015 M Na_2HPO_4, 0.012 M dextrose, 0.05 M HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.1]) at 23°C for 15 min before addition to the culture medium. After a 12-h incubation at 37°C, cultures were washed with phosphate-buffered saline (PBS), treated for 2 min with 15% glycerol, and washed twice with PBS, and medium containing 5 mM sodium butyrate was added. After 24 h, the sodium butyrate medium was removed and G418 selection was applied (0.4 mg/ml for Ltk$^-$ and Ly cells, 1 mg/ml for Vero cells) until resistant colonies appeared, in 4 to 6 weeks. For cloned cell lines, single colonies were isolated, grown to mass culture, and screened for synthesis of human IFN after HSV infection. Otherwise, between 20 and 100 colonies were pooled together.

Formation of CAT enzymatic activity was measured by incubating ^{14}C-labelled chloramphenicol with 10-fold dilutions of extracts prepared from 5×10^6 cells after freeze-thawing. Biologically active human IFN-β secreted into the culture medium from transfected cell lines or after microinjection of plasmid DNA into *Xenopus* oocyte nuclei was analyzed by protection of Vero or GM2504 cells from vesicular stomatitis virus cytopathic effects in serial dilution assays (24, 25, 27).

Transient DNA transfection procedure. For transient-expression assays in Vero cells (20, 21), 10 μg of target plasmid DNA was mixed with 20 μg of effector plasmid DNA and $CaCl_2$, coprecipitated in 3 ml of HEPES-phosphate buffer, and added to cells in 30 ml of medium in a 175-cm^2 culture flask. The glycerol shock step was carried out at 12 h, and HSV or CHX treatment was initiated at 36 h after transfection. Total cell RNA was harvested 48 h after transfection.

RNA isolation and S1 hybrid protection analysis. Total cellular RNA was prepared from lysates of cell monolayers by a guanidine thiocyanate cell method as described previously (18). Mapping of the 5′ ends of mRNA was performed by S1 nuclease analysis with radiolabeled single-stranded DNA or RNA probes. To detect correctly initiated IFN or CAT mRNA species, uniformly labeled, complementary RNA probes were synthesized by in vitro SP6 polymerase-directed transcription from the respective fragments subcloned into pGEM vectors (Fig. 1B). Equal quantities of the RNA samples (10 μg) were hybridized in buffer containing 0.02 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], 2 M NaCl, 0.005 M EDTA (pH 6.5), 80% formamide, and 1.5×10^5 cpm of radiolabeled probe in a 10-μl volume overnight at 50°C. RNase digestion of hybridization reactions was performed with 35 U of S1 nuclease in a 300-μl volume at 37°C for 30 min. Samples were ethanol precipitated, and the dried pellet was resuspended in 80% formamide-dye loading buffer, heated for 5 min at 95°C, fractionated by electrophoresis though a 6% polyacrylamide–7 M urea gel at 35 mA for 2 h, and exposed to X-ray film.

Nuclear run-on transcription assay. To measure the transcription of a specific gene by the nuclear nascent RNA

TABLE 1. IFN activity expressed from two different hybrid IE94-IFN genes after microinjection into *Xenopus laevis* oocytes

Hybrid gene	Plasmid DNA	IFN titer[a] (U/ml)					
		Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	Expt 6
TK-IFN	pGR192	60	85	50	—[b]	—	—
IE175-IFN	pGR238	320	2,860	1,700	—	—	—
IE94-IFN(204)	pTJ204	13,000	15,000	18,000	12,000	2,000	2,350
IE94-IFN(211)	pTJ211	—	—	—	—	12,000	21,000
Vector	pBR322	<40	<40	<40	<40	<80	<80

[a] Combined values from both the incubation medium and oocyte extracts. All samples were assayed first on Vero cells. However, the values for the TK-IFN and vector samples were below the threshold of detection (<200 U/ml), and they were reassayed on the more sensitive GM2504 cells. Experiments 5 and 6 were carried out in oocytes derived from a different animal from those used in experiments 1 to 4.

[b] —, not done.

run-on assay (25), nuclei were isolated from 5×10^7 cells at the indicated times after treatment and stored at −70°C. Frozen nuclei (200 μl) were thawed at 23°C and incubated for 30 min at 30°C after addition of 200 μl of 2× labeling reaction buffer (0.3 M KCl, 0.01 M Tris-HCl [pH 8.0], 0.005 M $MgCl_2$, 0.005 M dithiothreitol, 0.001 M each ATP, CTP, and GTP, and 10 μCi of [α-^{32}P]UTP [3,000 Ci/mmol, 10 mCi/ml]). The ^{32}P-labeled RNA was purified by the addition of 2 μl of 10-mg/ml RNase-free DNase I for 5 min at 30°C, followed by the addition of 200 μl of 0.5 M Tris-HCl–0.125 M EDTA–5% sodium dodecyl sulfate (SDS) buffer (pH 7.4) plus 10 μl of 20-mg/ml proteinase K for 30 min at 42°C. The sample was extracted with 1 ml of phenol-chloroform-isoamyl alcohol, and ethanol precipitated, resuspended in 200 μl of 10 mM Tris-HCl–1 mM EDTA–10 mM $MgCl_2$ (pH 8.0), treated a second time with DNase I plus proteinase K, and then extracted with phenol-chloroform. The RNA was precipitated from the aqueous layer by the addition of 50 μl of 50% trichloroacetic acid–2.5% sodium pyrophosphate–10 μl of 10-mg/ml carrier tRNA for 30 min on ice, resuspended in 0.1 M sodium acetate (pH 7.2), and ethanol precipitated. The pellet was dissolved in 200 μl of H_2O, and the amount of radioactivity incorporated was determined. Equal amounts of counts from each sample were used as probes to Southern blots containing five fragments of *Eco*RI- and *Hin*cII-cleaved pIFR (17) plasmid DNA on nylon filters (3,255, 1,087, 767, 452, and 352 bp in size). The 352-bp fragment represents the IFN-β promoter, the 767-bp fragment contains the IFN-β body and coding region, and the 1,087-bp fragment contains the IFN -β 3′ untranslated regions. Hybridization was carried out in an 800-μl volume with 50% formamide and 10% dextran sulfate for 3 days at 37°C. The nitrocellulose filter strips were washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–10% SDS at 60°C for 15 min each and then twice in 2× SSC, treated with 1 μg of RNase A per ml in 2× SSC at 37°C for 15 min, and exposed to X-ray film.

RESULTS

Confirmation of the validity of the IE94-IFN promoter constructions. To demonstrate that the IE94-IFN reporter genes do indeed have high basal expression properties, we compared the levels of human IFN-β produced from four different hybrid IFN reporter genes after microinjection into *Xenopus* oocytes (Table 1). The construction of plasmids containing the TK-IFN, IE175-IFN, IE94-IFN(204), and IE94-IFN(211) genes was described previously (14, 16), and a comparison of the structures of the two different versions of the IE94-IFN genes is included in the summary diagram in Fig. 1A. Our previous studies have indicated that the instability of IFN mRNA observed in mammalian cells is apparently not a factor in microinjected oocytes (16).

Hybrid genes containing IFN-β cDNA inserted in the sense orientation behind either the HSV TK promoter (pGR192) or the HSV IE175 promoter (pGR238) both yield biologically active human IFN in microinjected oocytes (18, 27). However, compared with the HSV hybrid genes in similar assays, IE94-IFN(204) produced much higher levels of IFN (Table 1, experiments 1 to 4). Over several experiments, the basal activity of IE94-IFN(204) relative to that of IEI75-IFN and TK-IFN expressed in the oocytes averaged approximately 250:25:1.

