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An Activity Specified by the Osteosarcoma Line U2OS Can Substitute Functionally for ICP0, a Major Regulatory Protein of Herpes Simplex Virus Type 1

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Among the five immediate-early regulatory proteins of herpes simplex virus (HSV) type 1, only ICP0 is capable of activating all kinetic classes of viral genes. Consistent with its broad transactivating activity, ICP0 plays a major role in enhancing the reactivation of HSV from latency both in vivo and in vitro. Although not essential for viral replication, ICP0 confers a significant growth advantage on the virus, especially at low multiplicities of infection. In this report we describe the expression of a novel activity by the osteosarcoma cell line U2OS that can substitute functionally for ICP0. Compared with Vero cells, both U2OS cells and cells of the ICP0-expressing line 0-28 significantly enhanced the plating efficiency of an ICP0 null mutant, 7134. In contrast, the plating efficiencies of the wild-type virus in all three cell types were similar. Single-step growth experiments demonstrated that the yield of 7134 in U2OS cells was severalfold higher than that in 0-28 cells and about 100-fold higher than that in Vero cells. In order to identify the viral genes whose expression is enhanced by the activity in U2OS cells, levels of expression of selected viral proteins in extracts of Vero and U2OS cells were compared by Western blot (immunoblot) analysis following low-multiplicity infection. At a multiplicity of 0.1 PFU per cell, the levels of expression of the immediate-early protein ICP4 and the early protein gD in 7134-infected U2OS cells were significantly higher than those in 7134-infected Vero cells. When infections were carried out at a multiplicity of 1 PFU per cell, however, no major differences in the levels of expression of these proteins in U2OS and Vero cells were observed. Cycloheximide reversal experiments demonstrated that the cellular activity expressed in U2OS cells that promotes high-level expression of ICP4 is not synthesized de novo but appears to exist as a preformed protein(s). To confirm this observation and to determine whether, like immediate-early genes, early, delayed-early, and late viral genes are also responsive to the cellular activity, transient-expression assays were performed. The results of these tests demonstrated that basal levels of expression from immediate-early, early, and delayed-early promoters, but not that from a late promoter, were significantly higher in U2OS cells than in Vero cells and that this enhancement occurred in the absence of viral proteins. Mutational analysis of the ICP4 promoter, the most responsive of the HSV type 1 promoters tested, has shown that the 139-bp sequence between the *Sph*I and *Sac*II sites is required for the high basal level of expression of ICP4 in U2OS cells.

During productive infection, the expression of herpes simplex virus type 1 (HSV-1) genes is regulated by four virus-encoded nuclear-phosphoproteins, ICPs 0, 4, 22, and 27 (2, 8, 12, 22, 24, 46, 56, 57, 61, 63, 64, 66, 68, 71). The genes for these proteins, termed immediate-early genes, are the first of over 70 viral genes to be expressed in virus-infected cells. The expression of the four immediate-early regulatory genes is activated by the virion-associated protein VP16 together with cellular transcription factors when the genome enters the nucleus (7, 10, 14, 17, 40, 54). The four immediate-early regulatory proteins serve to ensure that viral genes are selectively expressed relative to cellular genes and that they are expressed at the appropriate times and in the proper order. On the basis of the times of their maximum rates of expression, several classes of viral genes are recognized: immediate-early, early, delayed-early, and late genes (36, 37, 62).

Of the four immediate-early viral proteins, only ICP0 is able to activate all classes of HSV genes (8, 26, 27, 30, 31, 51, 52, 58). Consistent with its broad and potent transactivating activity, ICP0 dramatically enhances viral infectivity at low multiplicities of infection and promotes reactivation of HSV from latency, both in vivo and in vitro (8, 34, 43). Although the mechanism by which ICP0 activates gene expression is unclear, the available evidence suggests that this protein performs functions related to transcription (35, 74). ICP4 is the major transcriptional activator of the virus (20, 21, 24, 53, 56) and acts synergistically with ICP0 to significantly enhance the expression of early, delayed-early, and late genes (27, 28, 31, 51, 52). Recent evidence suggests that the synergistic effects of ICP0 and ICP4 may result from a direct interaction between these two proteins (73). ICP22 modifies the carboxy-terminal portion of RNA polymerase II (60), a modification that presumably favors transcription of viral genes, and is also able to modify the *trans*-regulatory activities of ICP0 and ICP4 (2). ICP27 regulates gene expression by posttranscriptional processing of viral messages (45, 48, 65). Thus, the four viral immediate-early regulatory proteins utilize a variety of mechanisms to ensure the timely and efficient transcription of viral genes and processing of viral transcripts.

Although the roles of individual immediate-early proteins in regulating HSV gene expression are becoming increasingly clear, the roles of cellular proteins in this process are not well understood. Several cellular transcription factors have been shown to enhance HSV-1 gene expression, but the identities of

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a number of other factors that actually mediate this enhancement and the manner in which they achieve this enhancement remain to be determined. The studies described here were initially undertaken to test the involvement of cell cycle regulatory factors in HSV-1 gene expression. The retinoblastoma susceptibility gene product (pRb) and p53 are among the most extensively studied proteins involved in the regulation of cell proliferation. These proteins function together to regulate progression through the cell cycle (3, 5, 6, 13, 19, 23, 29, 32, 38, 47). Studies of the transforming proteins of DNA tumor viruses have demonstrated that these proteins form stable complexes with either pRb or p53 (18, 25, 49, 70). It is believed that the formation of such complexes leads to functional inactivation of pRb and p53, allowing deregulation of normal cell cycle control (4, 15). Although HSV-1 is not a tumor virus, it is reasonable to assume that alterations in the expression of cellular proteins involved in cell cycle regulation may have measurable effects on HSV-1 gene expression and hence on its replication efficiency.

The human osteosarcoma cell lines SAOS2 and U2OS have been used extensively to study the molecular biology and functional properties of pRb and p53. These two cell lines differ from each other in the manner in which normal cell cycle regulation is disrupted. SAOS2 cells specify a functionally inactive form of pRb truncated at its carboxy terminus and contain a large deletion in the gene encoding p53, whereas U2OS cells encode wild-type pRb and p53 (23, 33, 38, 39, 42, 44, 67). Notably, the latter observation indicates that the loss of functional pRB and p53 is not the only avenue that leads to disruption of cell cycle regulation but that other properties unique to U2OS cells can produce the same result. Using these two cell types, we examined the effects of altered cell cycle regulation on the growth properties and patterns of gene expression of HSV-1.

In this report, we describe the expression of a novel activity in the osteosarcoma line U2OS that can substitute functionally for ICP0. Like ICP0, the cellular activity is able to enhance the plating and replication efficiencies of an HSV-1 ICP0 null mutant and the de novo synthesis of infectious virus after transfection of null mutant viral DNA. Specifically, data from single-step growth experiments showed that the yield of an ICP0 null mutant, 7134, was 100-fold higher in U2OS cells than in Vero cells. At low multiplicities of infection, the expression of HSV-1 immediate-early, early, and late genes was markedly enhanced in 7134-infected U2OS cells compared with Vero cells. The factors responsible for the activity specified in U2OS cells are not synthesized de novo upon virus infection but rather preexist in these cells. Consistent with its ability to enhance replication of an ICP0 null mutant, the activity is able to elevate basal levels of expression from viral immediate-early, early, and delayed-early promoters in the absence of any viral proteins. The ability of the cellular activity to substitute for ICP0 suggests either that the two are homologs or that they affect a common cellular pathway leading to gene activation.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney (Vero) cells, 0-28 cells (Vero cells stably transformed with the ICP0 gene) (8, 64), U2OS cells (American Type Culture Collection [ATCC], HTB96), HEp-2 cells (human epidermoid carcinoma 2 cells) (ATCC), and HeLa cells (ATCC) were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. SAOS2 cells (ATCC; HTB85) were grown in Dulbecco's modified Eagle's medium supplemented with 4.5 g of glucose per liter, 0.11 g of Na-pyruvate per liter, and 10% fetal bovine serum in the presence of 0.1 mM nonessential amino acids (Sigma Chemical Co., St. Louis, Mo.) and 1 μg of insulin (Sigma) per ml.

