Characterization of Regulatory Functions of the HSV-1 Immediate-Early Protein ICP22

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Previous work has shown that the 68-kDa immediate-early protein of herpes simplex virus type 1 (HSV-1), also known as ICP22, is involved in the control of viral gene expression, although the precise mechanism remains to be elucidated. In order to study the function(s) of this protein, we constructed expression vectors containing the coding sequence of the ICP22 gene placed under the control of the SV40 or HCMV promoter. After cell transfection, ICP22 synthesis was studied by immunoblotting, using a specific antiserum. In transient expression experiments in COS cells in which the ICP22 vector was under the control of the SV40 promoter, we found that ICP22 was able to inhibit chloramphenicol acetyltransferase (CAT) expression under the control of either the $\alpha 22$ (IE4) promoter or other immediate-early promoters, such as $\alpha 4$ (IE3), $\alpha 0$ (IE1), and $\alpha 27$ (IE2). CAT expression under the control of the $\alpha 4$ (IE3) promoter was inhibited in these cells by expression of ICP22 under the control of the HCMV promoter; it was also inhibited in RAT-1 cells by ICP22 expressed under the control of the SV40 or HCMV promoter. In contrast, CAT expression directed by the SV40 or HCMV promoters was only weakly or not inhibited by the ICP22 vectors. We also constructed an expression vector for UL13, a gene whose product is implicated in the phosphorylation of ICP22. Although CAT expression under the control of the $\alpha 4$ (IE3) promoter was also negatively regulated by the UL13 gene product, the effects of the ICP22 (directed by the SV40 or HCMV promoter) and UL13 vectors were not synergistic; furthermore, at a particular molar ratio of the two vectors, inhibition of CAT activity was partially reversed. The results in the present work suggest that ICP22 can negatively regulate the expression of immediate-early viral genes and that its phosphorylation by UL13 protein kinase might be involved in the modulation of its function. © 1996 Academic Press, Inc.

INTRODUCTION

In cells lytically infected with herpes simplex virus type-1 (HSV1), the first genes to be transcribed are designated α, or immediate-early (IE), genes and encode five proteins, designated ICP0, 4, 22, 27, and 47. Genetic studies have shown that the genes coding for ICP4 and 27 are essential for viral growth and exert a regulatory role. ICP4 is the major protein involved in transcription regulation in HSV-1 (Mackem and Roizman, 1982; Deluca *et al.,* 1985; Gelman and Silverstein, 1987); it is necessary during infection for the activation of the β, or early (E), and γ, or late (L), genes (Watson and Clements, 1980) and also represses the expression of all the α genes (O'Hare and Hayward, 1985). ICP27 appears to mediate the transition from the β to the γ phase of viral infection and also transactivates γ genes and represses α and β genes, in combination with ICP4 and ICP0 (Sekulovich *et al.,* 1988). Other IE genes are not essential for viral growth in all cell types, but have been implicated as playing a role in gene expression. In particular, ICP0 transactivates α, β, and γ genes and is thought to play

a major role in reactivation from latency (Cai *et al.,* 1993) (for review, see Roizman and Sears, 1991).

The situation with regard to ICP22 is not entirely clear. In transient transfection assays, ICP22, both alone and in combination with other immediate-early proteins, shows no ability to transactivate HSV1 promoters (O'Hare and Hayward, 1985). However, in certain cell lines infected with a virus containing a deletion in the ICP22 gene, late gene expression is reduced, suggesting a cell-type-dependent role for this protein in the activation of this class of genes (Sears *et al.,* 1985). More recently, ICP22 has been implicated in the modification of the host RNA polymerase II (Rice *et al.,* 1995). The ICP22 gene has some interesting characteristics. Its regulatory sequences, shared with the ICP47 gene, overlap those of the ICP4 gene and of the viral origin of replication, OriS. The gene consists of two exons and one intron, the entire coding sequence being located in exon 2 in the unique short sequence of the HSV-1 genome. The protein is guanylated, adenylated, and phosphorylated, and at least part of these posttranslational modifications involve the viral protein kinases encoded by UL13 and US3 and casein kinase II (Purves and Roizman, 1992; Blaho *et al.,* 1993; Purves *et al.,* 1993; Mitchell *et al.,* 1994). To study how ICP22 might modulate viral gene expression during infection, we constructed two expression vectors in

which the coding sequences of ICP22 and UL13 were placed under the control of the SV40 and/or HCMV promoters. Using transient expression assays, we then analyzed the effects of ICP22 vectors on IE viral gene expression in the presence and absence of UL13. The results indicate that ICP22 can repress the expression of CAT directed by IE promoters, including the α22 promoter, and that this inhibition can be partially reversed by the UL13 vector at a particular molar ratio of the two vectors.

MATERIALS AND METHODS

Cells, virus, and viral DNA

COS and Vero cell lines were obtained from the American Type Culture Collection and Rat-1 was from Weinberg's laboratory (MIT, Cambridge, MA). HSV1(F) (Heine et al., 1974) was provided by Dr. B. Roizman (University of Chicago). Viral DNA was purified from the cytoplasm of infected Vero cells by banding in NaI equilibrium gradients (Walboomers and Ter Scheggett, 1976).