Measurements on basal expression from the second IE94-IFN(211) hybrid gene, which lacks the large NH_2-terminal spliced segment within the IE94 coding region (introns 1 and 2), gave IFN titers approximately eightfold greater than those obtained with IE94-IFN(204) when microinjected into a parallel set of oocytes from the same animal (Table 1, experiments 5 and 6). This difference correlates with similar higher levels of induced IFN expression in IE94-IFN(211) than in IE94-IFN(204) cell lines (24). Although the absolute levels of IFN activity produced by pTJ204 in experiments 5 and 6 was nearly an order of magnitude lower than that in experiments 1 through 4, we conclude that the IE94-IFN(211) gene produces between 200- and 2,000-fold higher expression of biologically active IFN than does the TK-IFN hybrid gene.

Note that IE94-IFN(204) contains the entire 140-bp IE94 5′ untranslated leader region together with splicing signals from introns 1 and 2 plus a 3′ poly(A) signal from the SV40 late genes, whereas IE94-IFN(211) contains only 30 bp of IE94 leader sequence without any splice signals but retains the natural 3′ regions and poly(A) signal from the IE94 gene. Despite these differences, the IE94-IFN(204) and IE94-IFN(211) constructions behaved similarly with regard to both very high basal expression in oocytes and low basal expression combined with high inducibility by HSV infection in DNA-transfected mammalian cell lines (16). Therefore, the high expression obtained with the IE94-IFN constructions implies that strong basal expression is an intrinsic property of the IE94 promoter-enhancer region.

Integrated IE94-CAT hybrid genes are constitutively expressed and do not respond to HSV infection. To ask whether another integrated target reporter gene under the transcriptional control of the same SCMV IE94 promoter region was inducible by HSV, we tested two hybrid CAT reporter gene plasmids containing different lengths of 5′ upstream IE94 sequences: IE94(−990/+30)-CAT in plasmid pTJ278 and IE94(−300/+30)-CAT in plasmid pTJ280 (9). These hybrid gene plasmids were used to establish permanent DNA-

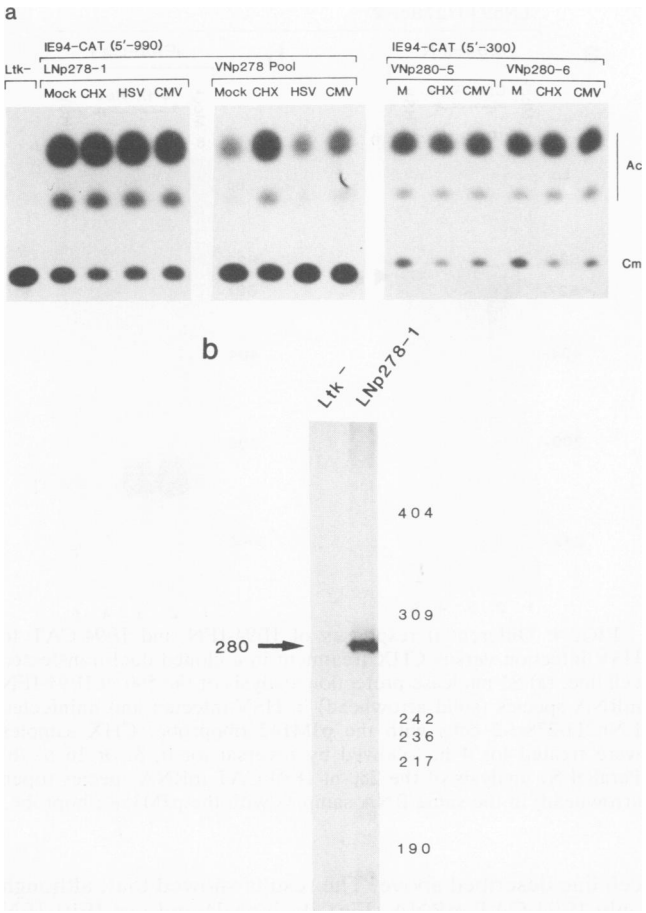

FIG. 2. Constitutive expression of IE94-CAT in permanent cell lines. (a) Autoradiographs of the results of CAT assays with extracts from four stable G418-resistant DNA-transfected cell lines that received coselected IE94-CAT plasmid DNA. Two different input plasmids were used; pTJ278, containing IE94(−990/+30)-CAT, and pTJ280, containing IE94(−300/+30)-CAT. One cell line was derived from mouse Ltk$^-$ cells (L), and the others were derived from monkey Vero cells (V). Three of the cell lines were derived from single colonies, and the fourth represented a pool of >50 colonies. Cell cultures were either given fresh medium for 8 h (Mock or M), treated with CHX for 4 h (CHX), infected with HSV-1(MP) for 8 h (HSV), or infected with SCMV(Colburn) for 48 h (CMV). (b) S1 nuclease protection analysis of correctly initiated IE94-CAT mRNA constitutively expressed from the IE94(−990/+30)-CAT gene in cell line LNp278-1. The control lane shows the absence of this mRNA species in the parent Ltk$^-$ cells. The probe represented a 5'-end-labeled 1,250-bp *SalI-Eco*RI DNA fragment from plasmid pTJ278. Sizes are shown in nucleotides.

transfected Vero and Ltk$^-$ cell lines by G418 coselection. Surprisingly, in all of these cell lines (whether derived from single or pooled colonies), strong constitutive expression of CAT activity was observed (Fig. 2a), which was not affected by HSV or SCMV infection and was affected only minimally by cycloheximide reversal treatment. Furthermore, analysis of stable CAT mRNA produced in one of these cell lines by an S1 nuclease protection assay with a ^{32}P-labeled DNA probe (Fig. 2b) revealed abundant constitutive expression of correctly initiated IE94-CAT mRNA (280-nucleotide [nt] band). These results resemble those observed with IE94-CAT in transient assays (9) but contrast dramatically with the earlier data for LTK$^+$-coselected cell lines containing the hybrid IE94-IFN(204) and IE94-IFN(211) gene constructions (16).

Differential responses of IE94-IFN and IE94-CAT genes to HSV infection in a dual-transfected cell line. To directly compare the responses of the hybrid IFN and CAT coding regions, we cotransfected both genes simultaneously into the same cells, thereby minimizing any variation due to host cell background effects. Plasmids containing the IE94-CAT(278) and IE94-IFN(211) genes were cotransfected together with pSV2-NEO into Ltk$^-$ cells by calcium phosphate precipitation. Single G418-resistant colonies were isolated and screened for retention of DNA sequences by the ability to induce human IFN-β after HSV infection. The results of testing for correctly initiated mRNA in one of the positive cell lines (LNp211/278cl-2) by S1 nuclease protection assays with appropriate riboprobes is shown in Fig. 3. IE94-CAT(278) mRNA was expressed constitutively, whereas basal expression of stable IE94-IFN(211) mRNA was again undetectable (Fig. 3a and b, mock lanes). Infection with HSV-1 resulted in stimulation of IFN mRNA in a dose-dependent manner that reached a 200-fold increase over the minimal detectable level at the highest MOI used (Fig. 3a). In contrast, a maximum three- to fourfold increase in CAT mRNA was observed under the same conditions (Fig. 3b). Accumulation of the 590-nt protected IFN mRNA was strongly dependent on the amount of input HSV used for infection (Fig. 3a), but both the level of 280-nt protected CAT mRNA (Fig. 3b) and the CAT enzyme activity (Fig. 3c) showed no more than a three- to fourfold increase over a 600-fold range in MOI.

Relative instability of human IFN-β mRNA compared with CAT mRNA in DNA-transfected cell lines. Since the integrated IE94-CAT and IE94-IFN target genes contain essentially the same promoter elements, the large differences in their basal expression and HSV inducibility could be related to differences in the relative stability and half-life of the IFN and CAT mRNAs in mammalian cells. For example, we have previously shown that the intact genomic human IFN-β gene, integrated into a G418-resistant cotransfected Vero cell line, produced mRNA that was rapidly degraded, with a half-life of less than 30 min (17). This is similar to the half-life of endogenous IFN-β mRNA observed in human cells (24). In contrast, a hybrid human IFN-β-CAT construction, in which the IFN coding sequences were replaced by the CAT coding region, produced much more stable mRNA in similar cotransfected cell lines (17). The strategy in those experiments was to induce the human IFN-β promoter with poly(rI):poly(rC) in the presence of CHX, which results in a transient, rapidly reversible enhancement of transcription and stabilization of endogenous IFN-β mRNA in human cells (25). CHX superinduction without reversal results in maximal accumulation of human IFN-β mRNA, and an estimate of the RNA stability can be assessed by monitoring the relative amount of RNA remaining after CHX reversal.