Wild-type HSV-1 (strain KOS) and an ICP0 null mutant (7134), as well as ICP0 nonsense mutant viruses (*n*212, *n*428, *n*525, *n*680, *n*720, and *n*770) derived from KOS, were propagated and assayed in Vero cells as described previously (8).

Single-step growth curves. Vero, 0-28, and U2OS cells were seeded in 35-mm-diameter dishes (3×10^5 cells per dish). Twenty-four hours after being seeded, the cells were infected at a very low multiplicity, i.e., with 1,000 PFU of 7134 per dish (or 0.003 PFU per cell), based on the titer determined on Vero cell monolayers. After 1 h of absorption at 37°C, the cells were washed twice with Tris-buffered saline, overlaid with 4 ml of growth medium, and incubated at 37°C. Infected-cell cultures were harvested at 3, 6, 9, 12, 15, and 18 h postinfection, and viral titers were determined by standard plaque assay on Vero cell monolayers.

Viral protein synthesis. Petri dishes (100-mm diameter) were seeded with 4×10^6 Vero or U2OS cells per dish. The cells were infected with wild-type HSV-1 (strain KOS) or 7134 at a multiplicity of 0.1 or 1.0 PFU per cell 24 h after being seeded. Cultures were harvested 11 h after infection, and the syntheses of selected HSV-1 proteins representing specific kinetic classes were measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (immunoblot) analysis as described below. Synthesis of immediate-early proteins was determined by using the cycloheximide reversal procedure as follows. Vero and U2OS cells were seeded as described above. The cells were infected 24 h after being seeded with KOS or 7134 at a multiplicity of 0.1 PFU per cell in the presence of 100 μg of cycloheximide per ml. Five hours after infection, the cycloheximide was removed and the cells were washed with medium containing 10 μg of actinomycin D per ml. Medium containing the same concentration of actinomycin D was then added to cultures, and incubation was continued for an additional 6 h, after which cells were harvested and lysates were prepared.

SDS-PAGE and Western blot analysis. Proteins in infected-cell lysates were resolved by SDS-PAGE (7% acrylamide) as described previously (72), transferred to polyvinylidene difluoride membranes, and examined by Western blot analysis with rabbit polyclonal antibodies to individual viral proteins of the kinetic classes immediate early (ICP4), delayed early (gD), and late (gC) as previously described (72). The rabbit polyclonal antibodies directed against ICP4 (RO21) and gC were kindly provided by Richard J. Courtney (Pennsylvania State University College of Medicine, Hershey). The polyclonal rabbit serum specific for gD was generously provided by Gary Cohen (University of Pennsylvania, Philadelphia). Briefly, after 1 h of incubation with primary antibodies, the polyvinylidene difluoride membrane was washed with 1× NETG buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, and 0.25% gelatin) containing 0.1% SDS and 0.5% Triton X-100 and was reacted with goat anti-rabbit immunoglobulin G–horseradish peroxidase conjugate (Bio-Rad Laboratories, Hercules, Calif.) for 1 h at a 1:2,000 dilution in 1× NETG buffer containing 0.05% Nonidet P-40. The blot was then washed, reacted with chemiluminescence protein detection reagent (DuPont, NEN Research Products, Boston, Mass.) according to the manufacturer's instructions, and exposed to Kodak X-AR5 film.

Plasmids. pSH (8) and pn11 (21) are plasmids that express ICP0 and ICP4, respectively. pIE3CAT-AN6 contains the ICP4 promoter upstream of the gene encoding chloramphenicol acetyltransferase (CAT) in vector pGEM-7Zf(+) (kindly provided by Ahn Nguyen-Huynh, Harvard Medical School, Boston, Mass.), whereas pTKCAT (20), pgBCAT (8), and UL10CAT (gift of Khandan Baradaran, Harvard Medical School) contain CAT-coding sequences under control of the HSV-1 thymidine kinase (early), gB (delayed-early), and UL10 (late) promoters, respectively.

To construct ICP4 promoter deletion mutant plasmids, pIE3CAT was digested with *Nar*I and end filled with the Klenow fragment of *Escherichia coli* DNA polymerase I. The linearized pIE3CAT was then digested with *Bss*HII, *Sph*I, or *Sac*II. The vector-containing fragments were isolated and recircularized with ligase. After transformation of *E. coli*, small batches of each plasmid were prepared, and their structures were verified by restriction enzyme digestion. The recombinants which contained deletions from *Nar*I to *Bss*HII, *Nar*I to *Sph*I, and *Nar*I to *Sac*II were designated pIE3CAT-ΔBN, -ΔSpN, and -ΔSaN, respectively. (A schematic diagram of these ICP4 promoter deletion mutants is shown in Fig. 5A.) To generate pIE3CAT-ΔSaS, a plasmid containing a 139-bp deletion between the *Sph*I and *Sac*II sites in the ICP4 promoter, pIE3CAT was doubly digested with *Sph*I and *Sac*II, and ends were made blunt by treatment with T4 DNA polymerase. The vector-containing fragment was then isolated and recircularized with T4 DNA ligase.

Purification of infectious viral DNA from HSV-1 virions. Vero cells were infected with either KOS or 7134 at a multiplicity of 0.01 PFU per cell. At 48 to 56 h postinfection, the cells were harvested, and HSV virions were purified as described previously (72). The purified virions were treated with proteinase K (200 μg/ml) in the presence of 0.5% Nonidet P-40 and 0.5% SDS at 37°C for 2 h. Virion DNA was then isolated following CsCl (57% [wt/wt] in 1× SSC [0.15 M NaCl plus 0.015 M sodium citrate]) density gradient centrifugation in Ti75 centrifuge tubes at 35,000 rpm for 68 h. The CsCl was removed from the viral DNA solution by dialysis against several changes of Tris-EDTA (pH 8.0) at 4°C.

Transfections and CAT assays. Dishes (100-mm diameter) were seeded with 2×10^6 Vero or U2OS cells per dish 24 h prior to transfection. Three hours before transfection, the medium was changed. DNA precipitates used in transfection assays were prepared by the calcium phosphate method in BES [*N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid] as described by Chen and Okayama (11). Specifically, 5 μg of pIE3CAT-AN6, pTKCAT, pgBCAT, or pUL10CAT was suspended in 500 μl of 2× BES buffer. Salmon testis DNA was added to yield 20 μg of total DNA, and the suspension was then mixed with an equal volume of 250 mM $CaCl_2$ for 20 min at room temperature. The resulting DNA-calcium phosphate coprecipitate was added to the cell culture medium. At 18 to 20 h posttransfection, cells were washed and refed with fresh medium. Cell

extracts were prepared in a volume of 150 μl of CAT buffer (0.25 M Tris-HCl, pH 7.8) at 44 to 48 h posttransfection. After protein concentrations were determined, CAT assays were performed by addition of 30 μl of 20 mM acetyl coenzyme A (Sigma) and 0.1 μCi of [^{14}C]chloramphenicol (DuPont, NEN Research Products). The reactions were carried out at 37°C for 45 min, and the various acetylated products were separated and measured as described previously (20). CAT assays were performed two to four times, and similar results were obtained in all cases.