Construction of plasmids

The fragment BamHI–BstEII, containing the α22 gene (pBN/1) (Garcin et al., 1990), was subcloned into pBluescript (SK, Stratagene) between the EcoRI and ClaI restriction sites. The 5' noncoding sequence of this gene, including the first exon and part of the intron, was deleted by exonuclease III and mung bean nuclease digestion. Various deleted plasmids were isolated and sequenced and one, designated p22, containing eight additional nucleotides outside the open reading frame, was selected for further cloning. The fragment BamHI–SalI, containing the α22 gene, was filled in with Klenow enzyme and cloned at the SmaI site of plasmid pcEXV3 (Miller and Germain, 1986) (referred to as pEX), downstream of the SV40 promoter; the product was designated pEX22. pCMV/CAT (Foecking and Hofstetter, 1986) was digested with HindIII and EcoRI to delete the CAT cDNA. Following Klenow treatment, the filled ends were either directly ligated, producing pCMV, or ligated to the blunt-ended BamHI–SalI fragment described above, producing pCMV22. To construct the UL13 vector, a BglII fragment from HSV1 (F) DNA (Perry and McGeoch, 1988) was cloned into the BamHI site of SK plasmid. A KpnI fragment was then deleted, producing pUL13, and a KpnI–XbaI fragment was cloned into the SmaI site of pEX, placing UL13 under the control of the SV40 promoter. Finally, the region between the BalI and SpeI sites was deleted, producing pEXUL13.

The plasmids pIGA72, 65, 106, and 98, containing the CAT gene under the respective control of the α4, 0, 22/47, and 27 regulatory-promoter sequences were obtained from S. Silverstein (Gelman and Silverstein, 1987). pSV2CAT (Gorman et al., 1982) was used as a control. pUT535, containing LacZ under the control of the HCMV promoter, was obtained from Dr. G. Tiraby (Baron et al., 1992).

Transfection assays

Cells (0.6×10^6) were seeded in cell culture flasks and then transfected 8 hr later, using the calcium method (Graham and Van Der Eb, 1973) or lipofectamine (Gibco-BRL), with 15 μg of herring sperm (HS) DNA or a mixture of plasmid DNAs (15 μg of DNA) plus HS.

Immunoblotting

Cells were transfected with plasmid DNA, as previously described. Twenty and 72 hr after transfection, they were washed with PBS and then sonicated, and the debris was removed by centrifugation. The proteins in the supernatant were separated by electrophoresis on 10% SDS–polyacrylamide gels, transferred electrophoretically to nitrocellulose membranes, then washed with water. To prevent nonspecific antibody binding, the membrane was treated with 5% fat-free milk in PBS, before being incubated for 1 hr with a specific rabbit anti-ICP22 antiserum provided by B. Roizman (Ackerman et al., 1985). The blots were washed three times with PBS containing 0.2% Triton X-100 and then incubated for 1 hr with goat anti-rabbit immunoglobulin antibodies conjugated to alkaline phosphatase. After three washes as above, the color reaction was developed using nitroblue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) as substrates (Garcin et al., 1990).

CAT and LacZ assays

Seventy-two or 96 hr after transfection, the cell lysates were analyzed for CAT activity, as described by Gorman et al. (1982), using 0.2 μCi of [^{14}C]chloramphenicol (60 mCi/mmol, NEN). All quantitative comparisons were made by measuring the mono- and diacetylated derivatives of chloramphenicol on the linear part of the product–enzyme concentration graphs. LacZ activity was determined colorimetrically by the absorbance at 570 nm using chlorophenol red–β-D-galactopyranoside (CPRG) as substrate (Eustice et al., 1991).

In vitro transcription and translation and immunoprecipitation of the translation product

In vitro transcription reactions were performed in a volume of 50 μl containing 0.5 μg of pUL13 or p22 and using T3 or T7 polymerase, respectively, followed by in vitro translation in the presence of 40 μCi of [^{35}S]-methionine (1000 Ci/mmol, NEN). RNA and protein syntheses were carried out using TNT-coupled reticulocyte lysate systems (Promega), following the manufacturer's instructions.

Immunoprecipitation of in vitro-translated UL13 protein was achieved using Staphylococcus aureus (Pansorbin,

Calbiochem), previously activated in RIPA buffer (10% SDS, 0.1% Tween, 1% DOC, 0.15 M NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, and 0.25 mM PMSF), diluted in the same buffer and immunoprecipitated with anti-UL13 polyclonal rabbit antiserum provided by A. Davidson (Cunningham *et al.,* 1992), as described by the authors.

Protein kinase assay

Proteins were extracted from the nuclei of mock-infected or HSV-1-infected cells and from pEX- or pEXUL13-transfected cells, using salt washes, followed by ammonium sulfate precipitation (Piette *et al.,* 1985). Nuclear extracts, or aliquots of previously washed immunoprecipitates (three times with PBS), were suspended in 50 μl of protein kinase buffer (20 mM Tris–HCl, pH 8, 1.5 M NaCl, 50 mM MgCl$_2$, 1 mM dithiothreitol, 0.1% Nonidet P40); 1.5 M casein was added to some samples (Cunningham, *et al.,* 1992). The assay was initiated by the addition of 1 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; NEN). After incubation of the reaction mixture at 37°C for 30 min, the nuclear extracts or immunoprecipitates were washed three times with PBS buffer, boiled for 5 min in denaturing buffer, and analyzed by 10% SDS–polyacrylamide gels. To quantify the radioactivity in nuclear extracts, aliquots were precipitated with 10% TCA in the presence of cold ATP.

RESULTS

Analysis of ICP22-encoding plasmids

COS cells were either infected with HSV1(F) or transfected with pCMV22, pEX22, or pBN/1, corresponding, respectively, to the ICP22 coding sequence under the control of the HCMV, SV40, or natural promoter. Twenty-four hours later, the cells were lysed and the soluble protein extracts analyzed by Western blots, using a specific anti-ICP22 antiserum (Fig. 1).

