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Role of Protein Kinase A and the Serine-Rich Region of Herpes Simplex Virus Type 1 ICP4 in Viral Replication

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Efficient expression of herpes simplex virus genes requires the synthesis of functional ICP4, a nuclear phosphoprotein that contains a prominent serine-rich region between amino acids 142 and 210. Residues in this region not only are potential sites for phosphorylation but also are involved in the functions of ICP4. By comparing the growth of a virus in which this region is deleted (*d*8-10) with wild-type virus (KOS) in PC12 cells or PC12 cells that are deficient in cyclic AMP-dependent protein kinase (PKA), two observations were made: (i) the growth of wild-type virus was impaired by 1 to 2 orders of magnitude in the PKA-deficient cells, indicating the involvement of PKA in the growth cycle of herpes simplex virus type 1, and (ii) while the growth of *d*8-10 was impaired by almost 2 orders of magnitude in wild-type cells, it was not further impaired (as was that of wild-type virus) in PKA-deficient cells, implicating the region deleted in *d*8-10 as a possible target for cellular PKA. In trigeminal ganglia of mice, the *d*8-10 mutant virus grew poorly; however, it established latency in nearly 90% of ganglia tested. Studies of the phosphorylation of wild-type and *d*8-10 ICP4 proteins revealed that the serine-rich region is a major determinant for phosphorylation of ICP4 in vivo and that the phosphorylation state could change as a function of the PKA activity. Consistent with this observation, the serine-rich region of ICP4 was shown to be a target for PKA in vitro. While intact ICP4 was readily phosphorylated by ICP4 in vitro, the *d*8-10 mutant ICP4 was not. Moreover, a synthethic peptide representing a sequence in the serine tract that is predicted to be a substrate for PKA was phosphorylated by PKA in vitro, having a K_m within the physiological range. These data suggest that PKA plays a role in viral growth through phosphorylation of one or more sites on the ICP4 molecule.

Infected-cell polypeptide 4 (ICP4) is the major transcriptional regulatory protein of herpes simplex virus type 1 (HSV-1). It is intimately involved in the control of viral gene expression during the course of infection. HSV-1 gene expression is regulated in an ordered fashion (23, 24, 50), such that three phases of gene expression can be distinguished: those of the immediate-early, early, and late genes (24). During this process, ICP4 acts as a negative regulator of immediate-early gene expression and as a positive transactivator of early and late gene expression (6, 12, 30, 47, 48, 61). Because of its role in activation, ICP4 is absolutely required for viral growth (12, 47).

ICP4 is a phosphorylated DNA-binding protein with an apparent molecular mass of 175 kDa (6, 14, 32, 46, 63). It can bind specifically to DNA, and its binding site is relatively degenerate (14, 16, 26, 28, 32, 33, 38–40). ICP4 also appears as three electrophoretic species on sodium dodecyl sulfate (SDS)-polyacrylamide gels (46) and as seven species on two-dimensional isoelectrofocusing gels (1). The fastest-migrating form seen on SDS gels accumulates in the cytoplasm, while the two slower-mobility species are localized in the nucleus (46, 63). The observed differences in mobility are, in part, due to different extents of phosphorylation. The extent of phosphorylation of ICP4 has been shown to affect its DNA-binding properties (39) and potentially its interaction with cellular protein-viral DNA complexes (41). Both serine and threonine residues were found to be phosphorylated in a temperature-sensitive mutant form of ICP4 labeled at the nonpermissive temperature (15). ICP4 can also be ADP-ribosylated (49) or adenylated and guanylated (2) in isolated nuclei, yet the significance of this observation in regard to infected cells has not been established. It is possible that different forms of ICP4 have different activities. Regulation of ICP4 activity may be one mechanism to ensure that viral proteins are produced in the proper quantity and at the proper time during the lytic infection, resulting in the efficient production of infectious progeny. Additionally, such mechanisms may lead to dramatically altered gene expression, resulting in restricted replication or latency.

Clustered regions in the ICP4 primary structure are conserved among the ICP4 homologs of related alphaherpesviruses (4, 21, 37, 60), suggesting the presence of functionally important domains. Genetic analysis of mutant ICP4 polypeptides has identified several such regions of conserved primary sequence that are required for the ICP4 regulatory activities (11, 43, 57). One of these regions, between amino acid residues 263 and 487, specifies the primary structure important for ICP4 DNA-binding activity (42, 57). This DNA-binding domain is clearly of functional importance, as both transactivation and repression mediated by ICP4 require its integrity, and most mutations which destroy the ability of ICP4 to bind to DNA render the protein nonfunctional or render the virus unable to grow (43, 57). Another region, between amino acid residues 142 and 210 (Fig. 1), contains a stretch of 24 residues, 19 of which are serines and 1 of which is threonine. This stretch, termed the serine-rich region, is well conserved among the sequenced alphaherpesviruses and is flanked by seven basic residues on the amino-terminal side and eight acidic residues on the carboxy-terminal side. The serine-rich region has been shown to be important for the induction of early and leaky late gene expression, particularly in mutants lacking the C-terminal sequences (42, 57), and has been genetically implicated as a

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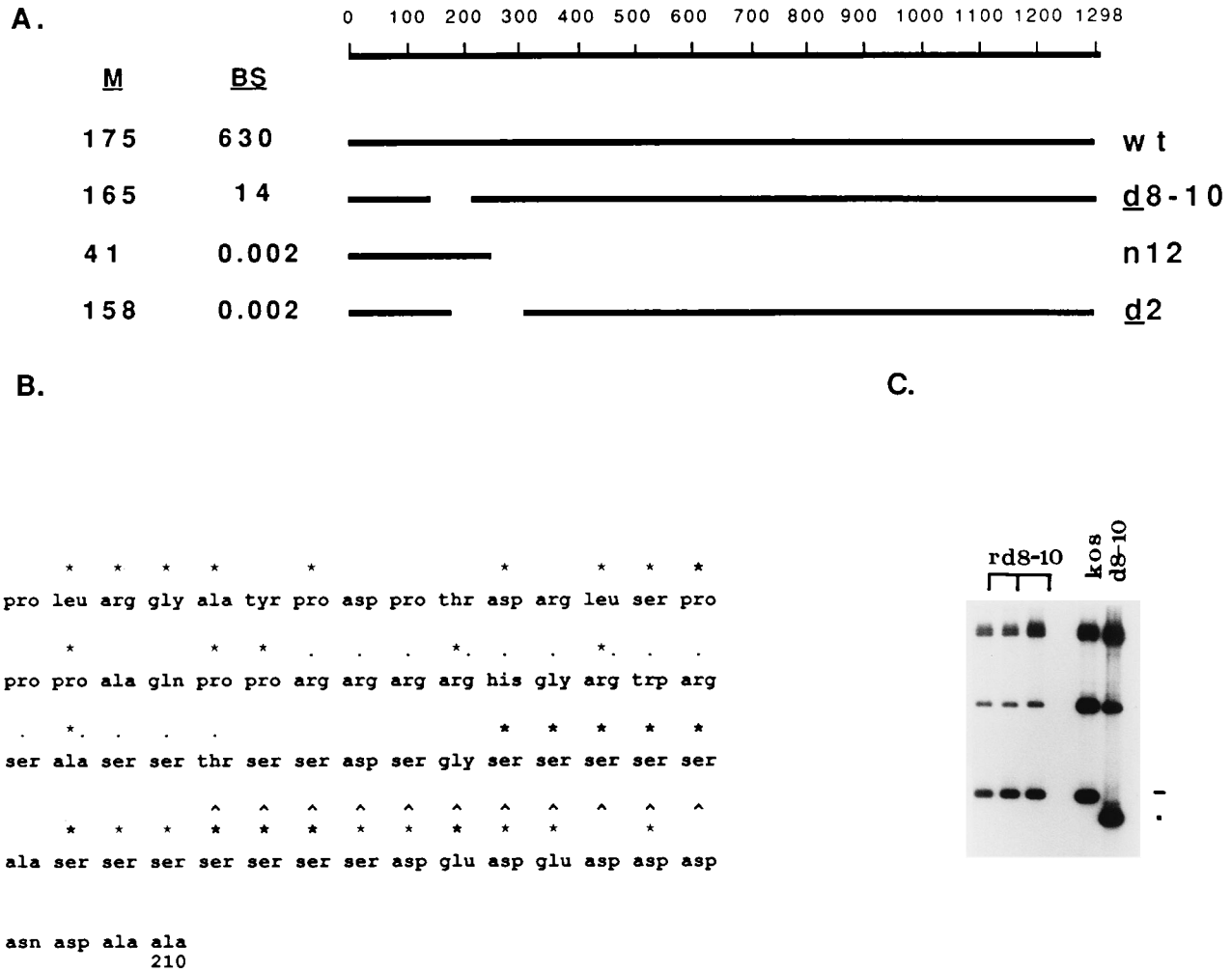