To identify the *cis*-acting sequences within pIE3CAT responsible for the high basal level of CAT expression of pIE3CAT in U2OS cells, 24-h-old U2OS cell monolayers were transfected with 2.5 μg of pIE3CAT, pIE3CAT-ΔBN, pIE3CAT-ΔSpN, or pIE3CAT-ΔSaN as described above. Forty-four hours after transfection, cell extracts were prepared and CAT activities were determined.

DNA slot blot analysis. Petri dishes (100-mm diameter) were seeded with 2×10^6 Vero or U2OS cells per dish. The cells were mock transfected or transfected with 10 μg of pIE3CAT-AN6 24 h after being seeded. Twenty-four hours after transfection, cell nuclei were isolated by the method of Andrews and Faller (1) and nuclear DNA was obtained as follows. Nuclei were resuspended in lysis buffer (10 mM Tris-HCl [pH 7.6], 50 mM EDTA, 0.1% SDS) in the presence of 200 μg of proteinase K per ml and incubated overnight at 37°C. DNA was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1); this was followed by two chloroform-isoamyl alcohol (24:1) extractions. After ethanol precipitation, the DNA was resuspended in 400 μl of 10 mM Tris-HCl (pH 7.6)–50 mM EDTA in the presence of 200 μg of RNase A per ml, incubated for 2 h at 37°C, and recovered by extraction with phenol-chloroform-isoamyl alcohol and ethanol precipitation. For slot blot analysis, 10 μg of nuclear DNA isolated from mock-transfected or pIE3CAT-transfected Vero or U2OS cells was transferred to nitrocellulose by vacuum transfer. Blots were hybridized with nick-translated, ^{32}P-labeled pIE3CAT-AN6 as described previously (16).

RESULTS

An activity expressed in U2OS cells enhances the plating and replication efficiencies of 7134. To assess the role of cell cycle-regulated factors in the *trans* regulation of HSV-1 gene expression, we first examined the plating efficiencies of an ICP0 null mutant (7134) and wild-type HSV-1 (strain KOS) on monolayers of SAOS2 and U2OS cells. SAOS2 cells are pRb^- $p53^-$ and U2OS cells are pRb^+ $p53^+$, yet both exhibit alterations in cell cycle regulation. If cell cycle-associated factors are involved in the expression of viral genes, and if these factors act through ICP0, the abilities of an ICP0 null mutant to form plaques on monolayers of these two cell types might differ relative to one another and relative to Vero cells.

To test this possibility, 24-h-old monolayers of SAOS2, U2OS, Vero, and ICP0-expressing Vero (0-28) cells were infected with either 100 PFU of wild-type virus per dish or 10 PFU of the ICP0 null mutant, 7134, per dish. The numbers of input PFU were calculated from viral titers obtained with 24-h-old Vero cell monolayers. The intentional difference in input multiplicity was based on the fact that stocks of 7134 contain large numbers of genome-containing virions that are unable to produce plaques on 24-h-old Vero cell monolayers (9). Thus, on Vero cell monolayers the physical particle-to-PFU ratio of 7134 stocks is 20- to 50-fold higher than that of wild-type virus, whereas on 0-28 cell monolayers the physical particle-to-PFU ratios of 7134 and wild-type virus are nearly equal. Notably, the entries of 7134 and wild-type virus into Vero cells are equally efficient (9).

As shown in Table 1, the plating efficiencies of wild-type virus were similar on monolayers of Vero, 0-28, and U2OS cells and ~50% lower on SAOS2 cell monolayers. As shown previously (8, 64), the plating efficiency of 7134 was enhanced significantly (~32-fold) on 0-28 cell monolayers compared with Vero cell monolayers. The plating efficiency of 7134 was enhanced approximately 10-fold on SAOS2 cell monolayers and about 100-fold on U2OS cell monolayers relative to that on Vero cell monolayers. In contrast to the case for the ICP0 null mutant (7134), the plating efficiencies of viruses with mutations in other nonessential viral genes (those encoding ICP22 and the latency-associated transcripts) were approximately equal on Vero, U2OS, and 0-28 cells (data not shown). As expected, none of the cell types tested supported plaque formation by mutant viruses defective in essential replication genes, i.e., those encoding ICP4 and ICP27 (data not shown). Furthermore, to determine if the cellular activity observed in U2OS cells represents a unique property associated with these cells, the plating efficiencies of 7134 on 24-h-old HEp-2 and HeLa cell monolayers were also tested. The results of these tests demonstrated that although both cell types can enhance the plating efficiency of 7134 to some degree (HEp-2, 4-fold; HeLa, 10-fold [relative to that with Vero cells]), this enhancement was still 10-fold less than that on U2OS cells. These findings suggest that U2OS cells express an especially high level of an activity that increases the plating efficiency of an ICP0 null mutant.

TABLE 1. Plating efficiencies of wild-type and ICP0 null mutant viruses in Vero, 0-28, SAOS2, and U2OS cells[a]

Cells	No. of plaques produced[b] with:			
	KOS		7134	
	Expt 1	Expt 2	Expt 1	Expt 2
Vero	98, 116	94, 95	9, 11	9, 10
0-28	99, 104	75, 82	490, 505	520, 540
SAOS2	39, 42	42, 50	110, 114	99, 102
U2OS	72, 90	86, 90	1,160, 1,180	1,050, 1,100

[a] Vero, 0-28, SAOS2, and U2OS cells were seeded at 4×10^6 cells per 100-mm-diameter dish. At 24 h postseeding, cells were infected with either wild-type virus (100 PFU per dish) or an ICP0 null mutant, 7134 (10 PFU per dish). After 1 h of adsorption, methylcellulose was added, and plaques were counted at 96 h postinfection. The number of input PFU was calculated by using titers obtained on 24-h-old Vero cell monolayers.

[b] Results of duplicate determinations are shown.

To further test the ability of U2OS cells to support 7134 replication, single-cycle growth experiments were carried out with Vero, 0-28, and U2OS cells at a very low multiplicity of infection (0.003 PFU per cell). Consistent with the plating efficiency data shown in Table 1, the growth curves shown in Fig. 1 demonstrate that 7134 replicated most efficiently in U2OS cells, less efficiently in 0-28 cells, and least efficiently in Vero cells. The 18-h yield of 7134 was severalfold higher in U2OS cells than in 0-28 cells and approximately 100-fold higher in U2OS cells than in Vero cells. In addition, the 9- to 12-h delay in the onset of new virus synthesis seen in Vero cells was not observed in U2OS or 0-28 cells, in which progeny virus was first evident between 6 and 9 h postinfection.