Following transfection, the expression vectors containing the HCMV or SV40 promoters produced detectable amounts of ICP22. Using the specific antibody, several immunoreactive bands with apparent molecular weights between 70 and 90 kDa were seen in both Vero and COS cells; more ICP22 was produced by the SV40 promoter-containing vector than by that containing the HCMV promoter. Several higher molecular weights bands were also seen, although at a lower intensity than in infected cells. In pCMV22-transfected COS cells, a weak 70-kDa immunoreactive band was seen. Under the control of its natural promoter (pBN1), no ICP22 synthesis was detectable, although the plasmid contains regulatory sequences located both upstream (Roizman and Sears, 1991) and downstream (Greco *et al.,* 1994) of the transcription initiation site. A faint nonspecific high molecular weight band was seen in all samples from COS-transfected cells.
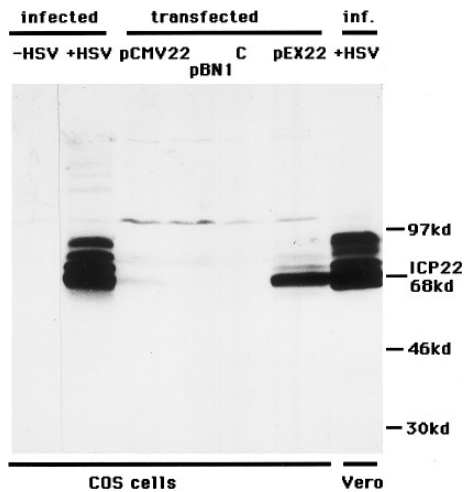

FIG. 1. Immunoblot of ICP22 extracted from infected and transfected cells. COS cells were infected with 20 PFU/cell of HSV1(F) or transfected with 15 μg of pCMV22, pBN/1, herring sperm DNA (C), or pEX22 DNA, and the proteins were separated by electrophoresis, transferred to nitrocellulose membranes, and treated with anti-ICP22 antiserum. HSV1-infected Vero and COS cells were used as controls. On the right, the position of the main form of ICP22 and molecular weight markers are shown.

The ICP22 vector inhibits CAT expression from regulatory regions

We then analyzed the regulation in COS cells of the HSV-1 α4 promoter by the pEX22 and pCMV22 expression vectors, using a plasmid containing α4 regulatory sequences upstream of the reporter CAT gene (pIGA72). Figure 2A shows the percentage of mono- and diacetylated chloramphenicol produced versus the concentration of pEX22 or pCMV22 used; dose-dependent inhibition of CAT expression can be seen. Using 10 and 5 μg, respectively, of the vectors pEX and pCMV (lacking the α22 coding sequence), there was essentially no inhibition of CAT activity (data not shown).

To confirm that the inhibition of IE gene expression was specific, three further experiments were carried out. First, COS cells were transfected with pIGA72 plus a series of mixtures of pEX + pEX22 (2 μg total) or pCMV + pCMV22 (5 μg total), a protocol designed to add increasing amounts of the α22 coding region while keeping the total amount of regulatory region constant to ensure that any effects seen were due to ICP22 ORF; a dose-dependent inhibition of CAT expression similar to that shown in Fig. 2A was observed (Fig. 2B). No difference between cells transfected with either pIGA72 alone or pIGA72 in the presence of pEX or pCMV was detected; the percentages of acetylated CAT, before (Fig. 2B) normalization, were 91.7 ± 0.2 and 86.7 ± 4.3%, with or without pEX, and 93 ± 0.5 and 89.2 ± 2.1%, with or without pCMV, respectively, suggesting that the ICP22 vectors lacking the coding sequence have no effect on the α4 promoter. Second, the ability of increased
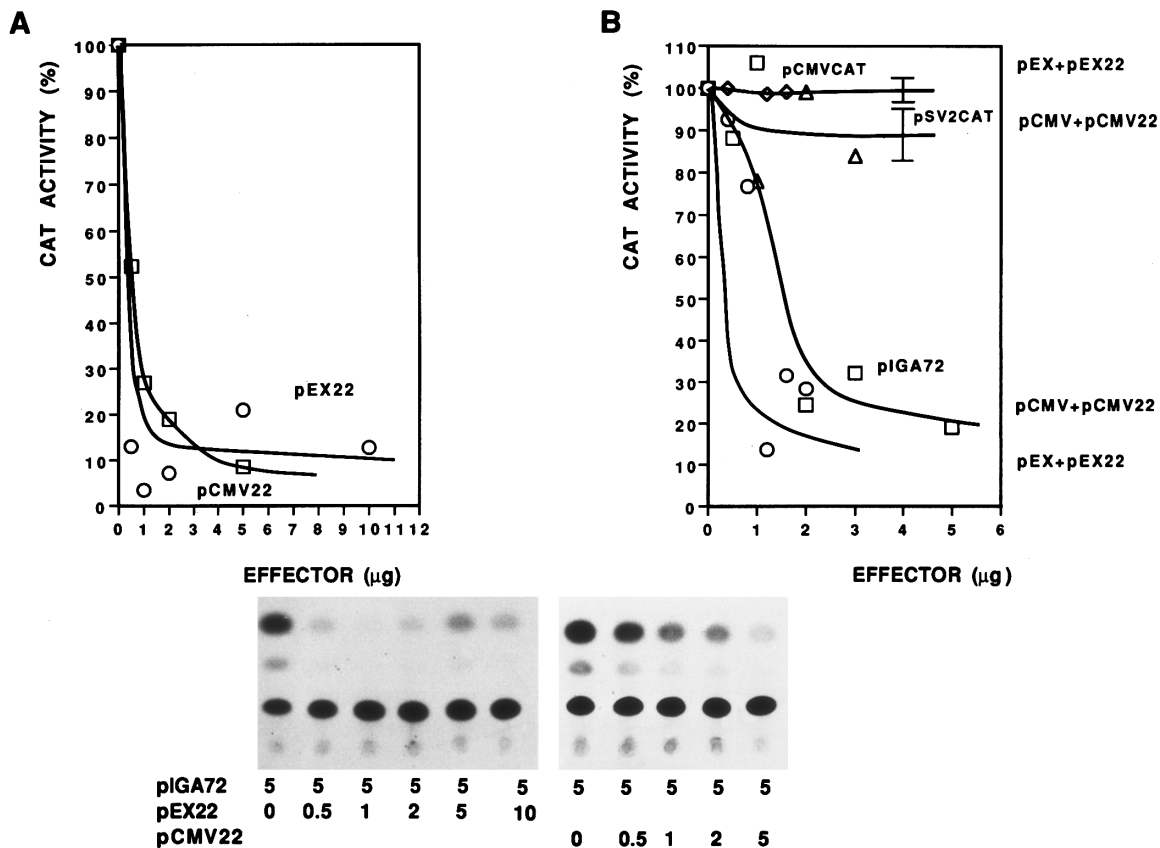
FIG. 2. Inhibition of CAT expression by ICP22 under the control of immediate-early or heterologous promoters. COS cells were cotransfected: (A) with 5 μg of pIGA72 (containing α4 promoter) and increased concentrations of pEX22 or pCMV22 as effectors; (B) with 5 μg of pIGA72, pCMVCAT, or pSV2CAT and 2 μg of the mixtures pEX+pEX22 or pCMV+pCMV22 as effectors. The circles represent the effect of pEX22 or pEX+pEX22 on PIGA72, the squares represent that of pCMV22 or pCMV+pCMV22 on pIGA72, the diamonds represent that of pEX+pEX22 on pCMVCAT, and the triangles represent that of pCMV+pCMV22 on pSV2CAT. The values shown are the percentage of acetylated chloramphenicol versus the concentration of the effector, ICP22 (pEX22 or pCMV22). Standard deviations are reported for 4 μg of expression vectors. The autoradiograms of the chromatograms for A are shown at the bottom of the figure.