FIG. 1. Structures of ICP4 deletion (d) and nonsense (n) mutations used in this study. (A) The amino acid sequences expressed in the wt and ICP4 mutant viruses are depicted by a solid line. The apparent molecular masses (M) of mutant peptides are shown in kilodaltons, and burst sizes (BS) are shown as PFU per Vero cell for each of the mutants and for the wt virus. For the burst size determination for *d*8-10, Vero cells were infected at an MOI of 5 PFU per cell, harvested at 18 h postinfection, and assayed on E5 cells. The burst sizes of the rest of the mutant viruses were previously determined (11). (B) Amino acid sequence of the deleted region in the *d*8-10 ICP4 protein. The HSV-1 amino acid residues marked with stars are conserved with the ICP4 homologs of varicella-zoster virus (37) or pseudorabies virus (4). The amino acids conserved in all three proteins are indicated by boldface stars. Those marked with dots or carets constitute the consensus sites for PKA or CKII phosphorylation, respectively. (C) Genotypes of *d*8-10 and its rescuant viruses. The indicated viral DNAs (*d*8-10, KOS, and three r*d*8-10) were digested with *Bam*HI, separated by electrophoresis, transferred to a nitrocellulose filter, and then probed with ^{32}P-labeled pKC1 as described in Materials and Methods. Because of the deletion of the serine-rich region (amino acids 142 to 210), the wt 1.84-kb *Bam*HI Y fragment (indicated by a dash on the right) was digested into a smaller fragment (indicated by a dot) in *d*8-10.

target for phosphorylation (11). Computer modeling of the serine-rich region predicts a loop structure (5, 57).

Further examination of the serine-rich domain has revealed another feature of interest: the sequences flanking the polyserine tract constitute two consensus motifs for phosphorylation by cyclic AMP (cAMP)-dependent protein kinase A (PKA) at the N-terminal end or by casein kinase II at the C-terminal end (Fig. 1). The sites for PKA phosphorylation typically contain a cluster of arginines followed by the targeted serine or threonine residues (29, 45), while the sites for casein kinase II phosphorylation generally feature a block of glutamate and/or aspartate following the targeted serines or threonines (13, 45). Thus, it seems possible that ICP4 proteins may interact with these cellular protein kinases, and the resulting phosphorylation may be of functional significance. This study examines the properties and function of ICP4 as a function of the serine-rich region through analysis of a mutant virus expressing a form of ICP4 that has this region deleted. The results demonstrate that cellular PKA is involved in the HSV-1 growth cycle and suggest that phosphorylation of the serine-rich region of the major regulatory protein ICP4 may serve as a target for the action of PKA.

MATERIALS AND METHODS

Virus and cells. The KOS strain of HSV-1 was used as the wild-type (wt) virus (7). The procedures for the propagation and plaque assay of KOS on Vero cells (an African green monkey kidney cell line) were as described previously (12). The HSV-1 ICP4-deficient viruses *n*12 (11), *d*2 (11), and *d*8-10 (57) were propagated on E5 cells, a Vero cell-derived cell line that expresses complementing levels of the wt ICP4 upon infection (8, 11). The primary structures of the ICP4 molecules expressed from these viruses are represented in Fig. 1. PC12 cells (a rat adrenal pheochromocytoma cell line) and the PC12 cell-derived cellular PKA-deficient cell lines A123.7 and AB11 (20) were grown in Dulbecco's modified Eagle's medium (Flow Laboratories) containing 0.45% glucose, 0.03% L-glutamine, and 0.025% $NaHCO_3$ and supplemented with 10% fetal bovine

serum and 5% horse serum in a humidified 10% CO_2 environment. A123.7 and AB11 cells, as well as the parental PC12 cell line, were kindly provided by J. Wagner and D. Ginty (Harvard Medical School, Boston, Mass.). Media were changed every 2 to 3 days, and cells were harvested and subcultured once a week.

Virus yield assays. Approximately 5×10^5 cells in 35-mm-diameter petri dishes were infected at a multiplicity of infection (MOI) of 5 PFU per cell in 0.1 ml for 1 h at 37°C. Following incubation, the monolayers were washed three times with medium and incubated with 3 ml of medium for 18 h. Infected-cell monolayers were then harvested, and clarified lysates were assayed on either Vero or E5 cells to determine the total virus yield. Burst sizes for the individual viral strains were expressed as PFU per infected cell.

Radioactive labeling of viral proteins. The medium for labeling infected-cell proteins with [^{35}S]methionine consisted of methionine-free Dulbecco's modified Eagle's medium supplemented with 2% inactivated fetal bovine serum. The labeling medium for ^{32}P was phosphate-free Dulbecco's modified Eagle's medium supplemented with 2% inactivated fetal bovine serum. Cells to be labeled with ^{32}P were incubated in the phosphate-free medium for 4 to 5 h prior to labeling. During radiolabeling, the cells were incubated at 37°C in 2 ml of the appropriate medium containing either [^{35}S]methionine (20 μCi/ml) or carrier-free $^{32}P_i$ (50 μCi/ml) (New England Nuclear Corp., Boston, Mass.). The time period for ^{32}P labeling was normally 2.5 to 5.5 h postinfection; it was 6 to 9 h postinfection in cycloheximide reversal experiments and 2 to 12 h postinfection for the purification of the radiolabeled ICP4. At the end of the labeling, the cells were washed four times in either phosphate-buffered saline (PBS) or Tris-buffered saline containing both protease inhibitors TLCK (*N*α-*p*-tosyl-L-lysine chloromethyl ketone) (0.1 mM) and leupeptin (0.5 mg/ml) and phosphatase inhibitors sodium orthovanadate (0.1 mM) and sodium pp_i (5 mM). The cells were then scraped into the solution and stored as decanted pellets at −80°C. For cycloheximide reversal experiments, 10^6 Vero cells were preincubated for 60 min in phosphate-free medium containing cycloheximide (100 μg/ml) and then infected with the wt or mutant viruses at an MOI of 10 PFU per cell. After a 1-h adsorption period, phosphate-free medium containing 100 μg of cycloheximide per ml was added, and the cultures were incubated at 37°C for an additional 5 h. The cells were then washed three times with phosphate-free medium containing 10 μg of actinomycin D per ml and incubated with 100 μCi of $^{32}P_i$ in 2 ml of phosphate-free medium plus 10 μg of actinomycin D per ml for an additional 3 h. After incubation, the cells were washed with ice-cold PBS containing 0.1 mM TLCK and harvested directly in SDS sample buffer.

Purification of ^{32}P-labeled ICP4. Both wt and mutant ICP4 proteins were isolated as previously described (26) with minor modifications. Briefly, approximately 2×10^8 Vero cells were infected with wt of *d*8-10 virus at an MOI of 10 PFU per cell and then labeled with 0.5 mCi of carrier-free ^{32}P from 2 to 12 h postinfection. The cells were harvested and subjected to homogenization for the isolation of nuclei as previously described (26). The nuclei were then lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 0.5 M KCl, 2% Nonidet P-40, and 0.1 mM TLCK. The lysate was cleared by centrifugation for 1 h at 45,000 rpm in an SW50.1 rotor at 4°C. The supernatant was saved as the nuclear extract and subsequently fractionated on the basis of the size in a manner similar to that reported previously (55). The extract was applied to a fast protein liquid chromatography (FPLC) gel filtration column (52 by 2 cm) packed with Superose 6 (Pharmacia, Piscataway, N.J.), equilibrated with CB0.5 buffer {20 mM Tris-HCl [pH 8.0], 0.5 M KCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 0.01% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate [CHAPS], 0.1 mM TLCK}, and run at a rate of 0.2 ml/min. Fractions were assayed by dot immunoblot (26). ICP4-containing fractions were pooled and further fractionated by chromatography over a 1-ml Mono Q anion-exchange FPLC column (Pharmacia) at a rate of 1 ml/min and eluted with a 50 mM to 0.5 M KCl gradient. Again, the peak fractions were pooled and further purified on a specific DNA-binding affinity column which was constructed by using the oligonucleotides previously described (26) by the method of Kadonaga and Tjian (27). After this procedure, a single polypeptide band was observed in Coomassie blue-stained SDS-polyacrylamide gels.