Additional evidence that the cellular activity in U2OS cells can substitute functionally for ICP0 is shown in Table 2. In these tests, the plating efficiencies of a series of ICP0 nonsense mutant viruses (*n*212, *n*428, *n*525, *n*680, *n*720, and *n*770) on monolayers of Vero, 0-28, and U2OS cells were compared. Cai and Schaffer have shown that the introduction of nonsense mutations at codons 212, 428, 525, and 680 in the ICP0 open reading frame yields polypeptides that are severely impaired in their ability to activate HSV gene expression, whereas a nonsense mutation at codon 720 produces only a minor effect and a mutation at codon 770 shows no effect on ICP0's transactivating activity (8). As shown in Table 2, the plating efficiencies of nonsense mutants *n*212, *n*428, *n*525, and *n*680 on both 0-28 and U2OS cell monolayers were significantly enhanced relative to those on Vero cell monolayers. The plating efficiencies of *n*720 on monolayers of 0-28 and U2OS cells were similar and about twofold greater than that on Vero cell monolayers. The plating efficiencies of *n*770 on monolayers of all three cell types were essentially the same. These observations indicate that the ability of the cellular activity in U2OS cells to enhance the plating efficiencies of ICP0 nonsense mutants correlates di-

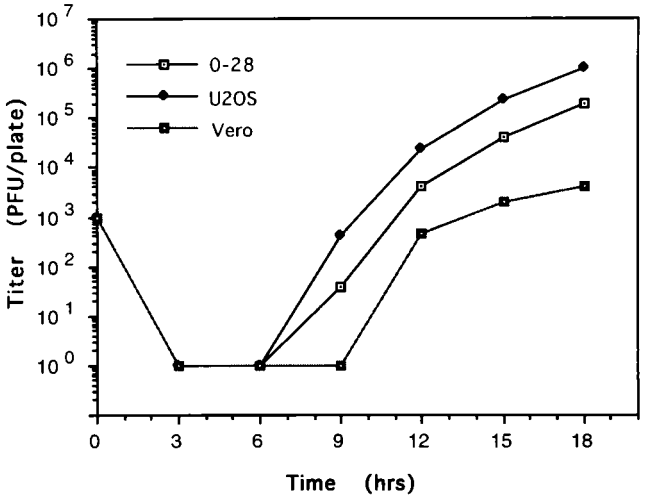

FIG. 1. Single-step growth curves for the ICP0 null mutant 7134 in Vero, 0-28, and U2OS cells. Cells of all three types were seeded at 3×10^5 cells per 35-mm-diameter dish. Twenty-four hours after being seeded, the cells were infected with 1,000 PFU of 7134 per dish (this dose was based on the titer in 24-h-old Vero cell monolayers). After 1 h of adsorption at 37°C, cells were washed with Tris-buffered saline, overlaid with 4 ml of growth medium, and incubated at 37°C. Infected cells were harvested at 3, 6, 9, 12, 15, and 18 h postinfection, and viral titers were determined by standard plaque assay on Vero cell monolayers.

rectly with the level of impairment in the transactivating activity of individual mutant forms of ICP0; i.e., the greater the impairment in the transactivating activity of the ICP0 mutant protein, the higher the plating efficiency of the corresponding mutant virus on U2OS cells.

Immediate-early viral gene expression is enhanced in 7134-infected U2OS cells. Cai and Schaffer have shown that the levels of early and late viral proteins are markedly reduced in ICP0 mutant virus-infected Vero cells at low multiplicities of infection (8, 9). It was not clear from those experiments, however, whether the reduction in expression of early and late viral genes was a consequence of the absence of ICP0 and its transactivating activity or of the reduced expression of other immediate-early genes, in particular, the major transcriptional activator, ICP4. To investigate the mechanism by which the activity in U2OS cells substitutes for ICP0 to enhance the plating and replication efficiencies of ICP0 mutant viruses, the levels of viral gene expression in 7134-infected Vero and U2OS cells at low multiplicities of infection were compared. Cells were infected with KOS or 7134 at multiplicities of 0.1 or 1 PFU per cell (the 7134 titer used was based on assays with 24-h-old 0-28 cell monolayers), and infected-cell proteins were resolved by SDS-PAGE. Because viral gene expression is very low at low multiplicities of infection, it is difficult to distinguish viral from cellular proteins on standard SDS gels. Therefore, the synthesis of viral proteins representing each of the three kinetic classes of HSV-1 proteins was examined by Western blot analysis with polyclonal antibodies directed against proteins representing three kinetic classes: ICP4 (immediate early), gD (early), and gC (late).

As shown in Fig. 2A, at a multiplicity of 0.1 PFU per cell, the levels of ICP4 expression in 7134-infected U2OS cells and KOS-infected Vero and U2OS cells were significantly higher than that in 7134-infected Vero cells. The synthesis of a representative early protein (gD) was also elevated in 7134-infected U2OS cells, probably because these cells express higher levels of immediate-early regulatory proteins. When infections were carried out at a multiplicity of 1 PFU per cell, no major difference in the levels of ICP4 in 7134- and KOS-infected Vero and U2OS cells was observed. The very high levels of gD and gC observed in 7134-infected U2OS cells are of special interest. In these cells, the synthesis of early and late viral proteins was more efficient than in KOS-infected U2OS cells, implying that ICP0 has the ability to repress the potent enhancing effect of the cellular activity on viral gene expression. This observation suggests that ICP0 can function not only as a strong transactivator but also as a fine-tuning factor that ensures optimal levels of viral gene expression at low multiplicities of infection. Collectively, these experiments demonstrate that (i) a cellular activity expressed in U2OS cells can substitute functionally for ICP0 to achieve elevated levels of viral gene expression and (ii) one function of ICP0 is to elevate the expression of immediate-early viral genes such as the ICP4 gene following low-multiplicity infection.

The cellular activity in U2OS cells is not induced by HSV-1 infection. HSV-1 virions contain at least 30 unique structural proteins (62). In addition to viral capsid proteins and envelope glycoproteins, a number of proteins are located within the virion tegument. The most extensively studied tegument proteins are VP16 (7, 10, 17, 40, 50, 54, 69), which acts as an inducer of immediate-early viral gene expression, and the virion host shutoff protein, Vhs (41). In addition to enhancing the expression of immediate-early viral genes (VP16) and repressing the expression of some host cell proteins (Vhs), these virion-associated factors may also be involved in the transcriptional activation of cellular genes upon HSV infection. To determine whether the cellular activity expressed in U2OS cells is activated by viral infection and whether activation occurs at the transcriptional level, infected U2OS cells were incubated in the presence or absence of specific inhibitors of protein synthesis (cycloheximide) and RNA synthesis (actinomycin D). Infected-cell proteins were resolved by SDS-PAGE followed by Western blot analysis with anti-ICP4, anti-gD, and anti-gC specific antibodies.

As shown in Fig. 2B, under cycloheximide reversal conditions, the level of ICP4 that accumulated in 7134-infected U2OS cells was significantly higher than that in 7134-infected Vero cells. In contrast to the case for infection of these cells in the absence of cycloheximide and actinomycin D (Fig. 2A), no synthesis of gD (early) and gC (late) proteins was detected. Because the synthesis of immediate-early viral proteins is to-

TABLE 2. Plating efficiencies of ICP0 nonsense mutant viruses in Vero, 0-28, and U2OS cells[a]

Virus[b]	No. of plaques produced[c] in:		
	Vero cells	0-28 cells	U2OS cells
*n*212	5, 5	260, 285	970, 948
*n*428	4, 5	58, 67	198, 203
*n*525	7, 6	54, 60	152, 143
*n*680	5, 4	35, 37	134, 150
*n*720	5, 6	10, 9	11, 10
*n*770	5, 6	5, 6	6, 7
KOS	6, 6	5, 4	4, 4

[a] Vero, 0-28, and U2OS cells were seeded in 100-mm-diameter dishes (4×10^6 cells per dish). Twenty-four hours after being seeded, the cells were infected with 5 PFU of ICP0 nonsense mutant viruses per dish (based on the titers determined on Vero cell monolayers). After 1 h of adsorption, methylcellulose was added, and plaques were counted 4 days after infection.