amounts of both plasmids (pEX22 and pCMV22) to inhibit CAT expression driven by heterologous promoters in COS cells was evaluated under the same conditions (Fig. 2B). With 4 μg of pEX22 on pCMVCAT and with 4 μg of pCMV22 on pSV2CAT, the results of three such experiments shown in Fig. 2B, gave respective CAT activities of 93 $\pm$ 2.5 and 89 $\pm$ 5%, suggesting that the ICP22 vector could either not inhibit or only weakly inhibit heterologous promoters. Third, the same specificity was seen for the HSV1 promoter in COS cells cotransfected with a mixture of CAT plasmid and LacZ plasmid under the respective control of the α4 promoter-regulatory region (pIGA72) and the HCMV promoter-regulatory region (pUT535). LacZ expression, determined colorimetrically, was not affected by increasing concentrations of pXE22, the absorbance at 570 nm (using CPRG as substrate) varying between 0.70 and 0.79 for amounts of pEX22 ranging from 0 to 2 μg. Thus, ICP22 is able to specifically block the α4 promoter.

Three other IE reporter plasmids, pIGA65, 106, and 98, which carry, respectively, regulatory sequences for α0,

22/47, and 27, were tested with pEX22. Dose-dependent inhibition was again seen, being more efficient with the α0 promoter (pIGA65) than with either the α22/47 (pIGA106) or α27 (pIGA98) promoters (Fig. 3A).

Finally, the ability of ICP22 to inhibit CAT expression was tested using pIGA72 in the Rat-1 cell line, which cannot complement a lack of ICP22 (Sears et al., 1985). The results, shown in Fig. 3B, demonstrate that, although higher concentrations of plasmid were required, both pEX22 and pCMV22 were able to inhibit CAT expression from pIGA72 in these cells. Thus, the ICP22 vector can inhibit all IE promoters, including itself, and is effective in two different cell types.

A plasmid expressing the UL13 gene product inhibits CAT expression from pIGA72 and can partly reverse the inhibitory effect of ICP22