In vitro phosphorylation reactions. (i) In vitro phosphorylation of purified ICP4 proteins with PKA. Approximately 20 ng of the purified ICP4 protein was added to a solution containing 10 mM Tris (pH 7.2), 10 mM $MgCl_2$, 50 mM NaCl, 10 mM dithiothreitol, and 20 mM [γ-^{32}P]ATP (0.15 mCi). The phosphorylation reaction was initiated by adding 60 U of PKA type I catalytic subunit purified from bovine heart (Sigma Chemical Co; 1,000 U/0.016 mg of protein). Reaction mixtures were incubated at 30°C for 30 min, and reactions were stopped by addition of SDS sample buffer. Equal portions of the reaction mixtures were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose sheet for exposure to Kodak X-AR film and for staining with an ICP4-specific antibody.

(ii) In vitro phosphorylation of a synthetic peptide. A peptide containing a potential PKA site in the serine-rich region of ICP4 (RRRRHGRWRPSASST, residues 165 to 179) was synthesized on an Applied Biosystems model 430A automated peptide synthesizer and purified by high-pressure liquid chromatography. Amino acid analysis confirmed the authenticity of this peptide, termed ICP4tide. ICP4tide (100 μM) or Kemptide (LRRASLG; Sigma), a commercially available substrate for PKA (100 mM), was then incubated at 30°C in a solution containing 24 mM MES (morpholineethanesulfonic acid) (pH 7.0), 60 mM ATP, and 5.6 nM [^{32}P]ATP (300 to 600 Ci/mmol). Each reaction was subsequently initiated by adding 80 U of PKA type I catalytic subunit purified from bovine heart (Sigma; 1,000 U/0.016 mg of protein) to a final volume of 52.5 μl. Following incubation for 4 min, 20 μl of the reaction mixture was spotted onto P81 phosphocellulose strips, and phosphopeptides were quantitated after three washes in 10 μl of 75 mM phosphoric acid as previously described (54). For kinetic studies, the concentration of ICP4tide was varied (see Fig. 9). ICP4tide was also phosphorylated in a manner similar to those described below for phosphorylation of purified ICP4 proteins with PKA except that synthetic peptide replaced the purified ICP4 and the ATP concentration was increased to 60 mM. Such phosphorylated peptides were resolved in an SDS–27% polyacrylamide gel.

Protein analyses. (i) SDS-PAGE. The harvested cell pellets were lysed in SDS-containing sample buffer (62.5 mM Tris-HCl [pH 6.8], 2.3% [wt/vol] SDS, 10% glycerol, 5.0% [vol/vol] β-mercaptoethanol, 0.00125% [wt/vol] bromophenol blue). The extracted viral polypeptides or purified ICP4 proteins suspended in SDS sample buffer were analyzed with SDS-polyacrylamide gels cross-linked with diallytartardiamide as described by Laemmli (34) with the modifications of Gibson and Roizman (19). When appropriate, the separated polypeptides were either stained with 0.25% Coomassie brilliant blue (Eastman; R250) or silver stained according to the protocol of the manufacturer (Bio-Rad Laboratories, Richmond, Calif.) and/or exposed to Kodak X-AR film.

(ii) Western immunoblot analysis. Separated polypeptides were electrophoretically transferred from gels onto nitrocellulose filters in a transfer apparatus according to the procedures of the manufacturer (Bio-Rad). ICP4 polypeptides were visualized by probing the filters with a 1:500 dilution of N15 polyclonal antibody directed against the N-terminal half of the ICP4 molecule (59). The primary antibody was detected by a 1:7,500 dilution of secondary anti-rabbit immunoglobulin G conjugated with alkaline phosphatase as indicated by the manufacturer (Promega Biotech, Madison, Wis.).

(iii) 2-D IEF. Two-dimensional isoelectrofocusing gel electrophoresis (2-D IEF) was performed with the Bio-Rad minigel system as prescribed by the manufacturer with some modifications. Briefly, the radiolabeled cell pellets were lysed, and the released polypeptides were solubilized in a urea-ampholine solution (pH 4 to 9 or pH 3 to 10) containing 2% CHAPS (Bio-Rad). The tube gels were preelectrophoresed at 200, 300, and 400 V for 15 min each and then washed carefully with chamber solutions (the upper chamber solution is degassed 20 mM NaOH, while the lower chamber solution is 10 mM H_3PO_4). Approximately 1.5 to 6 μg of solubilized polypeptides were loaded onto the tube gels, and the electrophoresis was conducted from the base to the acid for a total of 5,000 V-h. After equilibration in SDS sample buffer, the tube gel was placed horizontally over a stacking gel and overlaid with SDS sample buffer containing 1% agarose to fix the tube gel for electrophoresis in the second dimension. Electrophoresis was performed essentially as described above for SDS-PAGE. After electrophoresis, the gel was fixed in a solution of H_2O-methanol-acetic acid (6:3:1), dried, and exposed to Kodak X-AR film for autoradiography.

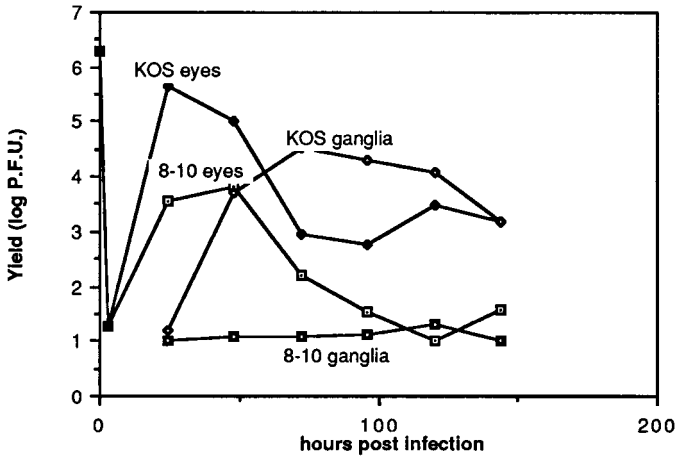
Marker rescue and viral DNA analysis. Approximately 10^6E5 cells were cotransfected with 1 μg of *d*8-10 viral DNA and 1 μg of *Bam*HI-digested plasmid containing a *Bam*HI Y fragment of the wt HSV-1 DNA by the calcium phosphate coprecipitation procedure (9). Because the genome of *d*8-10 contains a specific deletion of the serine-rich region in both copies of the ICP4 gene and the deletion results in a partial temperature-sensitive mutant phenotype, plaque assays of the harvested viral particles were conducted at both 39.6 and 37°C. Normal-size plaques at 39.6°C, indicative of reintroduction of wt sequences into the ICP4 gene, were isolated and subsequently plaque purified. The identity of the ICP4 allele was confirmed by Southern blot analysis (10).

Animal studies. The mouse eye model was used to compare the in vivo growth characteristics of *d*8-10 and KOS. The procedures for the inoculation of the mouse corneas, the assays for virus replicating at the site of inoculation and in the trigeminal ganglia, and the determination of reactivatable latent virus were as previously described (35).

RESULTS

Contribution of the serine-rich region of ICP4 in vitro and in vivo. To investigate the role of the highly conserved serine-rich region, a mutant virus, *d*8-10 (57), from which the entire serine-rich region between amino acids 142 and 210 of ICP4 has been deleted (Fig. 1A and B), was studied. When the growth characteristics of *d*8-10 were examined in Vero cells, it was found that the virus was able to form plaques, although they were somewhat smaller than those of KOS, indicative of a reduced virus yield. To quantify the growth deficiency of *d*8-10, both Vero and E5 cells were infected with KOS and *d*8-10 at an MOI of 2.5 PFU per cell, and viral yields were determined by plaque assay on E5 cells. As shown in Table 1, KOS and *d*8-10 had comparable yields in E5 cells, although the burst size of *d*8-10 was significantly reduced in Vero cells, indicating that the region deleted in *d*8-10 is important for viral growth and that functional ICP4 expressed in E5 cells can

A.