We have carried out similar experiments in Ltk$^-$ cells that received coselected intact human IFN-β or hybrid IFN-β-CAT genes. Following poly(rI):poly(rC) plus CHX treatment, the induced IFN mRNA was found to be rapidly degraded after a 30-min reversal step, whereas the IFN-CAT mRNA displayed a much longer half-life (4 to 8 h; not shown). These results suggest that human IFN-β mRNA, even when expressed from a gene introduced into Vero or Ltk$^-$ cell lines by DNA transfection, is regulated similarly to endogenous IFN mRNA induced in human cells.

CHX treatment activates expression of both IE94-IFN and

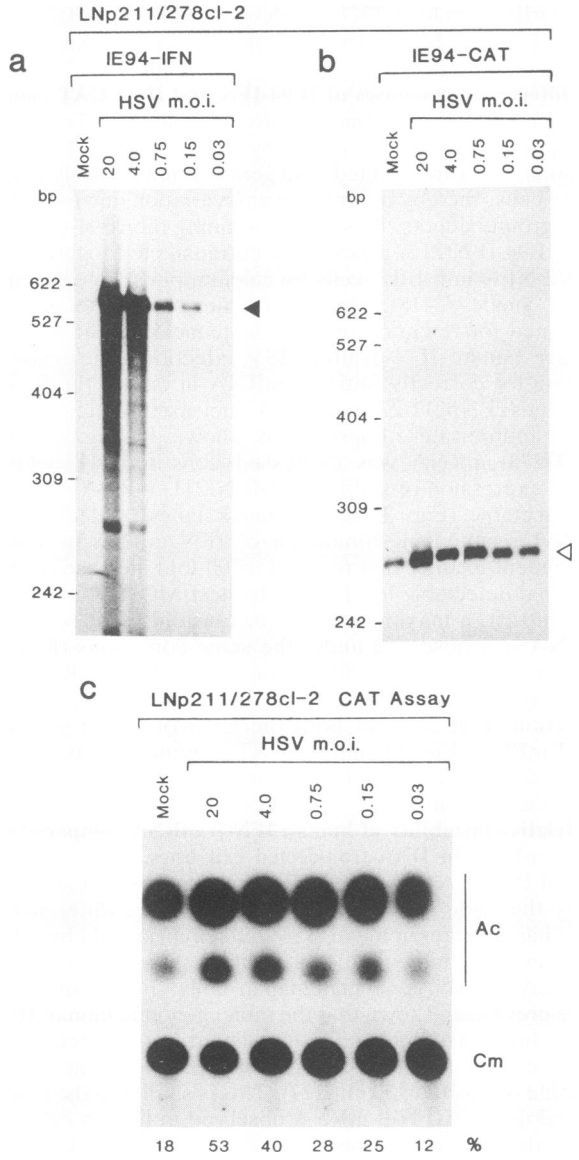

FIG. 3. Dependence for HSV induction of IE94-IFN mRNA on MOI and comparison with IE94-CAT expression in a cloned dual-transfected cell line. (a) S1 hybrid protection analysis of IE94-IFN(211) mRNA (solid arrowhead at 590 nt) with the pJM142 riboprobe in a cell line (LNp211/278cl-2) that received both pTJ211 (IE94-IFN) and pTJ278 (IE94-CAT) DNA as well as pSV2-NEO DNA. Whole-cell RNA samples were obtained after infection with HSV-1(MP) over a 600-fold range of different MOIs (PFU-per-cell ratios). (b) Parallel S1 protection analysis of IE94-CAT(278) mRNA (open arrowhead at 280 nt) with the pJM158 riboprobe in the same RNA samples used above. (c) CAT assay results on extracts of the same infected cell cultures used above. Percent conversion of [^{14}C]chloramphenicol (Cm) to acetylated derivates (Ac) is given below each lane.

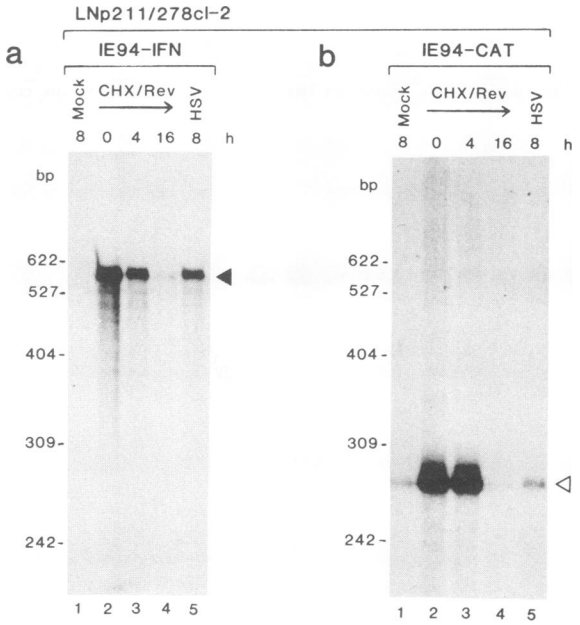

FIG. 4. Differential responses of IE94-IFN and IE94-CAT to HSV infection versus CHX treatment in a cloned dual-transfected cell line. (a) S1 nuclease protection analysis of the 590-nt IE94-IFN mRNA species (solid arrowhead) in HSV-infected and uninfected LNp211/278cl-2 cells with the pJM142 riboprobe. CHX samples were treated for 4 h, followed by reversal for 0, 4, or 16 h. (b) Parallel S1 analysis of the 280-nt IE94-CAT mRNA species (open arrowhead) in the same RNA samples with the pJM158 riboprobe.

IE94-CAT. In our previous studies, the levels of steady-state IE94-IFN mRNA (but not of IE175-IFN RNA) in Ltk$^+$ cell lines were also strongly induced by CHX treatment in the absence of HSV infection (16). To determine whether this type of induction is a promoter-specific phenomenon rather than reporter gene specific, we carried out similar experiments in the dual-transfected cloned G418-resistant Ltk$^-$ cell line described above. The results showed that, although only IE94-CAT mRNA (Fig. 4b, lane 1) and not IE94-IFN mRNA (Fig. 4a, lane 1) was present constitutively, the CHX treatment (4 h) significantly increased the levels of both correctly initiated CAT and IFN mRNAs. Again, infection of parallel samples with HSV strongly stimulated the IE94-IFN mRNA levels (Fig. 4a, lane 5) but had no significant effect on the steady-state level of IE94-CAT mRNA (Fig. 4b, lane 5). Maximal RNA accumulation was observed before removal of the inhibitor. However, reversal of the CHX block for 4 h resulted in a two- to threefold decrease in IFN mRNA levels with little effect on CAT mRNA levels, and 16 h after reversal, no CAT or IFN mRNA could be detected. Therefore, because CHX affected both CAT and IFN mRNA levels, we assume that it may have a direct effect on the rate of transcription from the IE94 promoter in addition to stabilization of IFN mRNA transcripts.