It has previously been noted that the protein kinase encoded by the UL13 gene of HSV1 can influence the posttranslational processing associated with phosphory-
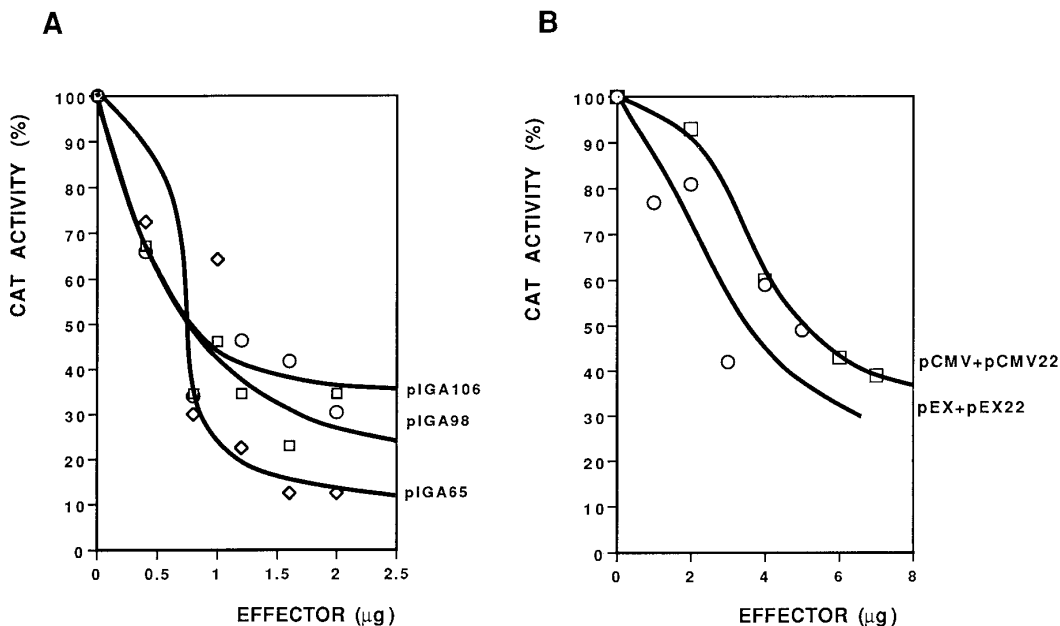
A

B

FIG. 3. Inhibition of CAT expression under the control of immediate-early promoters by ICP22. (A) COS cells were cotransfected with 5 μg of pIGA98 (squares), 65 (diamonds), or 106 (circles), corresponding to the α27, 0, and 22 promoters, respectively, and with 2 μg of the mixture pEX+pEX22. (B) Rat-1 cells were cotransfected with 5 μg of pIGA72 and 2 μg of the mixture pEX+pEX22 (circles) or pCMV+pCMV22 (squares). The values shown are the percentages of acetylated chloramphenicol versus the concentrations of the effector, ICP22 (pEX22 or pCMV22).

lation of ICP22, thereby altering its function (Purves and Roizman, 1992). To study whether the UL13 gene product might exert an effect on the inhibitory effect of ICP22 on CAT expression, we constructed two vectors expressing UL13 under the control of either the T3 promoter (pUL13) or the SV40 promoter (pEXUL13).

The pUL13 and p22 vectors, containing the coding sequences for UL13 and ICP22 directed by the T3 and T7 promoter, respectively, were transcribed *in vitro* and translated in the presence of [^{35}S]methionine. The results (Fig. 4, left) show that, in the case of ICP22, three bands were seen, corresponding to three different open reading frames, the largest with the same molecular weight as ICP22 (lane 1), while, for UL13, three bands were also seen (lane 2), but, after immunoprecipitation with UL13-specific antiserum, only one band with a molecular weight of 57 kDa (identical to that of UL13) was detected (lane 3).

Aliquots of unlabeled UL13 immunoprecipitates were incubated with ^{32}P-labeled γ-ATP in protein kinase buffer in the presence of casein, a known heterologous substrate for UL13 (Cunningham *et al.*, 1992) or of *in vitro*-translated ICP22 (Fig. 4, right). Highly efficient phosphorylation of casein (lane 2) and weak autophosphorylation of UL13 were seen (lane 3). Various labeled bands, with molecular weights higher than the 68 kDa marker, corresponding to the range of molecular weights noted in infected cells (Fig. 1), were seen with ICP22 in the presence of UL13 (lane 4). No casein phosphorylation occurred in the absence of UL13 (lane 1); thus UL13 possesses functional protein kinase activity.

Finally, the protein kinase activity of pEXUL13 was tested by its autophosphorylation by ^{32}P-labeled γ-ATP using nuclear extracts of COS cells transfected with 10 μg of either pEX or pEXUL13; the TCA precipitable radioactivity was 3638 cpm/μg of protein for pEXUL13, 534
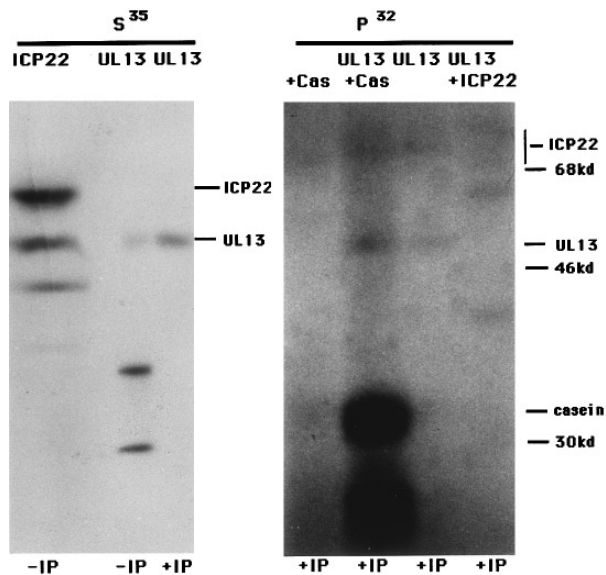

FIG. 4. Identification and enzymatic activity of the UL13 protein. (Left) Autoradiograph of *in vitro* translation in reticulocyte lysates after *in vitro* transcription of p22 (left side) or UL13, before (−IP) or after (+IP), immunoprecipitation with specific anti-UL13 antibodies. (Right) *In vitro* phosphorylation of casein alone and either casein, *in vitro*-translated buffer alone or *in vitro*-translated ICP22 with immunoprecipitated UL13 protein. Molecular weight markers are shown on the right.