B.

Reactivation
(ganglia reactivated/ total ganglia)

Virus	no addition	+ DMSO
KOS (wild-type)	12/12 (100%)	9/9 (100%)
d8-10	6/38 (16%)	8/9 (89%)

FIG. 2. Growth of *d*8-10 in mice. To examine the growth of HSV-1 in an in vivo latency model as a function of the region deleted in *d*8-10, mice were infected with KOS and *d*8-10 on the corneas with an input dose of 2×10^6 PFU per eye. (A) Viral growth in both the eyes and the trigeminal ganglia of infected mice was monitored as a function of time postinfection by assay of PFU. (B) At 30 days postinfection, ganglia were explanted and cocultivated with E5 cells in the presence or absence of dimethyl sulfoxide (DMSO) to determine if latent *d*8-10 was present.

complement the growth deficiency of *d*8-10. These results were consistent with the observations made by Paterson and Everett for a similar virus (44).

To determine if the growth deficiency was due solely to the deletion of the serine-rich region, we conducted a marker rescue experiment to reintroduce the deleted region into the ICP4 gene of the *d*8-10 genome. Monolayers of Vero cells were cotransfected with equal amounts of infectious *d*8-10 viral DNA and a *Bam*HI-digested plasmid containing the 1.84-kb *Bam*HI Y fragment of HSV-1 DNA. The *Bam*HI Y fragment possesses the sequence across the deleted codons. The *d*8-10 mutant virus forms significantly smaller plaques at 39.6°C. Normal-size plaques at 39.6°C resulting from the plating of the transfection progeny on Vero cells were isolated, and the identity of the ICP4 allele was confirmed by Southern blot analysis (Fig. 1C). A representative rescuant was amplified and designated r*d*8-10. Vero cells and E5 cells were then infected with KOS, *d*8-10, and r*d*8-10, and the plaquing efficiencies and burst sizes were determined as described above and are summarized in Table 1. As expected, r*d*8-10 had a burst size similar to that of wt virus. Taken together, the complementation of *d*8-10 in E5 cells (Table 1) and the marker rescue data strongly suggested that the growth deficiency of *d*8-10 was due solely to the deletion of the serine-rich region. Therefore, although the serine-rich region of ICP4 is not essential for the viral growth in tissue culture, it does play a significant role in determining viral yield.

TABLE 1. Growth of *d*8-10 in Vero and E5 cells[a]

Cells	Virus	Titer (PFU/ml)	Burst size[b]
Vero	KOS	2.15×10^8	977
	*d*8-10	3.10×10^6	14
	r*d*8-10	1.85×10^8	841
E5	KOS	1.95×10^8	886
	*d*8-10	1.40×10^8	636
	r*d*8-10	1.30×10^8	591

[a] Vero and E5 cells were infected with the indicated virus at an MOI of 5 PFU per cell. At 18 h postinfection, the infected cells were scraped into the medium, frozen and thawed, and briefly sonicated, and the resulting suspension was clarified by low-speed centrifugation. The clarified suspension was assayed for PFU on E5 cells.

[b] Calculated as the total number of PFU per infected cell.

The serine-rich region is not essential for viral growth in tissue culture, but because of the conservation of this region in the neurotropic herpesviruses, it seemed reasonable to explore the possibility that this region may be important for the replicative cycle of HSV in vivo. Accordingly, *d*8-10 was introduced into mice by infection of scarified corneas with an input dose of 2×10^6 PFU per eye. Viral growth at the site of inoculation

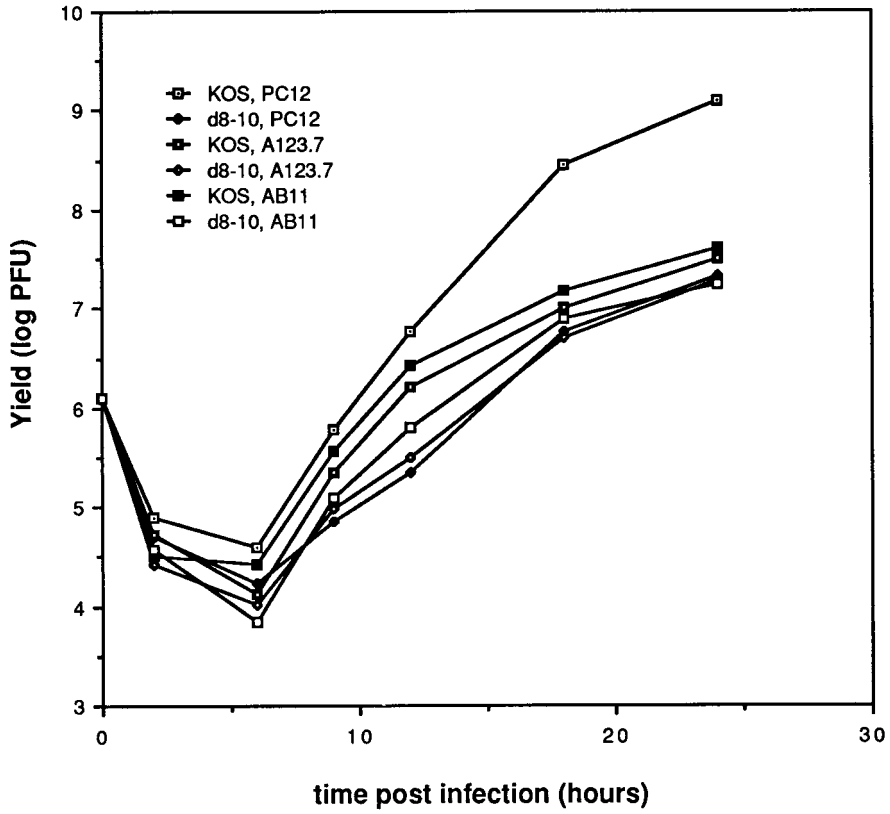


FIG. 3. Single-step growth curves of wt and *d*8-10 viruses. Nerve growth factor-differentiated PC12 cells and PKA-deficient cells were infected in parallel in 35-mm-diameter plates with KOS and *d*8-10 at an MOI of 2.5 PFU per cell. At the indicated times, the cells were scraped into the medium and sonicated, and total virus was determined by plaque assay on E5 cells.

and in trigeminal ganglia was then monitored by eye swabs and by assay of PFU in ganglial homogenates as a function of time postinfection. Figure 2A shows that *d*8-10 was able to grow, but less efficiently than KOS, in the eyes of the mice. This was consistent with the relative growth properties of KOS and *d*8-10 in cultured cells (Table 1). However, in the trigeminal ganglia, the growth of *d*8-10 was even more substantially impaired, suggesting that the serine-rich region of ICP4 is more important for growth in sensory neurons. The growth defect of *d*8-10 in the ganglia provided a basis for the conservation of the serine-rich region in the related alphaherpesviruses. The intact ganglia were also explanted at 30 days postinfection and cocultivated with E5 cells to determine the ability of *d*8-10 to establish latency and reactivate. Figure 2B shows that 16% of the explanted ganglia from mice previously infected with *d*8-10 reactivated on their own, and 89% of them could be reactivated in the presence of dimethyl sulfoxide, a reagent that has been widely used to enhance the sensitivity of the reactivation process (62). Therefore, *d*8-10 was able to establish latency despite the apparent neuronal growth defect.