[b] The three-digit number in the designation of each nonsense mutant represents the codon where a nonsense mutation was introduced in the open reading frame of the ICP0 gene.

[c] Results of duplicate determinations are shown.

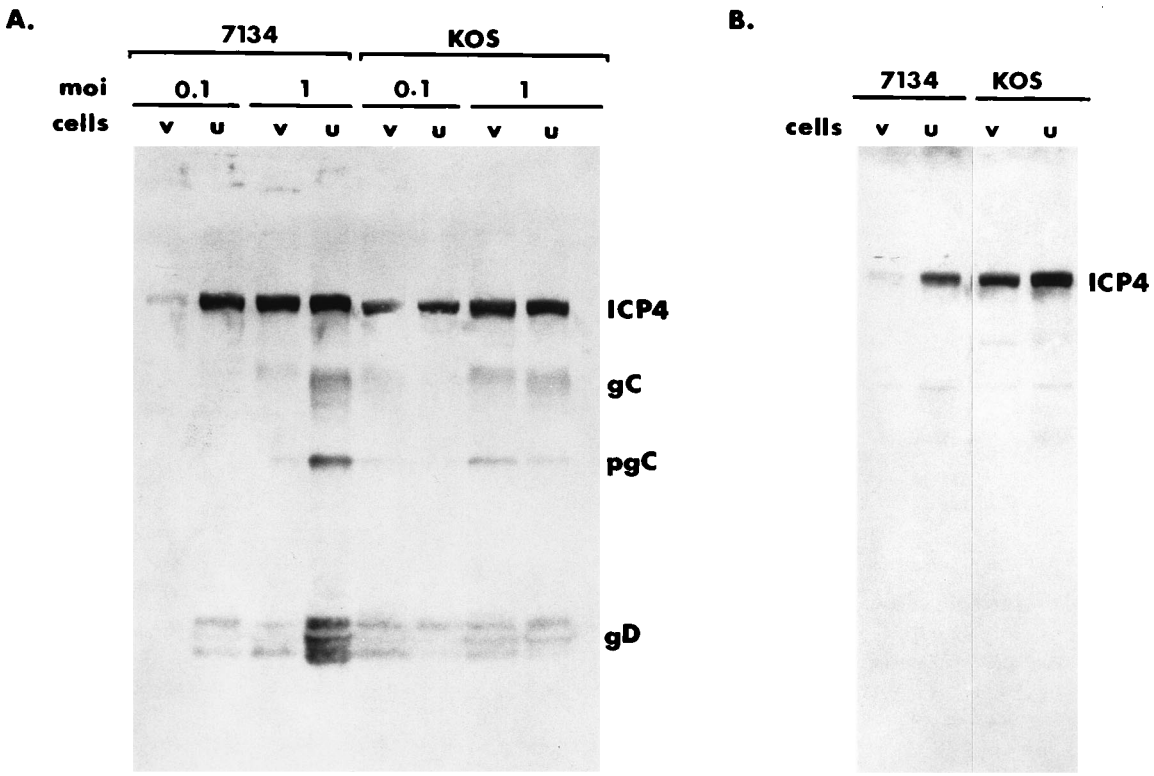

FIG. 2. Viral gene expression in wild-type and ICP0 null mutant virus-infected Vero (v) and U2OS (u) cells. (A) Vero cells and U2OS cells were seeded at 4×10^6 cells per 100-mm-diameter dish. Twenty-four hours after being seeded, the cells were infected with 0.1 or 1.0 PFU of KOS or 7134 per cell. Infected cells were harvested 12 h after infection, and proteins from infected-cell lysates were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and examined by Western blot analysis with anti-ICP4 (immediate-early), anti-gD (early), and anti-gC (late) antibodies. moi, multiplicity of infection. (B) Twenty-four-hour-old Vero and U2OS cells were infected with 0.1 PFU of KOS or 7134 per cell in the presence of 100 μg of cycloheximide per ml. At 5 h postinfection, the cycloheximide was removed and the cells were washed with medium containing 10 μg of actinomycin D per ml. Medium containing the same concentration of actinomycin D was then added to the cultures, and incubation was carried out for an additional 6 h. Viral proteins in infected-cell extracts were examined by Western blot analysis with anti-ICP4, anti-gD, and anti-gC antibodies. This experiment was conducted simultaneously with the experiment shown in panel A. Therefore, the no-treatment controls at 0.1 PFU per cell are shown in panel A.

tally dependent upon the activities of preexisting cellular and virion-associated factors in cycloheximide- and actinomycin D-treated cells, the factors that promote the high level of ICP4 expression in U2OS cells are not produced de novo but rather appear to exist as preformed proteins. This conclusion was further supported by the results of the following experiment. Twenty-four-hour-old Vero and U2OS cell monolayers were transfected with the ICP4-expressing plasmid pn11. Cells were harvested at 44 h posttransfection, and the expression of ICP4 was assayed by immunoprecipitation and Western blot analysis with an anti-ICP4 polyclonal antibody (Fig. 3A). In the absence of other HSV-1 proteins, the level of ICP4 expressed in transfected U2OS cells was significantly higher than that in transfected Vero cells. Moreover, slot blot analysis of nuclear DNA isolated from pIE3CAT-transfected Vero and U2OS cells demonstrated that the transfection efficiencies of plasmid DNA in Vero and U2OS cells were similar (Fig. 3B). Therefore, the cellular activity specified by U2OS cells that is responsible for the high-level expression of ICP4 preexists in these cells.

As noted above, the results of cycloheximide-actinomycin D reversal experiments (Fig. 2B) also showed that the level of ICP4 detected in wild-type-virus-infected Vero cells was much higher than that in 7134-infected Vero cells. Since ICP0 is present within HSV-1 virions (72), this observation suggests that at low multiplicities of infection, virion-associated ICP0, like VP16, may function as an activator to enhance the expression of immediate-early viral genes.

Like ICP0, the cellular activity expressed in U2OS cells can enhance the de novo synthesis of virus following transfection of infectious viral DNA. Previous studies have demonstrated that ICP0 plays a critical role in the de novo synthesis of virus following transfection of Vero cells with infectious viral DNA (8). Specifically, these tests showed that the synthesis of progeny virus was significantly delayed and reduced in Vero cells transfected with infectious 7134 DNA compared with cells transfected with infectious wild-type viral DNA. To determine whether the cellular activity expressed in U2OS cells can substitute for ICP0 to enhance the de novo synthesis of virus from transfected viral DNA, Vero and U2OS cells were transfected with purified, infectious 7134 or KOS DNA in the presence or absence of an ICP0-expressing plasmid, pSH. As shown in Table 3, infectious 7134 DNA produced large numbers of plaques on U2OS cell monolayers but no plaques on Vero cell monolayers. Cotransfection of 7134 DNA with pSH facilitated plaque formation on Vero cells and further enhanced the efficiency of plaque formation on U2OS cells by about twofold. For infectious KOS DNA, plaque formation was twofold greater on U2OS than on Vero cell monolayers, and like for 7134 DNA, cotransfection with an ICP0-expressing plasmid enhanced plaque formation by KOS DNA significantly on both Vero and U2OS cells. The results of these tests demonstrate that like ICP0, the activity in U2OS cells can enhance the efficiency of plaque formation by infectious 7134 and KOS DNAs. The ability of ICP0 to further enhance plaque forma-