Further analysis of IE94-IFN and IE94-CAT reporter genes in dual-transfected pooled cell lines. To confirm the results obtained with the single cloned Ltk$^-$ cell line described above, we established five additional dual-cotransfected cell lines, two derived from Ltk$^-$ cells, one from murine Ly cells, and two from simian Vero cells. Three received the IE94-CAT(278) plasmid together with IE94-IFN(211) and SV2-NEO, and two received a new IE94-CAT construction in plasmid pJM50. The latter IE94-CAT(50) gene was intended to be more directly comparable to IE94-IFN(211), with the CAT coding region cassette (including SV40-derived splice signals) inserted into the same genetic background and between identical 3′ and 5′ transcriptional control signals that were all derived from the IE94 gene (Fig. 1A). In addition, the two Vero cell lines received a modified IE94-IFN(211mod) gene, in which the reading frame of the

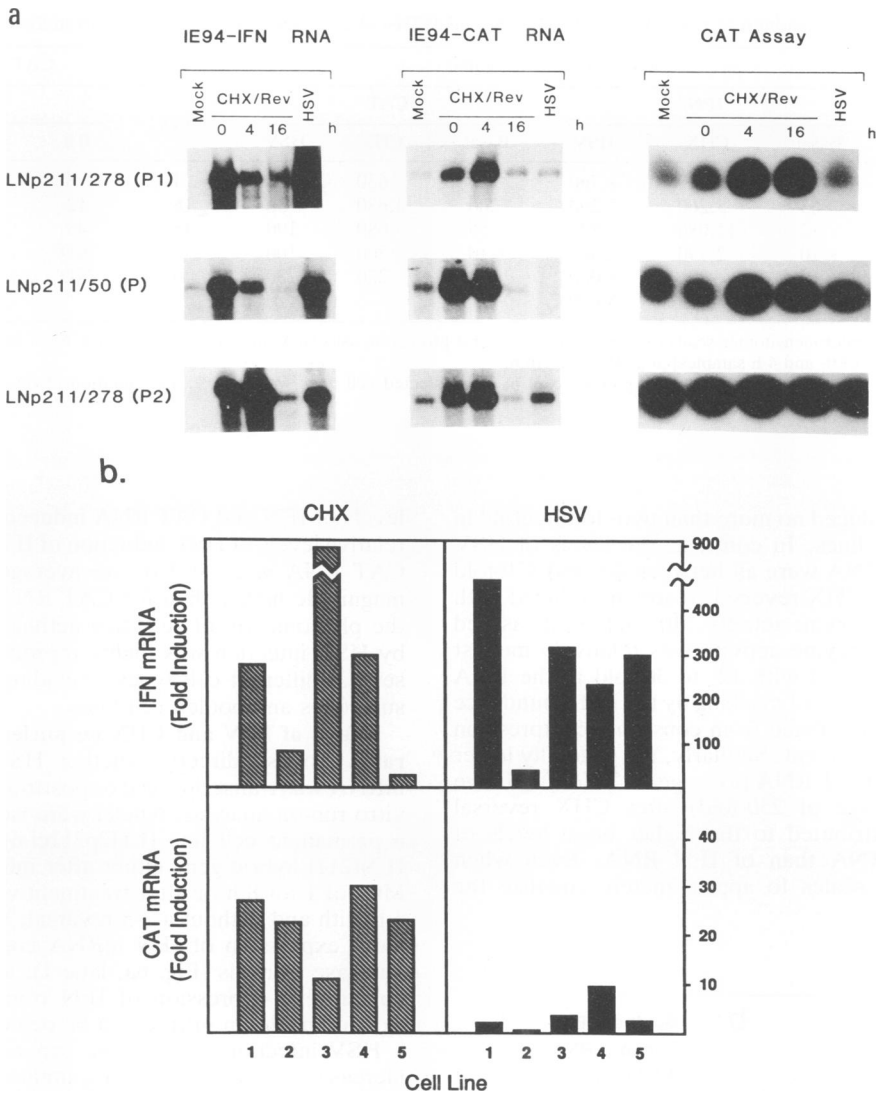

FIG. 5. HSV-induced expression of IE94-IFN but not IE94-CAT in multiple dual-transfected pooled cell lines. (a) Autoradiographs of riboprobe S1 assays comparing the patterns of steady-state 590-nt IE94-IFN and 280-nt IE94-CAT protected mRNA present before and after HSV infection or CHX reversal protocols. The 3′-acetylated chloramphenicol spots from parallel CAT assays carried out on the same culture samples are given on the right. Results for three pooled G418-resistant Ly (P1) or Ltk$^-$ (P and P2) cell lines that were transfected with various combinations of IE94-IFN (pTJ211) and IE94-CAT (pTJ278 or pJM50) plasmids are shown. (b) Histograms summarizing the fold induction of protected IE94-IFN (hatched bars) and IE94-CAT (solid bars) mRNA after HSV infection (8 h) or CHX reversal protocols (average of 0- and 4-h samples) in five pooled dual-transfected Ltk$^-$ or Vero cell lines (see Table 2). 1, LYNp211/278 (P1); 2, Ltk$^-$Np211/50 (P); 3, Ltk$^-$Np211/278 (P2); 4, VNp211mod/50 (P); 5, VNp211mod/278 (P).

IFN coding sequence was disrupted by insertion of a linker sequence near the NH_2 terminus to eliminate possible autoregulatory or deleterious effects of constitutive production of biologically active IFN. All of these cell lines represented mass cultures derived from pools of up to 100 individual G418-resistant colonies to minimize the effects of unusual copy number or the influence of particular integration sites, etc., which might be seen in single isolated cloned cell lines. Note that the IFN and CAT S1 assays were carried out in parallel from the same infected cell cultures and with the same RNA preparations.

Measurements of IFN and CAT mRNA levels from before and after HSV infection or CHX treatment again revealed low or undetectable basal expression of IE94-IFN in all five cell lines, with strong induction (averaging approximately 300-fold) by both HSV infection for 8 h and CHX reversal treatment (Fig. 5 and Table 2). In several cases, the induction by HSV was considerably greater than that by CHX, whereas in the others, the fold induction by CHX was equal to or greater than that by HSV. In two of the mouse cell lines and in both Vero cell lines, the half-life of CHX-induced IE94-IFN was less than 4 h, as measured by the persistence of S1-detectable mRNA after CHX reversal, although on this occasion, the level of IFN mRNA increased after a 4-h reversal in one cell line.

Four of the five dual-transfected cell lines produced detectable basal IE94-CAT mRNA, and all produced constitutive CAT enzyme activity (Fig. 5a and Table 2). Significantly, CAT enzyme activity was not appreciably induced in any of the cell lines after HSV infection, and the levels of

TABLE 2. Differential induction responses of IE94-IFN and IE94-CAT to HSV infection in dual-transfected pooled cell lines

Cell line	RNA[a] (absorbance units)						CAT assay[b] (%)				
	IFN			CAT			Mock	CHX			HSV
	Basal	CHX	HSV	Basal	CHX	HSV		0 h	4 h	16 h	
LYNp211/278 (P1)	<10	2,860	4,760	23	630	41	4	8	64	65	4
Ltk⁻Np211/50 (P)	63	5,260	2,290	200	4,680	30	16	12	68	31	19
Ltk⁻Np211/278 (P2)	12	11,080	3,840	55	680	190	48	48	69	46	44
VNp211mod/50 (P)	<10	2,990	2,300	<10	300	100	5	ND[c]	24	19	3
VNp211mod/278 (P)	10	300	3,000	90	220	230	6	ND	ND	21	6
LH2p211cl-6	155	3,660	6,690								

[a] Absorbance units from microdensitomer scans of bands from S1 hybrid protection assays. Values are shown for the basal level and the levels after CHX reversal treatment (average of 0- and 4-h samples) and HSV infection.

[b] Percent acetylation of chloramphenicol. The background level in untransfected cell lines was <0.1%. The duration of reversal after CHX treatment is indicated.

[c] ND, not done.