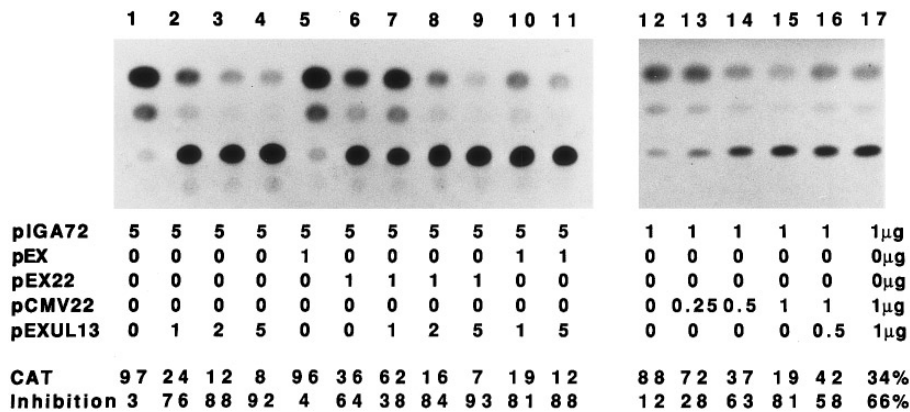|	1	2	3	4	5	6	7	8	9	10	11		12	13	14	15	16	17
pIGA72	5	5	5	5	5	5	5	5	5	5	5		1	1	1	1	1	1µg
pEX	0	0	0	0	1	0	0	0	0	1	1		0	0	0	0	0	0µg
pEX22	0	0	0	0	0	1	1	1	1	0	0		0	0	0	0	0	0µg
pCMV22	0	0	0	0	0	0	0	0	0	0	0		0	0.25	0.5	1	1	1µg
pEXUL13	0	1	2	5	0	0	1	2	5	1	5		0	0	0	0	0.5	1µg
CAT	97	24	12	8	96	36	62	16	7	19	12		88	72	37	19	42	34%
Inhibition	3	76	88	92	4	64	38	84	93	81	88		12	28	63	81	58	66%

FIG. 5. Effect of UL13 on the inhibitory effect of ICP22 on immediate-early promoters. COS cells were transfected (left) using the calcium method with 5 μg of pIGA72 and various amounts of pEX (lanes 5), pEXUL13 (lanes 2–4), or pEX22 (lane 6) or various proportions of the mixtures pEX22+pEXUL13 (lanes 7–9) or pEX+pEXUL13 (lanes 10 and 11); (right) using lipofectamine with 1 μg of pIGA72 and various amounts of pCMV22 alone (lanes 13–15) or 1 μg of pCMV22 in combination with variable amounts of pEXUL13 (lanes 16 and 17). The upper part of the figure shows the autoradiograms of chromatograms, while the percentages of acetylated chloramphenicol and pIGA72 inhibition are given below the figure.

cpm/μg for pEX-transfected cells, 65,441 cpm/μg for lysates of infected cells (24 hr after infection with 20 PFU/cell of HSV1), and 585 cpm/μg for lysates of uninfected cells. This high level of phosphorylation is relevant in terms of the stimulation of several protein kinases following viral infection (Lemaster and Roizman, 1980).

Since our UL13 vectors clearly expressed a functional protein kinase which was previously observed to phosphorylate ICP22 (Purves and Roizman, 1992), the effect on pIGA72 expression of the simultaneous presence in COS cells of the plasmids pEX22 and pEXUL13 was analyzed; the results are shown in Fig. 5 (left). The UL13 gene product strongly inhibited CAT expression under the control of α4 promoter, the effect being dose-dependent (columns 1 to 4). One microgram of UL13 vector (pEXUL13) inhibited pIGA72 activity by 76% (column 2), while the same amount of pEX22 gave 64% inhibition (column 6). The combination of 1 μg each of pEXUL13 and control plasmid pEX (column 10) resulted in 81% inhibition, while that of 1 μg each of pEXUL13 and pEX22 (column 7) resulted in only 38% inhibition. When pEXUL13 was present in excess over pEX22, the inhibition results were similar to those seen with pEXUL13 alone (columns 8 and 3, 9 and 4). A similar degree of reversal of ICP22 inhibition occurred when the amount of SV40 promoter driving ICP22 and UL13 was normalized for all samples using a plasmid containing only the SV40 promoter (pEX) (data not shown). The mean inhibition produced by 5 μg of pIGA72 in three different experiments was 74 $\pm$ 2.6% for 2 μg of pEX22, 77.7 $\pm$ 2.4% for 1 μg of pEXUL13, and 10.3 $\pm$ 2.3% for 2 μg of pEX22 in the presence of 1 μg of pEXUL13.

To confirm these results, pCMV22 and pEXUL13 were tested in COS cells for their ability to inhibit CAT expression under the control of pIGA72; the results are shown in Fig. 5 (right). Increased inhibition of CAT expression was seen with increasing concentrations of pCMV22 (columns 12–15). The 81% inhibition produced by 1 μg of pCMV22 fell to 58% on addition of 0.5 μg of pEXUL13 (column 16). As in the previous experiment, increasing the amount of pEXUL13 resulted in further inhibition of pIGA72 (column 17). These observations suggest either that, at a certain specific ICP22/UL13 ratio, the UL13 vector can partially reverse the inhibitory effect of ICP22 on IE gene expression or that ICP22 can partially reverse the inhibitory effect of UL13.

DISCUSSION

Our data indicate that, under the experimental conditions used in this work, ICP22 inhibits CAT expression under the control of HSV-1 IE promoters, including the α22 (IE4) promoter itself. The effect seems to be HSV1 promoter-specific, since ICP22 did not inhibit the SV40 and HCMV promoters, and it occurred when the protein was expressed under the control of two different heterologous promoters (SV40 and HCMV) in two different cell lines (COS and Rat-1).