The serine-rich region of ICP4 has been genetically implicated as a site for phosphorylation that is important for ICP4 activity and HSV growth. This raised the possibility that one or several kinases may regulate the activity of ICP4 and growth of HSV by altering the modification state of the serine-rich region. Cellular PKA is a ubiquitous serine/threonine-specific kinase, and an ICP4 temperature-sensitive mutant protein is predominantly phosphorylated at serines and threonines (15). Therefore, were studied the possible involvement of PKA in ICP4 phosphorylation and HSV growth. In the inactive state, PKA consists of a complex of two regulatory subunits and two catalytic subunits. Binding of cAMP alters the conformation of the regulatory subunits, causing them to dissociate from the complex. The released catalytic subunit are thereby activated to phosphorylate a variety of protein substrates that contain specific consensus motifs for PKA. Two PKA-deficient cell lines, AB11 and A123.7, have been described; these cell lines were derived from PC12 cells and created by transformation with a gene containing different point mutations in the regulatory subunits (20). These subunits cannot bind cAMP, and as a consequence, the catalytic subunits cannot be released and PKA remains inactive. The growth of *d*8-10 relative to that of wt virus in parental PC12 cells and PKA-deficient cells was tested in a single-step growth experiment. The results (Fig. 3) illustrate that although both KOS and *d*8-10 were able to grow in PC12 cells, the yield of *d*8-10 was significantly lower (20- to 65-fold less) than that for KOS over the time course of the experiment. This was consistent with the observations with Vero cells (Table 1) and BHK cells (44). Interestingly, when the two different PKA-deficient cell lines were used for infection, the growth of the wt was reduced to the level observed (1 to 2 orders of magnitude) with the mutant *d*8-10 on normal PC12 cells. However, the growth of *d*8-10 was not further impaired in PKA-deficient cells relative to the growth in PC12 cells, implying that the deletion of the serine-rich region reduces the importance of PKA activity for *d*8-10 growth. This provides a connection between the serine-rich region of ICP4, the activity of cellular PKA, and HSV-1 growth. A simple explanation would be that the serine-rich region is a functional target for cellular PKA. We also utilized a third PKA-deficient

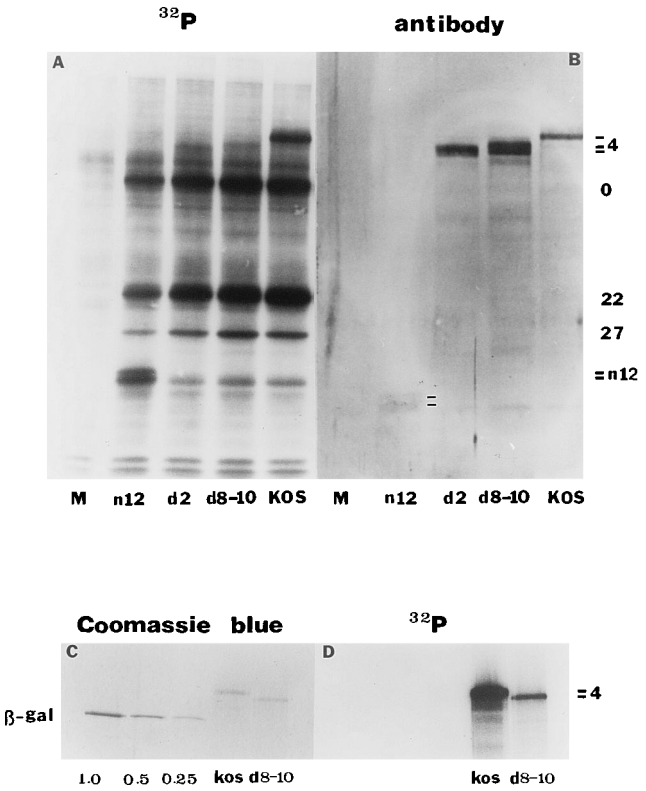


FIG. 4. Phosphorylation of ICP4 as a function of the serine-rich region. (A) Vero cells were infected with the indicated ICP4 mutant viruses, or the wt virus, at an MOI of 10 PFU per cell and metabolically labeled with ^{32}P in a cycloheximide reversal experiment. Whole-cell lysates were solubilized in SDS, electrophoretically separated on a 9% polyacrylamide gel, and transferred onto nitrocellulose filter paper. The filter was exposed to X-AR5 film to generate the autoradiogram shown. M, markers. (B) Following the exposure, the filter was probed with antibody against ICP4 and processed as described in the text. (C and D). ^{32}P-labeled ICP4 protein purified from KOS- and *d*8-10-infected Vero cell extracts were electrophoretically separated in an SDS–9% polyacrylamide gel also containing 1.0, 0.5, or 0.25 μg of β-galactosidase (β-gal). The gel was stained with Coomassie blue, dried, and exposed to X-AR5 film. Shown are a photograph of a stained gel (C) and the autoradiographic images of the same gel (D). The numbers on the right represent the positions (ICP designations) of ICP4 proteins and the other immediate-early ICPs.

cell line, A126, which was isolated following nitrosoguanidine mutagenesis to ensure that the growth difference was not due to the cell clonal variation. The use of these cells yielded similar results (64).

Phosphorylation of the serine-rich region. Previous studies have shown that ICP4 exists in cells as multiple forms possibly due to different phosphorylation states (46, 63). Genetic analysis has implicated the serine-rich region in the ICP4 molecule as one of the phosphorylation sites (11). Using different ICP4 mutant viruses (Fig. 1A), we have analyzed the phosphorylation patterns of the corresponding ICP4 polypeptides by SDS-PAGE. Vero cells infected with wt or ICP4 mutant viruses were metabolically labeled with $^{32}P_i$ under cycloheximide reversal conditions. The crude cell extracts were separated by SDS-PAGE and subsequently transferred onto a nitrocellulose sheet. The autoradiographic images of the labeled bands on the blot were made on Kodak X-AR film (Fig. 4A) prior to visualization of the ICP4 polypeptides with an ICP4-specific antibody to indicate the amount of protein applied to each lane (Fig. 4B). This order of analysis was necessary because the labeled phosphate groups on the ICP4 polypeptides can be removed by alkaline phosphatase conjugated with the secondary antibody. As shown in Fig. 1A, the *n*12 nonsense mutant virus (11) produced a truncated ICP4 molecule just long enough to include the serine-rich region. The stop codon in the *n*12 ICP4 gene is at codon 251. The ICP4 polypeptides expressed by *n*12 were highly phosphorylated and displayed several related migrating forms (Fig. 4A). The *d*2 and *d*8-10 deletion mutant viruses specify ICP4 polypeptides lacking defined regions of the serine tract (Fig. 1A). Despite the presence of greater quantities of the *d*2 and *d*8-10 ICP4 relative to the wt (fig. 4B), the extent of phosphorylation of the *d*2 and *d*8-10 ICP4 polypeptides was dramatically reduced compared with that of the wt ICP4 and the *n*12 ICP4 (Fig. 4A).

To quantitatively examine the differences in phosphorylation between the ICP4 proteins with or without the serine-rich region, ^{32}P-labeled ICP4 proteins were purified from KOS- and *d*8-10-infected Vero cell extracts. The amounts of purified proteins were then quantified on SDS-polyacrylamide gels by using 1, 0.5, and 0.25 μg of β-galactosidase as standards. The gel was stained with Coomassie brilliant blue (Fig. 4C) to indicate the amounts of the *d*8-10 and the KOS ICP4 proteins. The same gel was also exposed to Kodak X-AR film (Fig. 4D), and the autoradiographic bands were scanned by densitometry. When similar amounts of the ICP4 proteins were examined, the amount of phosphorylation on *d*8-10 ICP4 was 6- to 10-fold less than that on wt ICP4. These analyses strongly suggested that the serine-rich region is a major determinant for phosphorylation of ICP4. There are also other phosphorylation sites in the molecule, because when this region was deleted, as in *d*8-10, the ICP4 was still phosphorylated, albeit to a much lesser extent.