CAT mRNA were induced no more than two- to threefold in four of the five cell lines. In contrast, the levels of HSV induction of IFN mRNA were all between 40- and 470-fold (Fig. 5b). As before, CHX reversal treatment induced both CAT RNA and CAT enzyme activity, although the measured stimulation of CAT enzyme activity was relatively modest (1.5- to 4-fold), compared with 12- to 30-fold at the RNA level, presumably because of masking by the high abundance of stable enzyme accumulated from constitutive expression in the cells prior to treatment. Similarly, the generally lower level of induction of CAT RNA (average of 20-fold) relative to IFN RNA (average of 250-fold) after CHX reversal treatment can be attributed to the higher basal levels of steady-state CAT RNA than of IFN RNA. Even when plotted on different scales to approximately equalize the levels of IFN and CAT RNA induced by CHX reversal, the relative levels of HSV induction of IFN RNA compared with CAT RNA were still on an average at least an order of magnitude higher than for CAT RNA (Fig. 5b). Therefore, the phenomenon of selective activation of IFN expression by HSV infection was highly reproducible and occurred in several different cell types, including multiple independent subclones and pooled cell lines.

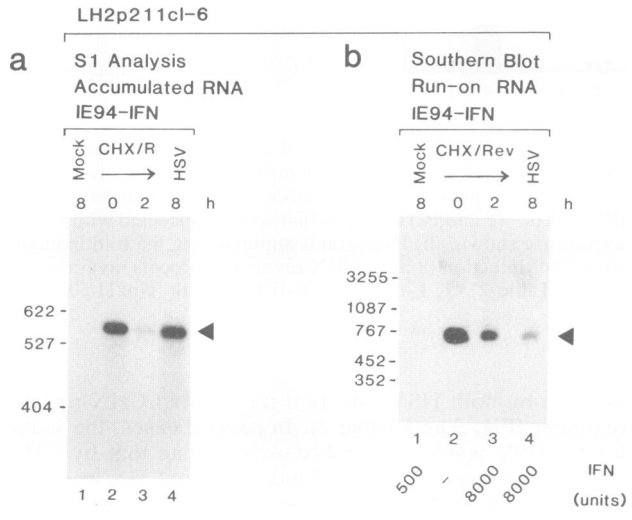

FIG. 6. Evidence that HSV induction of IE94-IFN mRNA occurs primarily at a posttranscriptional level. (a) S1 nuclease protection analysis of stable 590-nt IE94-IFN mRNA (arrowhead) in LH2p211cl-6 cells before and after induction by CHX and CHX reversal treatment or after HSV infection. (b) Measurement of transcription initiation levels by nuclear run-on analysis. ^{32}P-labeled mRNA synthesized in isolated nuclei from LH2p211cl-6 cells was hybridized to cleaved pIFR DNA on Southern blots. Levels of biologically active human IFN-β released into the medium of the same cell cultures are listed below the appropriate lanes. The autoradiograph was exposed for 2 h.

Effects of HSV and CHX on nuclear run-on transcription rates. To ask directly whether HSV stimulation of IFN mRNA was transcriptional or posttranscriptional, we used in vitro run-on analysis. Nuclei were isolated from cultures of a permanent cell line (LH2p211cl-6) containing the IE94-IFN(211) hybrid gene either after infection with HSV at an MOI of 1 for 8 h or after treatment with CHX (6 μg/ml) for 4 h with and without a 2-h reversal. Whereas essentially no basal expression of IFN mRNA could be detected by S1 nuclease analysis (Fig. 6a, lane 1), at least some low-level constitutive expression of IFN transcripts synthesized in isolated nuclei in vitro could be detected (Fig. 6b, lane 1).

HSV infection in the same experiment caused a 50-fold increase in steady-state accumulated S1-protected IFN mRNA (Fig. 6a, lane 4), but no more than a 3-fold increase in specific newly initiated mRNA transcripts in parallel samples of isolated nuclei (Fig. 6b, lane 4). Therefore, the nuclear run-on results indicated that HSV infection did not significantly affect the transcription initiation rate from the SCMV IE94 promoter and suggested that the HSV-dependent increase in IFN mRNA was caused by stabilization of the usually unstable IFN mRNA or some other posttranscriptional event mediated by HSV-encoded factors.

Treatment of the cells with CHX for 4 h without reversal induced an equivalent increase (50-fold) in accumulated steady-state IFN mRNA (Fig. 6a, lane 2) but also gave a 10-fold increase in specific newly synthesized mRNA transcripts in isolated nuclei (Fig. 6b, lane 2), showing that CHX-induced activation of IE94-IFN mRNA includes increased transcriptional initiation at the SCMV IE94 promoter. After a 2-h CHX reversal, a 10- to 20-fold decrease in the level of S1-detectable IFN mRNA was observed (Fig. 6a, lane 3), whereas run-on mRNA transcripts were reduced by only two- to threefold (Fig. 6b, lane 3). The significant drop in steady-state IFN mRNA without a correspondingly large decrease in IFN mRNA run-on transcripts after CHX release suggests that inhibition of IFN mRNA degradation must also play a role in the CHX effect. Both the HSV infection and the CHX reversal protocol yielded equivalent

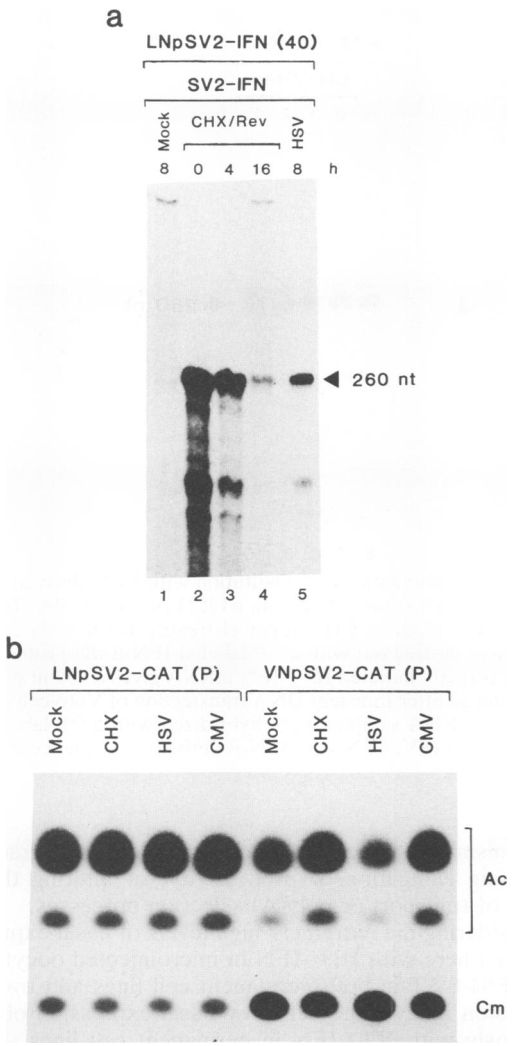

FIG. 7. HSV-induced stimulation of SV2-IFN mRNA expression but not of SV2-CAT expression in permanent cell lines. (a) S1 nuclease protection analysis of SV2-IFN mRNA in a G418-resistant pooled Ltk$^-$ cell line that received cotransfected pJM40 DNA. Samples were analyzed before or after HSV-1 infection and at various intervals after reversal of a 4-h CHX block. Total cell RNA was hybridized with a ^{32}P-labeled IFN-β riboprobe (pJM94). (b) CAT assay results showing constitutive expression of CAT enzyme in pooled G418-resistant Ltk$^-$ or Vero cell lines that received cotransfected pSV2-CAT DNA. Samples were examined from extracts of cells after HSV, SCMV, or mock infection and after CHX treatment for 4 h plus reversal for 4 h.

16-fold increases over the basal levels of biologically active IFN protein product.

Differential expression of SV2-IFN and SV2-CAT mRNA after HSV infection. We reasoned that if the major effect of HSV infection in cell lines containing the IE94-IFN hybrid gene was stabilization of the human IFN mRNA, then identical results should be obtained with a hybrid IFN construction containing another strong constitutive promoter and enhancer region, such as that from the well-characterized SV40 early gene. Expression of human IFN-β mRNA driven by the SV40 early promoter in permanent G418-resistant Ltk$^-$ cell lines containing the SV2-IFN hybrid gene proved to display the same features as that from the IE94-IFN hybrid. For example, basal levels of steady-state IFN-specific mRNA in the pooled LNpSV2-IFN(40) cell line were very low but could be activated strongly by both CHX treatment and HSV infection (Fig. 7a). Again, release from the CHX block reduced the amount of RNA detectable by 4 h and almost abolished it by 16 h. In dramatic contrast, parallel DNA-transfected neomycin-resistant pooled Ltk$^-$ or Vero cell lines receiving the SV2-CAT hybrid gene (LNpSV2-CAT[P] and VNSV2-CAT[P]) gave high-level constitutive CAT expression (Fig. 7b). Again, the basal levels of CAT activity could be induced slightly by CHX reversal treatment but not by HSV infection. These results support the conclusion that the mechanism for HSV stimulation of IFN expression is independent of promoter-specific functions other than the presumed general requirement for relatively high basal transcription initiation rates.