Expression of, and inhibition by, ICP22 was greatest when it was expressed in COS cells under the control of the SV40 promoter (pEX22), with 1 μg of pEX22 or 2 μg of pCMV22 being sufficient to produce 50% inhibition of pIGA72 in these cells (Fig. 2B). In Rat-1 cells, more pEX22 and pCMV22 were required to produce the same degree of inhibition (Fig. 3B). The data in the two cell types were qualitatively similar, despite the fact that COS and Rat-1 cells differ in their ability to support the growth of the ICP22-deleted mutant (Sears et al., 1985).

It is interesting to note that ICP22 and other alphaherpesvirus ICP22-like proteins (PRV, RSp40; EHV1, IR4 ORF; BHV1, BICP22; and VZV, gene 63) show some structural homology in their central part, a possible DNA-binding region. The kinetics of the expression of ICP22 homologs differ during the course of infection. In the case of

pseudorabies virus (PRV), the ICP22 homolog is an early gene (Zhang and Leader, 1990); in equine herpes virus 1 (EHV1), its homolog is expressed, under various promoters, depending on the phase of the permissive cycle, first as an early gene, then as a γ1 gene (Holden et al., 1992); in transient assays, it enhances the transactivation of IE, early, and late promoters when expressed in combination with others transactivators (Holden et al., 1995); and in bovine herpes virus 1 (BHV1), BICP22 is expressed with IE or L kinetics, under the control of a single promoter (Schwyzer et al., 1994). According to cycloheximide experiments, only BICP22 and gene 63, the ICP22 counterpart in VZV, are regulated in the same way as ICP22 of HSV1 (Schwyzer et al., 1994; Debrus et al., 1995). In transfection experiments, the gene 63 product inhibits IE promoters (Jackers et al., 1992). Although more recent experiments have not confirmed these results (Kost et al., 1995), the similar kinetics of VZV and HSV1 ICP22 homologs might be related to similar functions during the IE infection stage.

The product of the UL13 gene, a serine-threonine protein kinase, is reported to be involved in ICP22 maturation (Purves and Roizman, 1992). We therefore constructed vectors expressing this protein to determine whether its presence could modify the inhibitory activity of ICP22. The vector was found to be functional, since it was able to phosphorylate both itself and casein. Surprisingly, we observed that UL13 itself was able to inhibit CAT expression under the α4 promoter, suggesting that the UL13 gene product might act as a gene expression regulator, probably via phosphorylation of cellular transcriptional factors. Although either expression vector (pEX22 or pEXUL13) was able to inhibit CAT expression under the control of the α4 promoter, their effects were not additive. Furthermore, when present at a particular molar ratio, a reproducible significant reduction of CAT inhibition was seen. Similar results were seen when pCMV22 was used instead of pEX22. The ratio of the two proteins required to produce partial reversal of inhibition suggests a stoichiometric interaction, as seen with other protein kinases, such as casein kinase II (CK2) (Meisner and Czech, 1991) and cAMP protein kinase (Jesse Chan et al., 1982).

In our Western blot experiments, using specific antiserum, several forms of ICP22 were seen, with the band ratios differing between transfected and infected COS cells and the faster migrating band being dominant in transfected cells (Fig. 1); this band, corresponding to the unprocessed molecule, is the only form found in cells infected by a UL13-deleted mutant and in cells infected by wild-type virus in the presence of cycloheximide (Purves et al., 1993). Thus, in the transient experiments performed in the absence of UL13, ICP22 was either nonphosphorylated or less phosphorylated and had undergone less maturation than in infected cells.

During infection, the large subunit of polymerase II undergoes partial phosphorylation of its carboxy-terminal domain (CTD), producing a form which is probably more adapted to viral than to cellular transcription (Rice et al., 1994). More recently, this modification has been shown to require ICP22 synthesis, but it was not possible to relate this to the cell-type-dependent growth phenotype of ICP22-deleted mutants (Rice et al., 1995). The authors suggest that the UL13 protein may be an ICP22-dependent CTD kinase. Our data, and those of others, regarding the similarity of the phenotypes of UL13- and ICP22-deleted mutants (Purves et al., 1993) suggest that UL13 can modify the phosphorylation of ICP22, and thus its function, in a similar manner to CK2, which acts directly or indirectly on the regulation of many cellular or viral genes (Meisner and Czech, 1991; Allende and Allende, 1995).

ORF 47, the UL13 VZV homolog, although unable to phosphorylate ORF 63, the ICP22 homolog (Heineman and Cohen, 1995), can phosphorylate ORF 62, the ICP4 homolog (Ng et al., 1994). Moreover, ORF 63 can be phosphorylated by protein kinase CK2 (Stevenson et al., 1996). HSV1 ICP22 has 120 amino acids in its N-terminal region, which are absent in the PRV, EHV1, BHV1, and VZV homologs (Zhang and Leader, 1990; Schwyzer et al., 1994); these make up two acidic blocks, one N-terminal to residue 12 and the second between residues 36 and 69, containing a high proportion of serine/threonine residues (Fig. 6) and at least four S*/T*-X-X-D/E consensus sequences close to β-turns, potential targets for phosphorylation by protein kinase UL13 and/or CK2 (Pinna, 1990). Moreover, the R/P-R-A-P/S-R sequence, a site of nucleotidylylation of ICP22, an event which apparently takes place at the IE stage of infection (Mitchell et al., 1994), is also located in this part of the molecule between residues 76 and 80.