To further examine phosphorylation differences between the ICP4 proteins with or without the serine-rich region, we examined the ICP4 molecules by 2-D gel electrophoresis. Vero cells infected with wt or ICP4 mutant viruses were labeled with $^{32}P_i$ from 2.5 to 5.5 h postinfection. The whole-cell lysates were solubilized in a urea-containing ampholine solution and electrophoretically separated on acrylamide gels in the first dimension according to the corresponding points of isoelectrofocus (pIs) and in the second dimension according to relative molecular weights (Fig. 5). The identities of labeled ICP4 proteins were confirmed by Western blots (data not shown). The wt ICP4 was heavily phosphorylated in Vero cells (Fig. 5A), and the phosphoproteins were highly heterogeneous, with pIs ranging from approximately 7.8 to 5.5, and formed a series of at least eight spots (with ^{35}S-labeled cell extracts, the 2-D IEF provides a better resolution of different species of the ICP4 proteins). The polypeptides in these spots appeared to differ in charge rather than in apparent molecular weight, suggesting that they may be generated either by differential modifications or by the differential stabilities of phosphate groups on each molecule. The distribution of *d*8-10 isoforms was shifted toward more-basic zones between pI 8.1 and 5.9 (Fig. 5B). This is consistent with the previous 1-D gel analysis showing that the *d*8-10 ICP4 protein was underphosphorylated relative to the wt (Fig. 4C). In contrast, the *n*12 ICP4, which retains the serine-rich region and is extensively phosphorylated (Fig. 4A and B), is very acidic, with pIs ranging from 3.8 to 4.4 (Fig. 5C). Thus, the results from the 2-D IEF analysis further indicated that the serine-rich region of ICP4 could serve as a major site for phosphorylation or could promote phosphorylation of other regions of the molecule, or both. Like the wt ICP4, the mutant ICP4 polypeptides were also heterogeneous, and the polypeptides in these spots differed in charge. It should be pointed out that the measured pIs varied slightly from sample to sample,

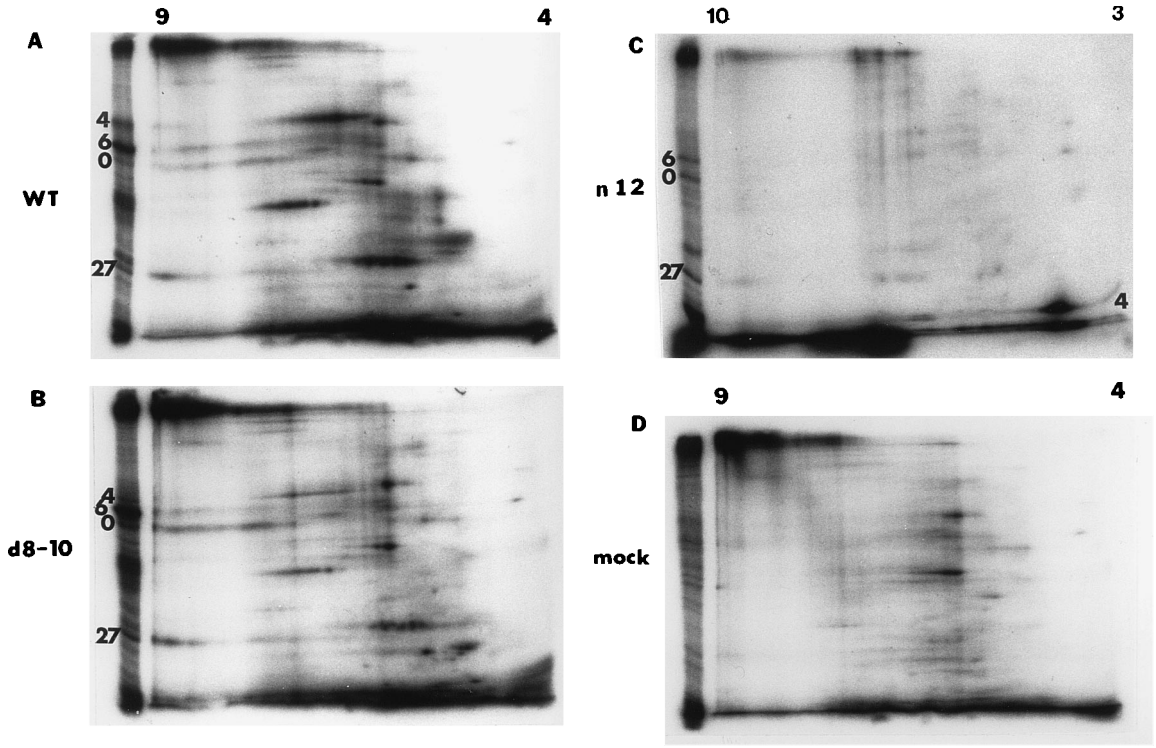


FIG. 5. 2-D IEF gel analysis of ^{32}P-labeled proteins in infected cells. Vero cells infected with wt virus and the indicated mutant viruses were labeled with $^{32}P_i$ from 2.5 to 5.5 h postinfection. The whole-cell lysates were subjected to 2-D IEF. Electrophoresis in the first dimension was from base to acid on a scale of pH 9 to 4 (A, B, and D) or pH 10 to 3 (C). (A to C) Autoradiographic images of labeled viral polypeptides (ICPs) expressed in wt (KOS) virus-infected cells (A), in *d*8-10 virus-infected cells (B), and in *n*12 virus-infected cells (C). See Fig. 1A for the primary structures of *n*12 and *d*8-10 relative to that of KOS. (D) Autoradiographic image of a mock-infected cell extract. On the left of each panel is a 1-D SDS-PAGE gel of the appropriate extract, labeled with the appropriate ICP numbers.

possibly because of the stabilities of phosphate groups on the molecules.

To more closely examine the effect of PKA on the modification of the serine-rich region of ICP4, we examined the ICP4 from PKA-deficient PC12 cells by 2-D gel electrophoresis. The parental PC12 and PKA-deficient cells infected with KOS or *d*8-10 were labeled with $^{32}P_i$ or [^{35}S]methionine as described in Materials and Methods. The resulting cell lysates were subjected to 2-D IEF to examine the phosphorylation of viral proteins as a function of the presence of PKA (Fig. 6). The wild-type ICP4 proteins in PC12 cells again exhibited great heterogeneity, with pIs ranging roughly from 7.7 to 5.8 (Fig. 6C), and formed a series of at least eight spots clearly detected by [^{35}S]methionine radioactivity (Fig. 6A). These results were basically consistent with the data in Fig. 5A and with the data obtained by Ackermann et al. (1). The number of actual ICP4 species might be underestimated because of the limitations of the detection method and the insoluble material at the origin. In comparison, the distribution of different phosphorylated species of the wt ICP4 proteins from PKA-deficient cells shifted toward higher pIs between approximately 7.8 and 6.3, and certain relatively acidic spots were missing (Fig. 6C and D). These results were consistent with the notion of the addition of fewer phosphate molecules onto the ICP4 protein when the activity of PKA is repressed.

[^{35}S]methionine-labeled cell extracts from *d*8-10-infected PC12 cells and PKA-deficient cells were also analyzed by 2-D IEF. Because of the low level of phosphorylation of *d*8-10 ICP4 and the decreased resolution of ^{32}P-labeled proteins on IEF, only the ^{35}S-labeled *d*8-10 ICP4 gave interpretable results. Figure 7A and B show the autoradiographic images of *d*8-10-infected cellular and viral protein patterns from PC12 cells and PKA-deficient cells, respectively. No difference in the phosphorylation states of the ICP4 proteins was apparent. Figure 7C and D are the autoradiographic images of the mock-infected cellular protein patterns from both cell types. Therefore, the 2-D gel analysis strongly suggested that the cellular PKA was involved in the phosphorylation of ICP4 and that the serine-rich region may serve as an in vivo target for PKA.

In vitro phosphorylation of ICP4 by PKA requires the serine-rich region. To demonstrate directly that PKA can phosphorylate ICP4, we incubated native ICP4 proteins purified from cells infected with wt or mutant *d*8-10 virus with the catalytic subunits of PKA. The reaction products were resolved by SDS-PAGE and transferred to a nitrocellulose filter paper for autoradiography (Fig. 8A) and Western blot analysis (Fig. 8B). The wt ICP4 protein was phosphorylated strongly by PKA (Fig. 8A, lane 2), while with the same amount of protein used (Fig. 8B, lanes 2 and 3), *d*8-10 ICP4 showed little, if any, phosphorylation by PKA (Fig. 8A, lane 3). Lane 4 of Fig. 8A contained only purified PKA. Several bands were phosphorylated, and these were possibly contaminants associated with PKA. Thus, ICP4 containing the serine-rich region is a substrate for PKA in vitro, and the serine-rich region greatly stimulates phosphorylation by PKA.