Induction of IFN-β mRNA by HSV and CHX in transient-expression assays. In previous experiments, activation of IE175-CAT, TK-CAT, and HIV long terminal repeat (LTR)-CAT after HSV infection produced essentially identical results in transient-expression assays in Vero cells and in permanent cell lines (14, 15, 18, 20). However, the two systems do not always give equivalent results; for example, with cytomegalovirus infection, IE175-CAT and TK-CAT respond only in transient-expression assays and not in permanent cell lines (15a, 18, 20). To ask whether HSV induction of a heterologous IFN reporter gene could also be demonstrated in transient-expression assays, the IE94-IFN(211) hybrid gene was transfected into Vero cells, and basal IFN mRNA levels were assayed after 48 h. The results showed that IE94-IFN(211) again gave low or undetectable basal expression (Fig. 8a, lane 1), but treatment with CHX initiated at 36 h after transfection induced accumulation of steady-state IFN mRNA, which was then shut off and degraded within 4 h after subsequent removal of the CHX block (Fig. 8a, lanes 2 to 5). Furthermore, just as in the permanent cell lines, infection with HSV at 36 h after target DNA transfection in transient-expression assays also resulted in substantial induction in the level of accumulated steady-state IE94-IFN mRNA by 8 h after infection (Fig. 8a, lane 6).

Role of 3′ sequences in SV2-IFN mRNA stability. The SV2-IFN(40) gene used above contains only the natural IFN-β gene-derived 3′ sequences and poly(A) signal. In contrast, both the IE94-CAT(278) and parent SV2-CAT genes contain the CAT-coding sequences fused to 850 bp of 3′ sequences derived from SV40, including the splice site and poly(A) signal from the early large T-antigen gene. To ask whether these SV40 3′ sequences may provide some or all of the increased stability of the CAT region cassette, a second hybrid gene, SV2-IFN(41), was prepared, in which the 3′ untranslated region of the IFN gene was replaced with the 3′ region from the SV40 T-antigen gene. We reasoned that similar to what has been shown for other genes with unstable mRNAs, the 3′ region may be critical for both degradation in uninfected cells and stabilization by HSV infection. The two forms of SV2-IFN genes were compared by S1 riboprobe protection analysis in transient-expression assays in Vero cells. The levels of IFN mRNA in cells transfected with the SV2-IFN(40) gene containing authentic IFN 3′ sequences were very low (Fig. 8b, lane 1) but were enhanced 5- to 10-fold by CHX treatment and then decayed within 1 to 2 h after reversal (Fig. 8b, lanes 1 to 5). In contrast, cells transfected with the SV2-IFN(41) gene containing SV40-derived 3′ sequences showed much higher basal IFN mRNA expression (Fig. 8b, lane 6) that was

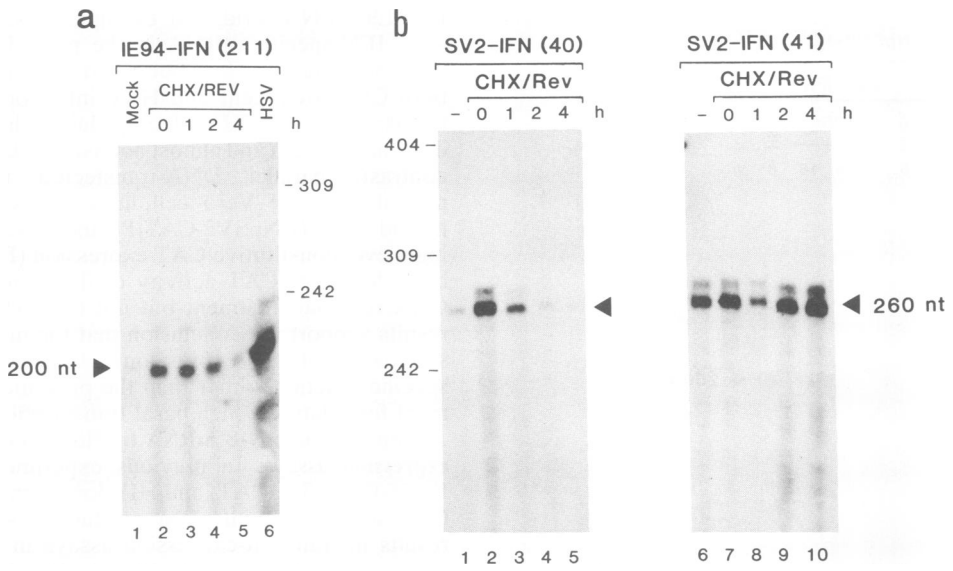

FIG. 8. Stimulation of IFN mRNA expression in transient-expression assays and stabilization by substitution with SV40-derived poly(A) and splicing signals. (a) S1 nuclease analysis of IE94-IFN mRNA in Vero cells 24 h after transfection with pTJ211 plasmid DNA. Total-cell RNA samples were extracted from cells either not treated with CHX (Mock, lane 1), after CHX reversal treatment for 0, 1, 2, or 4 h (CHX/Rev, lanes 2 to 5), or after HSV infection for 8 h (lane 6). Hybridization was carried out with a ^{32}P-labeled IFN-β riboprobe derived from plasmid pJM94 that recognized only the coding region of the IFN-β gene. Correctly initiated IE94-IFN mRNA gives a 200-nt protected RNA species. (b) S1 nuclease protection analysis of 260-nt SV2-IFN mRNA produced after transient DNA transfection of Vero cells with or without CHX treatment for 4 h followed by reversal for 0, 1, 2, or 4 h. Whole-cell RNA samples were hybridized with a ^{32}P-labeled IFN riboprobe transcribed from pJM94 DNA. Lanes 1 to 5, transfection with pJM40 DNA (SV2-IFN with IFN-β-derived 3′ sequences); lanes 6 to 10, transfection with pJM41 DNA (SV2-IFN with SV40-derived 3′ sequences).

relatively unaffected by CHX treatment, and the levels were maintained for greater than 4 h after CHX reversal (Fig. 8b, lanes 7 to 10). Therefore, linkage of the SV40 3′ signals to the IFN-coding region led to stabilization of the IFN mRNA. Since the CAT reporter gene cassette also contains the 3′ region from SV40, a similar effect probably accounts for some or all of the difference in responses between the IFN and CAT gene constructions used in these studies. Furthermore, since the strong constitutive SV40 early promoter should be equally active in both the SV2-IFN(40) and SV2-IFN(41) constructions, the 3′ sequences derived from the IFN gene probably contribute to the rapid degradation of the authentic IFN mRNA.

DISCUSSION

In a previous study, we reported that HSV infection induces a several-hundred-fold increase in the level of both steady-state accumulated IFN mRNA and biologically active IFN protein produced from cell lines containing integrated target human IFN-β reporter genes driven by the SCMV IE94 promoter-enhancer region (16). We have now extended this analysis to show that the novel HSV-dependent stimulation (i) acts specifically on the IFN mRNA coding region and its associated 3′ sequences and is not operative on a CAT reporter gene cassette with SV40-derived splicing and 3′ poly(A) sequences; (ii) occurs independently of the IE94 promoter-enhancer sequences to the extent that substitution with the SV40 promoter-enhancer produced the same effect; and (iii) represents primarily a posttranscriptional mRNA stabilization event giving rise to increased steady-state RNA levels and does not involve significantly increased transcription initiation rates. Additional experiments will be necessary to address whether the mechanism of stabilization involves preventing degradation, increasing elongation or processivity, or altering the efficiency of transport or poly(A) selection processes.