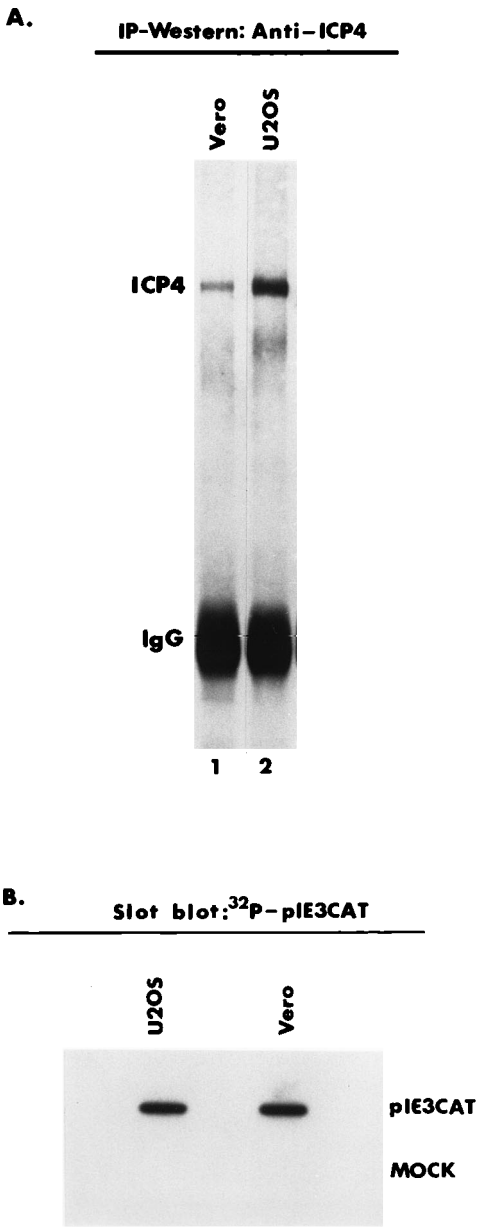

FIG. 3. (A) Expression of ICP4 in Vero and U2OS cells. Vero and U2OS cells were transfected with 10 μg of the ICP4-expressing plasmid pn11. Forty hours after transfection, cells were lysed and immunoprecipitated with an anti-ICP4 polyclonal antibody, RO21. The immunoprecipitated proteins were subjected to SDS-PAGE followed by Western blot analysis with the anti-ICP4 polyclonal antibody. (B) Transfection efficiencies of Vero and U2OS cells. Vero and U2OS cells were seeded at 2×10^6 cells per 100-mm-diameter dish. Twenty-four hours after being seeded, the cells were either mock transfected or transfected with 10 μg of pIE3CAT. Twenty-four hours after transfection, cell nuclei were isolated by the method of Andrews and Faller (1) and nuclear DNA was obtained. To compare the transfection efficiencies of Vero and U2OS cells, 10 mg of nuclear DNA from mock-transfected or pIE3CAT-transfected Vero or U2OS cells was analyzed by slot blot hybridization with nick-translated, ^{32}P-labeled pIE3CAT as a probe.

tion by both types of DNA in U2OS cells indicates that ICP0 and the cellular activity are distinct but are additive in these tests. As described above, the plating efficiency tests showed that the numbers of plaques produced by wild-type virus on Vero and U2OS cell monolayers were similar (Tables 1 and 2). The apparent difference in the ability of the cellular activity to

TABLE 3. Infectivities of 7134 and wild-type HSV-1 viral DNAs in Vero and U2OS cells[a]

DNA	ICP0	No. of plaques produced			
		Expt 1		Expt 2	
		Vero cells	U2OS cells	Vero cells	U2OS cells
7134	−	0	328	0	380
	+	75	605	82	648
KOS	−	185	406	138	252
	+	431	>1,000	263	>1,000

[a] Vero and U2OS cells were seeded at 4×10^6 cells per 100-mm-diameter dish. At 24 h postseeding, cells were transfected with 0.5 μg of infectious KOS DNA or 1 μg of infectious 7134 DNA in the absence or presence of 0.5 μg of the ICP0-expressing plasmid pSH. At 17 h posttransfection, cells were washed and 20 ml of methylcellulose was added to the cell monolayer. Plaques were counted 5 days after transfection.

enhance the infectivity of wild-type viral DNA following transfection but not infection can be explained by the hypothesis that in infection, virion-associated transactivators such as VP16 are sufficient to enhance the level of immediate-early viral gene expression such that productive infection can occur. In contrast, in transfection assays, virion-associated transcription factors are absent at the initial stages of infection. In this case, the likelihood that productive infection will be initiated from infectious viral DNA depends solely upon the ability of the cellular activity to elevate the expression of immediate-early viral genes to levels at which productive infection can occur. This hypothesis is strongly supported by the observation that the basal levels of expression of immediate-early viral genes for ICP4 (Fig. 3A) and ICP0 (data not shown) are significantly enhanced in U2OS cells relative to Vero cells.

The cellular activity in U2OS cells enhances basal levels of expression from representative immediate-early, early, and delayed-early promoters. Among HSV immediate-early regulatory proteins (ICPs 0, 4, 22, and 27), only ICP0 is capable of activating all kinetic classes of HSV genes. Although the cellular activity expressed in U2OS cells can substitute for ICP0 to enhance viral gene expression, the identities of the specific classes of viral genes activated by the cellular activity were unknown. To determine which kinetic classes of viral promoters are responsive to the cellular activity, transient-expression assays were performed with Vero and U2OS cells. For this purpose, cells were transfected with chimeric CAT constructs containing immediate-early (pIE3CAT-AN6), early (pTKCAT), delayed-early (pgBCAT), and late (pUL10CAT) promoters, and CAT activities were determined.

As shown in Fig. 4, the basal levels of expression of immediate-early, early, and delayed-early promoter/CAT plasmids were significantly lower in Vero cells than in U2OS cells. CAT expression from the late (UL10) promoter was very low in both cell types. Notably, the high basal level of CAT expression from the ICP4 promoter that occurred in U2OS cells was not observed in SAOS2 or HeLa cells (data not shown). Thus, the U2OS cell enhancing activity is unique for at least one viral promoter.

Localization of the *cis*-acting elements in the ICP4 promoter that respond to the cellular activity in U2OS cells. The results of the CAT assays just described indicate that basal expression from immediate-early and early promoters is strongly enhanced in U2OS cells relative to Vero cells. Because ICP4 is a transcriptional activator whose function is required to regulate the expression of all classes of viral genes, and because the promoter of the ICP4 gene has the highest basal level of activity among the several HSV-1 promoters tested in U2OS

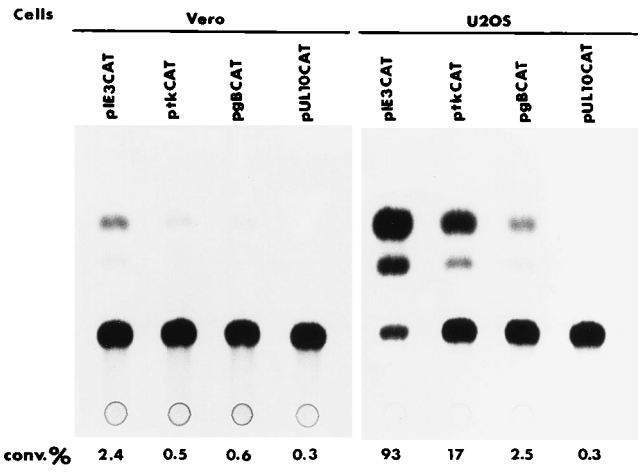

FIG. 4. Gene expression from the promoters of selected classes of HSV-1 genes in Vero and U2OS cells. Vero and U2OS cells were seeded at 2×10^6 cells per 100-mm-diameter dish. Twenty-four hours after being seeded, the cells were transfected with 5 μg of either pIE3CAT (immediate-early promoter), pTKCAT (early promoter), pgBCAT (delayed-early promoter), or UL10CAT (late promoter). At 40 to 44 h posttransfection, cells were harvested, extracts were prepared, and CAT activities were determined.

cells, we attempted to identify the *cis*-acting elements within the ICP4 promoter that respond to the activity in U2OS cells.