Examination of the primary amino acid sequences of the ICP4 proteins revealed a consensus motif for PKA phosphorylation in the serine-rich region. Accordingly, a peptide, named ICP4tide (RRRRHGRWRPSASST, residues 165 to 179), corresponding to this potential site was synthesized and incubated

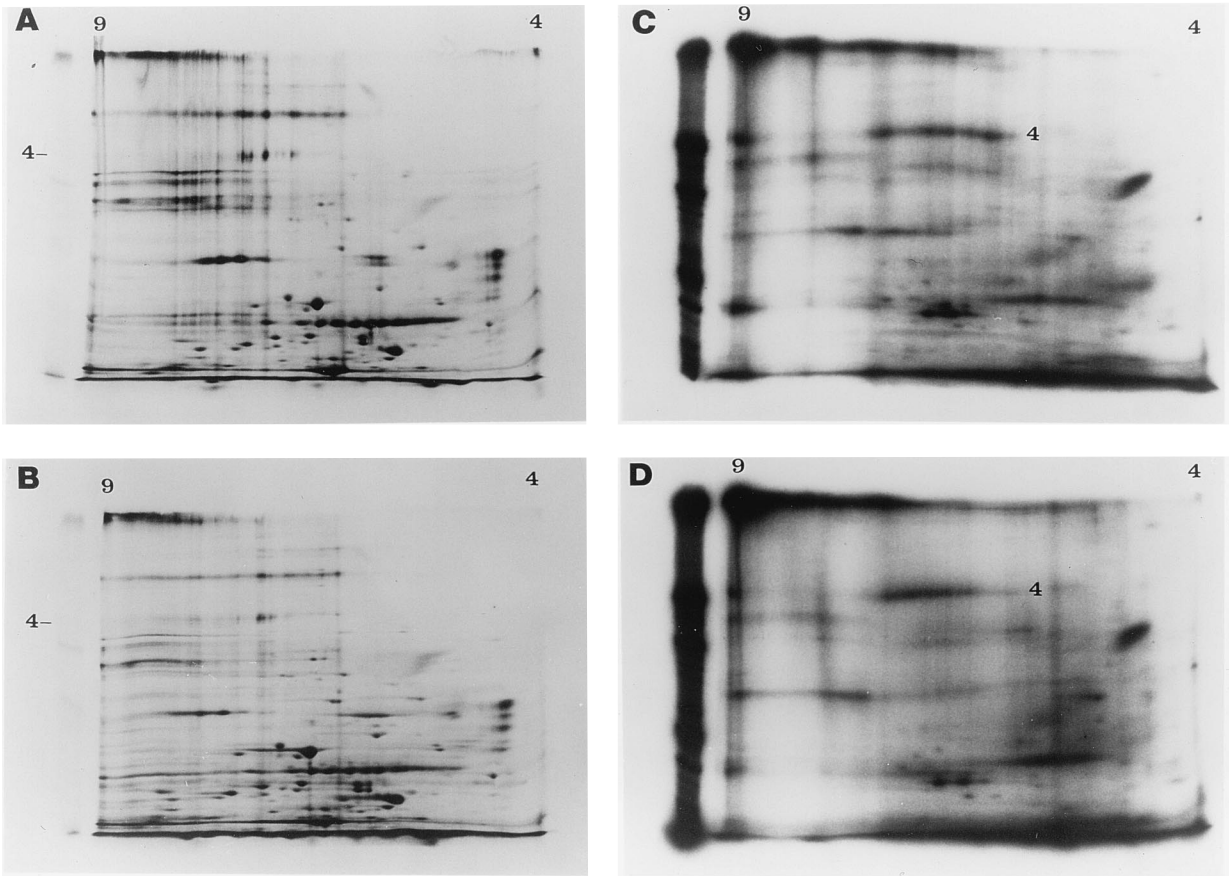


FIG. 6. 2-D IEF of KOS-infected PC12 and PKA-deficient cell extracts. PC12 cells (wt) (A and C) and PKA-deficient cells (A123.7) (B and D) were infected with KOS and labeled with ^{35}S (A and B) or ^{32}P (C and D) from 2.5 to 5.5 h postinfection. The whole-cell lysates were subjected to 2-D IEF gel analysis, and autoradiographic images of the expressed ICPs are shown. The heterogeneous ICP4 polypeptides are indicated by number.

in vitro with purified type I catalytic subunits of PKA and [γ-^{32}P]ATP. Kemptide (LRRASLG), a commercially available substrate for PKA, was used as a positive control. Compared with Kemptide, ICP4tide was also strongly phosphorylated by PKA (Table 2). To determine the K_m values of these reactions, we incubated different concentrations of ICP4tide with [γ-^{32}P]ATP and a fixed amount of PKA catalytic subunits (80 U each) for 4 min at 30°C. Figure 9 shows the velocity-substrate plot of the resulting data, and from the Lineweaver-Burk representation of these data, the K_m was determined as described previously (29). The K_m for Kemptide was 9 μM in our system (Table 2), which was similar to that reported previously (29, 52), while the K_m for ICP4tide was 39 μM, which was very comparable to those of physiological substrates of cellular PKA (54). Thus, the synthetic ICP4tide could be considered to be a good substrate for PKA in vitro. Additional studies demonstrated that the initial reaction rates at both the highest and lowest concentrations of substrate were in the linear range (64). The accompanying paper (65) further demonstrates, by trypic peptide, phosphoamino acid, and protein sequencing analyses, that the PKA site represented in ICP4tide is utilized in vivo.

DISCUSSION

Growth properties of mutant viruses lacking the serine-rich region. Previous studies have demonstrated that deletion of the conserved serine-rich sequence in the background of an ICP4 polypeptide truncated at residue 774 resulted in a loss of the ability to transactivate the early thymidine kinase gene promoter in transient assays (57). Paterson and Everett have also investigated the effect of mutations in the serine-rich region on the function of ICP4. They found that the deletion of the entire serine-rich region (codons 162 to 229) of ICP4 reduces the transactivation efficiency of the (gD) promoter in transient assays (42) and delays the synthesis of early and late viral proteins in mutant virus-infected cells (44). The growth efficiency of such mutant virus was also reduced more than 10-fold over a 24-h time course when tested on BHK cells (44). The results of the studies on growth and viral gene expression of *d*8-10 (with codons 142 to 210 deleted) in cultured cells reported here are consistent with those previous findings. In this work, the importance of the serine-rich region in viral growth was further demonstrated by tests in a mouse model. During acute infection in the eyes of the mice, the growth properties of *d*8-10 were similar to those seen in tissue culture. In contrast, viral growth in trigeminal ganglia was greatly decreased. It is possible that the reduced level of growth at the site of inoculation is responsible for the lack of growth in the trigeminal ganglia. However, these results demonstrate that the deleted serine-rich sequence does specify a function that is necessary for the activity of ICP4 and for the viral growth during a natural cause of infection. Other explanations that remain to be tested are the possibilities that on interaction(s) between a neuronal factor(s) and the serine-rich sequence is required for viral growth in murine trigeminal ganglion neu-