Considering the extremely high levels of basal expression observed here with IE94-IFN in microinjected oocytes and with IE94-CAT in both permanent cell lines and transient-expression assays, the very low basal expression obtained previously with IE94-IFN in permanent cell lines (16) appeared to represent a somewhat extraordinary and contradictory finding. However, this result has now been reproduced in multiple cell lines, and the difference can probably be attributed to the highly unstable properties intrinsic to human IFN-β mRNA in mammalian cells. Evidence that an SV2-IFN reporter gene behaves similarly to IE94-IFN, even in transient-expression assays, strongly supports this contention and implies that the effect is reporter gene specific and promoter independent. There is much data in the literature to indicate that IFN-β mRNA in human cells has a half-life of less than 30 min after poly(rI):poly(rC) induction, and our results suggest a similar stability for induced human IFN-β mRNA after introduction of the intact gene into DNA-transfected Vero and Ltk$^-$ cell lines. Although the CAT gene mRNA has only intermediate stability (relative to β-globin mRNA, for example), our measurements have clearly demonstrated that it has a considerably longer half-life than IFN-β mRNA (17).

Infection with HSV produced large increases in the levels of steady-state IE94-IFN or SV2-IFN mRNA in both permanent cell lines and transient-expression assays. This was especially dramatic in the dual-transfected cell lines, in which cotransfected IE94-CAT invariably failed to respond to infection. The best evidence that this induction represents a stabilization or posttranscriptional processing mechanism came from our nuclear run-on assays, which showed little

concomitant increase in IE94-IFN mRNA at the transcriptional initiation level. Unfortunately, the complication of CHX induction has so far prevented direct measurement of the half-life of the IE94-IFN mRNA synthesized after HSV infection. We cannot rule out the formal possibility of antitermination or increased processivity mechanisms similar to those suggested to operate for the c-*myc* gene after serum stimulation (2) and on the HIV LTR in the presence of the *tat* trans-activator protein (11), but if that were the case, stabilization of SV2-IFN by addition of the SV40 splicing and poly(A) regions should not have prevented HSV activation.

Our experiments imply that even the normally strong and constitutively expressed SV40 early and SCMV IE94 promoters can be at least partially repressed when integrated into the host chromatin structure. Downregulation of gene expression by labile cellular factors and derepression by CHX treatment has been demonstrated previously for the human IFN-β promoter (5, 28). Similarly, in the cell lines used here, the induction by CHX includes a transcription initiation component, as judged by nuclear run-on assays for both IE94-CAT and IE94-IFN expression, and is therefore probably due to removal of labile cellular repressors of the SCMV IE94 promoter. By monitoring levels of basal CAT expression from the promoters for the SV40 T antigen and SCMV IE94 genes in permanent cell lines, we have observed that permanent Vero and Ltk$^-$ cell lines containing hybrid CAT genes driven by these promoters also all exhibit a passage-dependent decrease in constitutive CAT expression (13a). The relatively slow acquisition of this repression contrasts with the more rapidly acting repression event that occurs immediately after the introduction of HIV LTR-CAT into permanent cell lines of this type (1, 14, 15). Interestingly, although HSV infection can also induce these repressed LTR-CAT constructions, that process occurs by a transcriptional initiation mechanism (15).

The ability to increase the half-life of SV2-IFN mRNA by replacing the natural 3′ IFN sequences with the same SV40-derived 3′ splicing and poly(A) signals that are present in all of our CAT cassette constructions implies either that the source of the instability lies within the 3′ portion of the IFN gene rather than within the IFN coding sequence itself or that the stabilizing effect of the SV40 3′ region is dominant. Considering that intact IE94 mRNA is thought to be highly stable (7, 8), inclusion of the 3′ noncoding sequences from the IE94 gene may also have contributed to stabilization of the CAT mRNA sequences in the IE94-CAT(50) target gene construction, which contains the splice signals but not the poly(A) signals from SV40 (Fig. 1). However, these same sequences clearly did not stabilize the mRNA produced from the IE94-IFN(211) construction. Similarly, this combination of the IE94 intron 1 splice signals and the SV40 late poly(A) region apparently did not stabilize the 560-bp IFN cDNA sequences present in the IE94-IFN(204) construction, as judged by the retention of HSV inducibility of the hybrid mRNA in permanent cell lines (16). Note that inspection of the hybrid gene constructions shown in Fig. 1A appears to implicate the small t-antigen splice signal as the common critical factor for conferring constitutive stabilization or increased steady-state accumulation. However, the identity of the HSV-responsive signal within the IFN gene coding sequences or 3′ region is not yet obvious. Human IFN-β mRNA contains an extremely AU-rich sequence (68 of 73 nt) that lies just upstream from and encompasses the first AATAAA motif and apparently plays a significant role in its lack of stability (33, 34). This sequence is included in all of the hybrid IFN constructions used in our experiments. Several other unstable cellular mRNA species, such as those for c-*myc*, c-*fos*, and granulocyte-macrophage colony-stimulating factor, have signals for instability that reside within AU-rich segments in their 3′ untranslated sequences (10, 31). Therefore, it will be interesting in the future to determine whether HSV-dependent mRNA accumulation might also be observed with these genes or whether some other features at the 3′ end of the IFN-β gene or the lack of splicing signals is responsible.

Several reports have described an HSV virion factor system, referred to as vhs, which is distinct from the VP16 transcription factor and is involved in the early general shut-off of host protein and RNA synthesis, including mRNA degradation, in infected cells (12, 23, 26, 30). However, the virion-mediated shut-off of host protein synthesis and degradation of host mRNA appears to be fundamentally different from the pathways effecting rapid turnover of cellular mRNAs encoding certain proto-oncogenes and growth factors. For example, whereas CHX treatment stabilizes mRNA transcripts for c-*fos*, c-*myc*, and IFN, CHX has no effect on HSV-induced degradation of cellular or IE mRNA (26). Indeed, we have found that the presence or absence of the vhs-1 mutation does not affect the observed stabilization of mRNA from our IE94-IFN constructions (data not shown).

Our results indicate that the reporter gene-specific effects are probably directed towards the specific types of 3′ noncoding, splicing, or poly(A) signals present rather than at the coding sequences themselves. In this regard, McLaughlan et al. (13) have recently presented evidence for selective poly(A) processing events in HSV-infected cells, which could also conceivably contribute to the reporter gene-specific effects described here. Alternatively, the fact that the most responsive target constructions used here involved mRNA species that lack introns and splice signals may be of potential significance. Perhaps this mechanism plays a role in preferential utilization of unspliced DE and late viral mRNAs versus spliced IE viral and cellular mRNAs at late stages of HSV infection. Although the particular HSV IE protein responsible for the induction of IFN mRNA levels in these experiments has not yet been identified, preliminary experiments suggest that cotransfection with the IE63 (or ICP27) gene can produce a similar effect in transient-expression assays (15a).

In conclusion, we have demonstrated, first, that differences in the stability or processing of target reporter gene mRNA species can play a prominent role in overall levels of gene expression; second, that the human IFN-β gene provides not only a useful reporter for measuring promoter regulation and expression at the transcriptional initiation level, but also an indicator of altered mRNA stability or posttranscriptional processing; and third, that HSV infection (despite the generalized shut-off of host mRNA synthesis) induces a factor that could potentially selectively stabilize other viral or cellular mRNAs in addition to that for IFN-β.