In addition, as found with EHV1 (Holden et al., 1992), a new transcript, 3′ coterminal with the mRNA encoded by the α22 gene and directing the synthesis of a new protein corresponding to the C-terminal part of ICP22, has recently been found (Carter and Roizman, 1996). The authors postulate that ICP22 encodes at least two sets of functions, the first mapping to the C-terminal half of the molecule and responsible for the wild-type phenotype absent from cells infected with R325 (Sears et al., 1985) or 22n199 (Rice et al., 1995) mutants and the second mapping in the N-terminal region, which may be modulated by nucleotidylylation by CK2 and phosphorylation by viral protein kinases (UL13 and US3). The modulation of IE expression by phosphorylation of ICP22, like the phosphorylation of the CTD domain of polymerase II involving ICP22, also appears to be cell-type-independent (since identical results were seen in both COS and Rat1 cells) and is probably related to the N-terminal part of ICP22.

On the basis of these and our own findings, we suggest that, at the IE infection stage, ICP22 is either non-
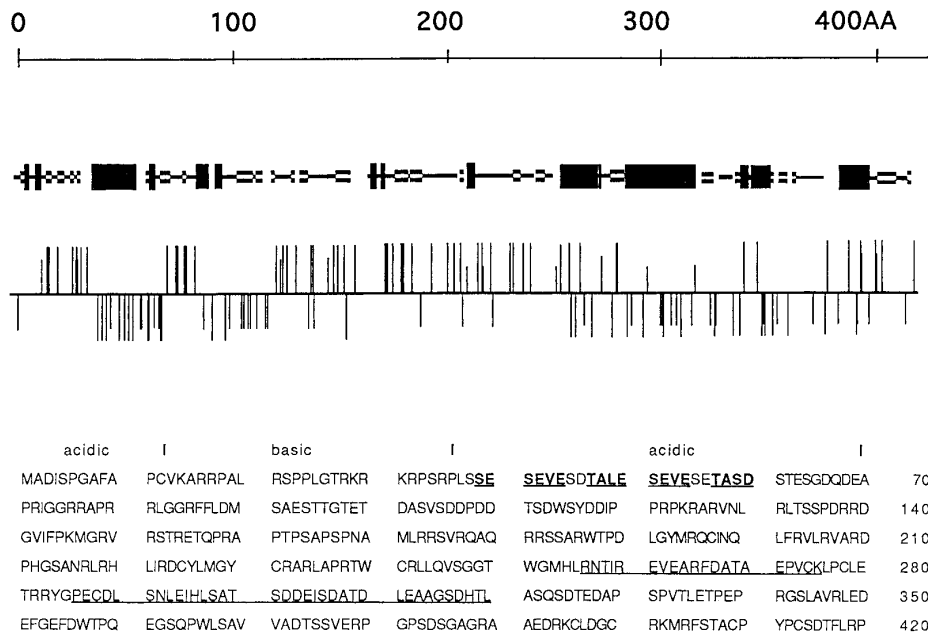
FIG. 6. Predicted secondary structure, distribution of acidic and basic residues, and amino acid sequence of ICP22. The amino acid sequences were analyzed according to Garnier *et al.* (1978). Three conformational states are shown: a helix (■), sheet (– – –), and β turn (===), with the blank regions being coil strands. The middle part of the figure shows basic and acidic residues (above and below the line, respectively). Individual residues are indicated by the length of the lines (E > D and R > K > H), as previously reported (Zhang and Leader, 1990). The lowest part of the figure shows the amino acid sequence of ICP22. Specific amino acids involved in helices are underlined and the S*/T*-X-X-E/D sequences (Meisner and Czech, 1991) are indicated in bold and underlined.

phosphorylated or only slightly phosphorylated and can act as a repressor of IE expression, while, at a later stage, it is modified, mainly by newly synthesized viral protein kinases UL13, resulting in both the loss of inhibitory effect and its involvement in late expression at the transcriptional or posttranscriptional level as previously observed (Purves *et al.,* 1993). This mechanism is not yet understood, but ICP22 might, like the ICP22 EHV1 homolog, increase the binding of ICP4 to viral promoters and be involved in the regulation of viral expression (D. J. O'Callaghan, personal communication) or increase the amplification of OriS (our unpublished observations). However, if ICP22 is a repressor of IE gene expression, ICP22-deleted mutants (R325 and 22/n199) would be expected to express IE proteins more efficiently; this does not seem to be the case (Purves *et al.,* 1993). In fact, such mutants express a truncated protein corresponding to the N-terminal part of ICP22. However, both cell-type-dependent growth and immediate-early gene expression are normal after cycloheximide reversion in the ICP22 null mutant (Poffenberger *et al.,* 1993). Thus, it appears that down-regulation of viral expression by ICP22 can be detected in transient experiments, but is difficult to see in viral infection. UL13 has been found in viral particles (Overton *et al.,* 1992) and is involved in the phosphorylation of the tegument protein, VP22 (Coulter *et al.,* 1993), but, at this stage, it appears not to be capable of phosphorylating ICP22 (Purves *et al.,* 1993). It is possible that the methods used were not sensitive enough to detect

low levels of phosphorylation. Our data probably demonstrate a mechanism which might be difficult to observe in infected cells, either because phosphorylation might be cycling on and off (Wilcox *et al.,* 1980) or because other viral factors might hide, or compensate for, the effect. In addition, the regulatory function of this protein is probably related not only to its posttranslational modification but also to its subcellular localization, which itself depends, either directly or indirectly, on these modifications.

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