As shown in Fig. 5A, pIE3CAT-AN6 contains the entire ICP4 promoter, which includes a variety of recognized *cis*-acting elements. Among these are TAATGARAT motifs, Sp1 binding sites, an NF-III (or OCT) binding site, TATA boxes, an origin of DNA replication (oriS), and a GCGGAAC motif. Previous studies have demonstrated that the TAATGARAT and GCGGAAC motifs play central roles in the induction of ICP4 gene expression by the virion-associated transactivator VP16 (69). To determine whether the cellular activity specified by U2OS cells mediates its enhancing activity through these or other promoter-associated elements in pIE3CAT-AN6, a series of CAT plasmids lacking defined promoter sequences was generated (Fig. 5A). The effects of these deletions on basal levels of CAT expression in Vero and U2OS cells were examined.

The results of CAT assays are shown in Fig. 5B. Although deletion of sequences between the *Nar*I and *Bss*HII sites and between the *Nar*I and *Sph*I sites had little effect on basal levels of CAT activity, deletion of sequences between the *Nar*I and *Sac*II sites abolished the responsiveness of pIE3CAT to the enhancing activity specified by U2OS cells. These observations suggest that the 139-bp region between the *Sph*I and *Sac*II sites in the ICP4 promoter plays a significant role in mediating the high basal level of expression of pIE3CAT in U2OS cells. Notably, deletion of the 139-bp *Sac*II-*Sph*I fragment also reduced the basal level of expression of pIE3CAT in Vero cells. However, the basal level of expression of CAT from pIE3CAT is considerably lower in Vero cells than in U2OS cells.

To confirm the role of the 139-bp region in the basal expression of pIE3CAT-AN6 in U2OS cells, plasmid pIE3CAT-ΔSaS was constructed. pIE3CAT-ΔSaS lacks only the 139-bp region between the *Sph*I and *Sac*II sites in the ICP4 promoter. The results of CAT assays (Fig. 6) demonstrate that, like pIE3CAT-ΔSaN, pIE3CAT-ΔSaS failed to respond to the enhancing activity specified by U2OS cells. These findings confirm the initial observation that the 139-bp region between the *Sph*I and *Sac*II sites in the ICP4 promoter is required for the high basal level of expression of pIE3CAT in U2OS cells. Notably, like pIE3CAT-ΔSaN, pIE3CAT-ΔSaS also exhibited a lower basal level of CAT expression than wild-type pIE3CAT-AN6 in Vero cells (data not shown), suggesting that factors that elevate ICP4 promoter activity in U2OS cells might also be expressed in Vero cells and that the levels and/or activities of these factors are simply higher in U2OS than in Vero cells.

DISCUSSION

The regulation of HSV gene expression involves a complex interplay between cellular and viral factors leading to either productive infection or latency. ICP0 has been shown to play a central role in enhancing viral gene expression following low-multiplicity infection, from infectious viral DNA and from quiescent genomes during reactivation of HSV-1 from latency in vivo and in vitro (8, 34, 43). Despite its long-recognized role in the life cycle of HSV-1, the mechanism of gene activation by ICP0 remains unclear. ICP0 is a 775-amino-acid nuclear phosphoprotein with an apparent molecular mass of 110 kDa. It is associated with chromatin in infected cells and binds to DNA in a manner that is not sequence specific (35, 55). Of the four immediate-early regulatory proteins, only ICP0 is capable of activating all classes of HSV genes as well as a number of heterologous viral and cellular genes and of inducing reactivation of HSV-2 from latency in an in vitro system (8, 26, 27, 30, 31, 34, 43, 51, 52, 58). Recent studies have shown that ICP0 may accomplish one of its regulatory functions through interactions with DNA and the TATA binding protein, TBP (74).

We have shown in this report that a cellular activity specified by U2OS cells can substitute for ICP0 to enhance the plating and replication efficiencies of ICP0 mutants and the de novo synthesis of virus following transfection of infectious viral DNA. Although the identity of the transacting factors that constitute this activity and the mechanism by which the cellular activity accomplishes these effects are unclear, the data presented above demonstrate that the activity involves enhancement of the expression of immediate-early, early, and delayed-early genes. The cellular activity is not induced by HSV-1 infection but apparently exists as a preformed protein(s). The available evidence indicates that the activation of at least one immediate-early gene, that for ICP4, by the cellular activity is promoter specific. Efforts are currently under way to determine whether other responsive genes are also activated through promoter-specific elements.

Cai and Schaffer have identified a cell cycle-specific function in Vero cells that can substitute for ICP0 to activate viral gene expression (9). Specifically, these investigators showed that the plating efficiency of the ICP0 null mutant 7134 is very low on standard 24-h-old Vero cell monolayers. In Vero cells growth arrested in the G_0/G_1 phase and then released from growth arrest, the plating efficiency of 7134 was increased 10- to 15-fold 8 h after release. This enhancement did not occur in cycling cells 24 h after release from growth arrest. Similarly, viral protein synthesis was significantly enhanced in 7134-infected cells at 8 h, but not 24 h, after release. Thus, an activity able to substitute for the transactivating activity of ICP0 is expressed in Vero cells following release from growth arrest in the G_0/G_1 phase of the cell cycle. A similar activity has also been observed in cells of neural origin, NB41A3 cells, in which this or another cellular factor expressed under the same conditions can enhance the basal level of gene expression from HSV immediate-early promoters in transient-transfection assays (59). Of special interest was the observation that the activating activity specified by NB41A3 cells was expressed in nonreplicating cells after release from growth arrest (59), supporting the initial observation of Cai and Schaffer that the activity is expressed only when cells transit the G_0/G_1 phase of the cell cycle and not in continuously cycling cells (9). Al-