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REFERENCES

1. **Bednarik, D. P., J. D. Mosca, and N. B. K. Raj.** 1987. Methylation as a modulator of expression of human immunodeficiency virus. J. Virol. **61:**1253–1257.
2. **Bentley, D. L., and M. Groudine.** 1988. Sequence requirements for premature termination of transcription in the human c-myc gene. Cell **53:**245–256.
3. **Chang, Y.-N., S. Crawford, J. Stall, D. Rawlins, K.-T. Jeang, and G. S. Hayward.** 1990. The palindromic series I repeats in the simian cytomegalovirus major immediate-early promoter behave as both strong basal enhancers and cyclic AMP response elements. J. Virol. **64:**264–277.

3a. **Chiou, C.-J., Y.-J. Chan, Y.-N. Chang, and G. S. Hayward.** Unpublished data.

4. **Fenwick, M. L., and J. Clark.** 1982. Early and delayed shut-off of host protein synthesis in cells infected with herpes simplex virus. J. Gen. Virol. **61:**121–125.
5. **Goodbourn, S., and T. Maniatis.** 1988. Overlapping positive and negative regulatory domains of the human β-interferon gene. Proc. Natl. Acad. Sci. USA **85:**1447–1451.
6. **Harris-Hamilton, E., and S. L. Bachenheimer.** 1985. Accumulation of herpes simplex virus type 1 RNAs of different kinetic classes in the cytoplasm of infected cells. J. Virol. **53:**144–151.
7. **Jeang, K.-T., G. Chin, and G. S. Hayward.** 1982. Characterization of cytomegalovirus immediate-early genes. I. Non-permissive rodent cells overproduce the IE94K protein from CMV-(Colburn). Virology **121:**393–403.
8. **Jeang, K.-T., M.-S. Cho, and G. S. Hayward.** 1984. Abundant constitutive expression of the immediate-early 94-kDa protein from cytomegalovirus (Colburn) in a DNA-transfected mouse cell line. Mol. Cell. Biol. **4:**2214–2223.
9. **Jeang, K.-T., D. R. Rawlins, P. J. Rosenfeld, J. H. Shero, T. J. Kelly, and G. S. Hayward.** 1987. Multiple tandemly repeated binding sites for cellular nuclear factor 1 that surround the major immediate-early promoters of simian and human cytomegalovirus. J. Virol. **61:**1559–1570.
10. **Jones, T. R., and M. D. Cole.** 1987. Rapid cytoplasmic turnover of c-*myc* mRNA: requirement of the 3′ untranslated sequences. Mol. Cell. Biol. **7:**4513–4521.
11. **Kao, S.-Y., A. F. Calman, P. A. Luciw, and B. M. Peterlin.** 1987. Anti-termination of transcription within the long terminal repeat of HIV-I by tat gene product. Nature (London) **330:**489–493.
12. **Kwong, A. D., J. A. Krupie, and N. Frenkel.** 1988. Herpes simplex virus virion host shutoff function. J. Virol. **62:**912–921.
13. **McLaughlan, J., S. Simpson, and J. B. Clements.** 1989. Herpes simplex virus induces a processing factor that stimulates poly(A) site usage. Cell **59:**1093–1105.

13a. **Mosca, J. D.** Unpublished data.

14. **Mosca, J. D., D. P. Bednarik, N. B. K. Raj, C. A. Rosen, J. G. Sodroski, W. A. Haseltine, G. S. Hayward, and P. M. Pitha.** 1987. Activation of human immunodeficiency virus by herpes virus infection: identification of a region within the long terminal repeat that responds to a trans-acting factor encoded by herpes simplex virus 1. Proc. Natl. Acad. Sci. USA **84:**7408–7412.
15. **Mosca, J. D., D. P. Bednarik, N. B. K. Raj, C. A. Rosen, J. G. Sodroski, W. A. Haseltine, and P. M. Pitha.** 1987. Herpes simplex virus type-1 can reactivate transcription of latent human immunodeficiency virus. Nature (London) **325:**67–70.

15a. **Mosca, J. D., and G. S. Hayward.** Unpublished data.

16. **Mosca, J. D., K.-T. Jeang, P. M. Pitha, and G. S. Hayward.** 1987. Novel induction by herpes simplex virus of hybrid interferon gene transcripts driven by the strong cytomegalovirus IE94 promoter. J. Virol. **61:**819–828.
17. **Mosca, J. D., and P. M. Pitha.** 1986. Transcriptional and posttranscriptional regulation of exogenous human beta interferon gene in simian cells defective in interferon synthesis. Mol. Cell. Biol. **6:**2279–2283.
18. **Mosca, J. D., G. R. Reyes, P. M. Pitha, and G. S. Hayward.** 1985. Differential activation of hybrid genes containing herpes simplex virus immediate-early or delayed-early promoters after superinfection of stable DNA-transfected cell lines. J. Virol. **56:**867–878.
19. **Nishioka, Y., and S. Silverstein.** 1977. Degradation of cellular mRNA during infection by herpes simplex virus. Proc. Natl. Acad. Sci. USA **74:**2370–2374.
20. **O'Hare, P., and G. S. Hayward.** 1984. Expression of recombinant genes containing herpes simplex virus delayed-early and immediate-early regulatory regions and *trans* activation by herpes virus infection. J. Virol. **52:**522–531.
21. **O'Hare, P., and G. S. Hayward.** 1985. Evidence for a direct role of both the 175K and 110K immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J. Virol. **53:**751–760.
22. **O'Hare, P., J. D. Mosca, and G. S. Hayward.** 1986. Multiple trans-activating proteins of herpes simplex virus that have different target promoter specificities and exhibit both positive and negative regulatory functions. Cancer Cells **4:**175–188.
23. **Oroscar, A. A., and G. S. Read.** 1989. Control of mRNA stability by the virion host shutoff function of herpes simplex virus. J. Virol. **63:**1897–1906.
24. **Raj, N. B. K., and P. M. Pitha.** 1981. Analysis of interferon mRNA in human fibroblast cells induced to produce interferon. Proc. Natl. Acad. Sci. USA **78:**7426–7430.
25. **Raj, N. B. K., and P. M. Pitha.** 1983. Two levels of regulation of β-interferon gene expression in human cells. Proc. Natl. Acad. Sci. USA **80:**3923–3927.
26. **Read, G. S., and N. Frenkel.** 1983. Herpes simplex virus mutants defective in the virion-associated shut-off of host polypeptide synthesis and exhibiting abnormal synthesis of alpha (immediate-early) viral polypeptides. J. Virol. **46:**498–512.
27. **Reyes, G. R., E. R. Gavis, A. Buchan, N. B. Raj, G. S. Hayward, and P. M. Pitha.** 1982. Expression of human β-interferon cDNA under the control of a thymidine kinase promoter from herpes simplex virus. Nature (London) **297:**598–601.
28. **Ringold, G. M., B. Dieckmann, J. L. Vannice, M. Trahey, and F. McCormick.** 1984. Inhibition of protein synthesis stimulates the transcription of human β-interferon genes in Chinese hamster ovary cells. Proc. Natl. Acad. Sci. USA **81:**3964–3968.
29. **Sacks, W. R., C. C. Greene, D. P. Aschman, and P. A. Schaffer.** 1985. Herpes simplex virus type 1 ICP27 is an essential regulatory protein. J. Virol. **55:**796–805.
30. **Schek, N., and S. L. Bachenheimer.** 1985. Degradation of cellular mRNAs induced by a virion-associated factor during herpes simplex virus infection of Vero cells. J. Virol. **55:**601–610.
31. **Shaw, G., and R. Kamen.** 1986. A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell **46:**659–667.
32. **Smibert, C. A., and J. R. Smiley.** 1990. Differential regulation of endogenous and transduced β-globin genes during infection of erythroid cells with a herpes simplex virus type 1 recombinant. J. Virol. **64:**3882–3894.
33. **Whittemore, L.-A., and T. Maniatis.** 1990. Postinduction turnoff of beta-interferon gene expression. Mol. Cell. Biol. **10:**1329–1337.
34. **Whittemore, L.-A., and T. Maniatis.** 1990. Postinduction repression of the β-interferon gene is mediated through two positive regulatory domains. Proc. Natl. Acad. Sci. USA **87:**7799–7803.