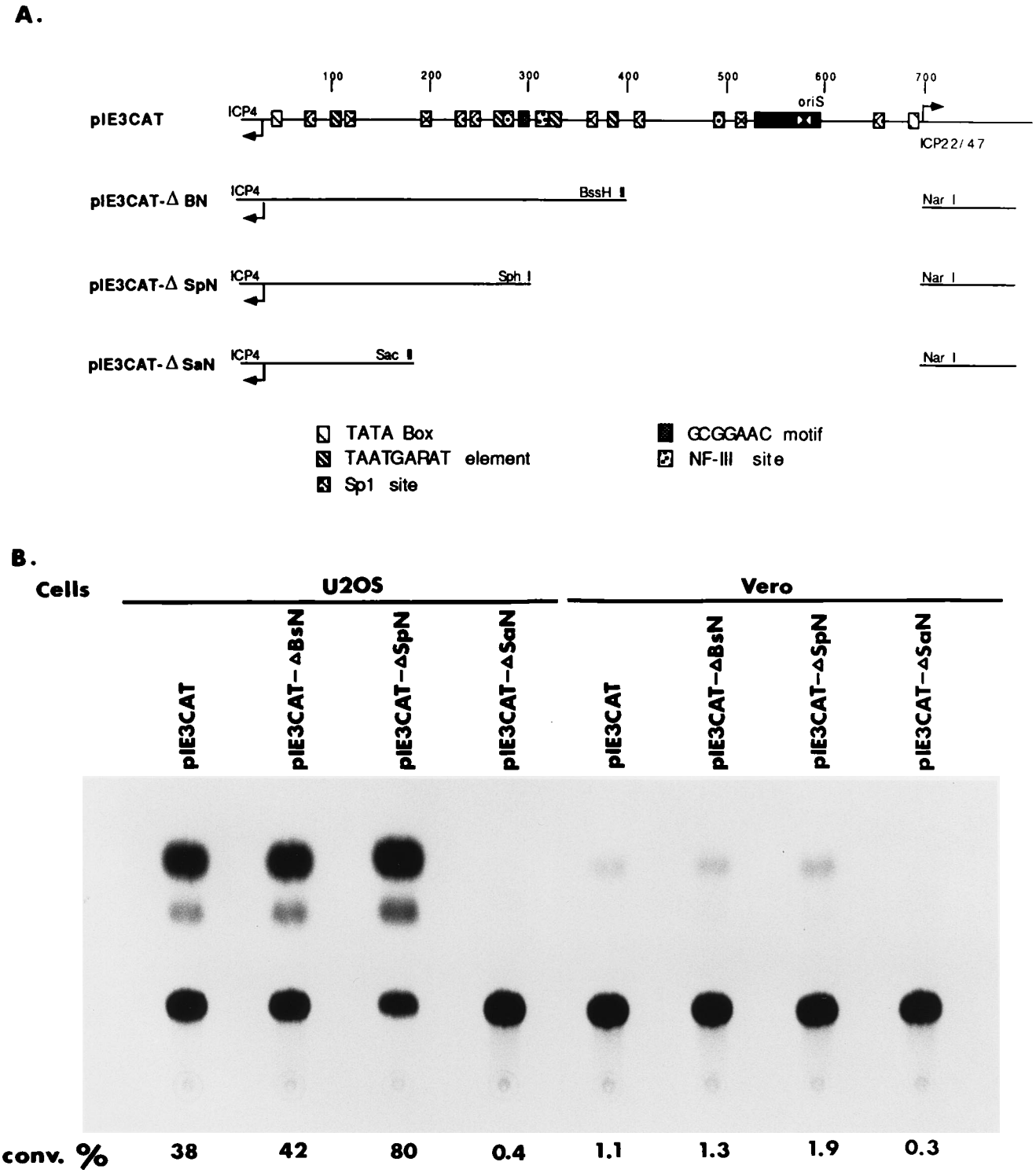

FIG. 5. Identification of the *cis*-acting sequence within the ICP4 promoter that responds to the activity in U2OS cells. (A) Schematic diagrams of the ICP4 promoter and of the mutant plasmids used in these studies. Binding sites for known viral and cellular transactivating proteins within the ICP4 promoter are shown. Relevant restriction sites used in generating mutant plasmids are also indicated; these include *Nar*I, *Bss*HII, *Sph*I, and *Sac*II sites. (B) Twenty-four-hour-old Vero and U2OS cells were transfected with 2.5 μg of the indicated plasmids. Forty-four hours after transfection, the cells were harvested, cell extracts were prepared, and CAT activities were determined.

though the cell cycle-regulated activities specified by Vero and NB41A3 cells share some properties in common with the cellular activity expressed continuously in U2OS cells, it is unclear whether these are the same or different activities. Should we succeed in identifying a specific *cis*-acting element(s) in the promoters of viral genes that respond to the activity in U2OS cells, we could mutate these elements and ask whether responsiveness to the Vero- and NB41A3-associated cellular activities

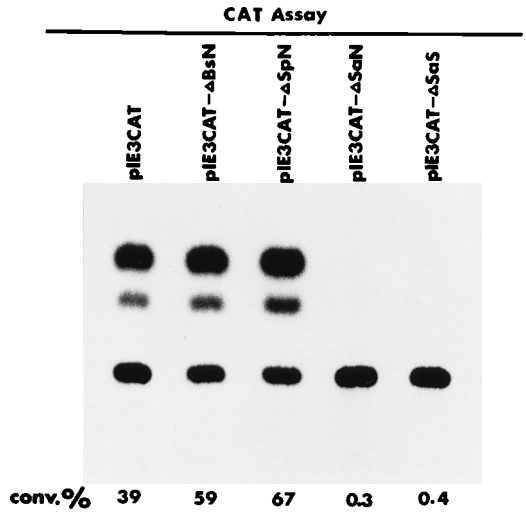

FIG. 6. Effect of deletion of the 134-bp region on the expression of pIE3CAT in U2OS cells. Twenty-four-hour-old U2OS cells were transfected with 2.5 μg of the wild-type and mutant plasmids shown in Fig. 5A or with plasmid pIE3CAT-ΔSaS, which contains a 134-bp deletion between the *Sph*I and *Sac*II sites in the ICP4 promoter. Forty-four hours after transfection, the cells were harvested, cell extracts were prepared, and CAT activities were determined.

is affected by the mutation. In this way it will be possible to determine whether the response elements, and in all likelihood the proteins that bind to them, are the same in all three cell types.

Efforts to identify *cis*-acting elements in the ICP4 promoter that respond to the activity specified by U2OS cells have shown that elements within the 139-bp region between the *Sph*I and *Sac*II sites mediate the high basal level of expression. A number of recognized binding sites for viral and cellular proteins have been identified within this region in the ICP4 promoter (Fig. 5A). Whether any of these elements mediate high-level basal expression from the ICP4 promoter is currently under investigation. Similar attempts to identify target sequences in other viral promoters that respond to the cellular activity are under way.

The observation that a novel activity expressed in mammalian cells can substitute functionally for ICP0 may have important implications both for enhancing the efficiency of infection (especially at low multiplicities) and for understanding the mechanism underlying reactivation of HSV-1 from latency. Our studies have shown that the cellular activity in U2OS cells is not induced by HSV infection and that like ICP0, this activity is able to enhance (i) the expression of HSV-1 immediate-early genes at low multiplicities of infection, (ii) the plating efficiency of an HSV-1 ICP0 null mutant, and (iii) de novo synthesis of virus from infectious viral DNA in vitro. Moreover, the cellular activity is able to promote high-level expression from immediate-early, early, and delayed-early, but not late, classes of HSV promoters in the absence of any viral proteins. Of special interest is the observation that ICP0 functions cooperatively with the cellular activity in U2OS cells to further enhance the de novo synthesis of virus from infectious viral DNA in vitro (Table 3) but also functions to repress very high levels of the cellular activity in virus-infected cells (Fig. 2A). Because immediate-early viral promoters are inactive during latent infection and the cellular activity expressed in U2OS cells can elevate gene expression from immediate-early viral promoters to very high levels, one can speculate that if a similar cellular activity was induced in nondividing, latently infected neurons, it might facilitate viral gene expression leading to reactivation.

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