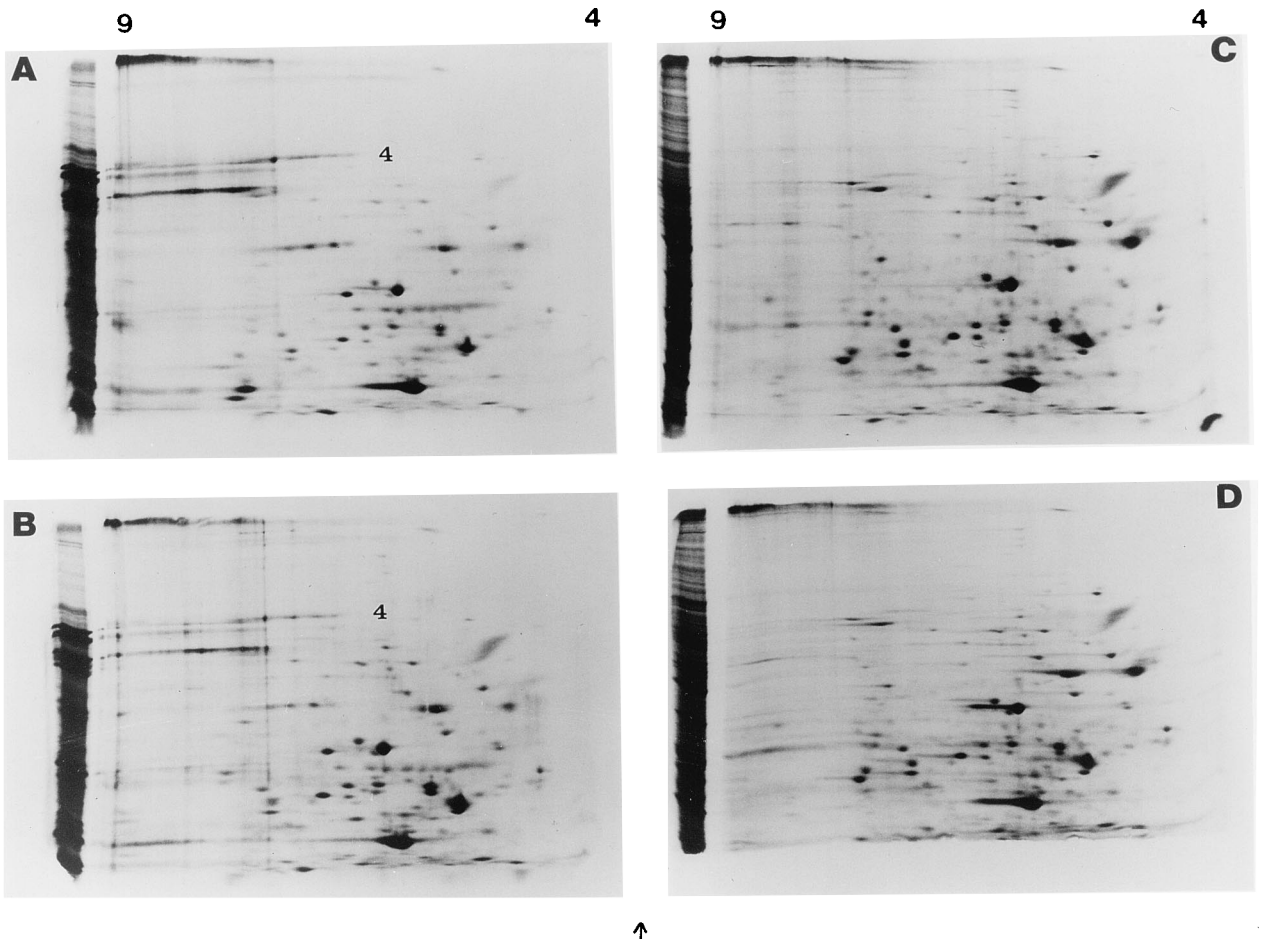


FIG. 7. 2-D IEF of *d*8-10-infected PC12 and PKA-deficient cell extracts. PC12 cells (wt) (A and C) and PKA-deficient cells (A123.7) (B and D) were mock infected (C and D) or infected with *d*8-10 (A and B). The infected cells were then labeled with [^{35}S]methionine from 2.5 to 5.5 h postinfection, and then cell lysates were prepared and subjected to 2-D IEF. Autoradiographic images of the expressed ICPs are shown. The heterogeneous *d*8-10 ICP4 polypeptides are indicated by number.

rons or that phosphorylation of the serine-rich region of ICP4 by signal transduction pathways may provide a mechanism to regulate the activity of ICP4 and hence viral growth as a function of external stimuli.

Phosphorylation of ICP4 polypeptides. Many of the nonstructural proteins encoded by HSV, such as ICP4, are phosphorylated in infected cells (46, 63). Both wt and mutant forms of ICP4 are phosphorylated in a very complex manner and exhibit multiple electrophoretic forms on SDS gels (48, 55, 56). The modification state of ICP4 may be important for its function or vice versa. However, how these differently migrating species are generated and what functions they might have are unknown. In this study, a major determinant for phosphorylation of ICP4 was localized to the prominent serine-rich region through genetic analysis and biochemical characterization. In addition it was determined that PKA can phosphorylate ICP4 as a function of the serine tract, in vivo and in vitro. Deletion of the serine-rich domain reduces the phosphorylation of ICP4 by at least 10-fold as determined by analysis of ICP4 protein in crude cell extracts, and it appears to reduce the phosphorylation by 6- to 10-fold with the purified ^{32}P-labeled proteins. This difference may be due to the action of one or more kinases directly on the serine tract or to structural differences between wt ICP4 and *d*8-10 that result in altered phosphorylation at sites outside the serine tract. It is also possible that the phosphorylation of the serine tract may affect phosphorylation at distant sites.

PKA has been reported to serve as a phosphate donor to correct a decreased-mobility change of phosphorylated ICP4 polypeptides after dephosphorylation with phosphatases (41). In the nucleus, PKA is known to associate with the transcriptional machinery and modify the activities of proteins that bind to DNA (3, 18, 36, 53). In this study, the growth of wt virus in PKA-deficient cells was reduced to the levels seen with *d*8-10 in PKA-proficient and -deficient cells. This result provides genetic evidence that the serine tract is a functional target of PKA and that interaction between PKA and ICP4 is important for optimum viral growth. It was also found that the presence of PKA in infected cells affected the isoelectric points of wt ICP4 on 2-D gels but had little effect on the *d*8-10 ICP4 protein. In vitro, PKA phosphorylated wt ICP4 but was unable to phoshorylate the *d*8-10 protein. Moreover, a synthetic peptide representing a sequence in the serine tract that resembles a PKA phosphorylation site was phosphorylated by PKA, having a K_m in the physiological range. Therefore, while PKA may influence many events in viral infection, the data presented in this paper strongly suggest that PKA may influence the activity of ICP4 by directly phosphorylating the serine-rich region.

It is possible that different phosphorylation states of ICP4 could also affect its DNA-binding affinity for different viral

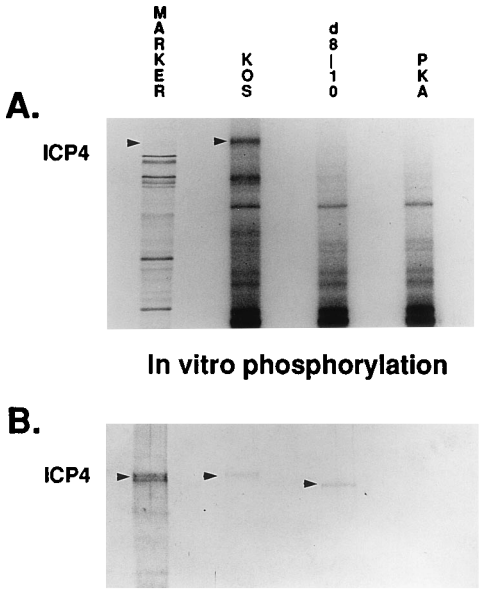


FIG. 8. Phosphorylation of purified wt and *d*8-10 ICP4 proteins with PKA. ICP4 proteins purified from KOS- or *d*8-10-infected Vero cells were incubated with [γ-^{32}P]ATP in the presence of PKA at 30°C for 30 min as described in the text. The reaction products were analyzed on an SDS–9% polyacrylamide gel and transferred to a nitrocellulose paper. (A) The radioactivity labeled proteins were visualized by autoradiography. As a control, lane 4 contained no ICP4 but did contain the catalytic subunit of PKA. (B) After the exposure in panel A was obtained, the same filter paper was reacted with the N15 ICP4-specific polyclonal antibody, incubated with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G, and processed as described in the text. A [^{35}S]methionine-labeled cell extract infected with KOS was used as a marker. The arrowheads indicate the position of ICP4.

promoters, as previously proposed (38, 41). The negative charges resulting from phosphorylated serines and a threonine and from the adjacent eight acidic residues may together serve as a transactivation domain in a manner similar to that of the acidic regions identified in other eukaryotic transactivators (25, 51). Alternatively, alterations in the phosphorylation state may result in allosteric changes within ICP4 that alter protein-protein interactions with other cellular molecules. Such interactions would be important for the regulatory activity and hence the viral growth. The *d*8-10 ICP4 protein is reduced about fourfold in the ability to form tripartite complexes on DNA with TBP and TFIIB (22, 58). While this is sufficient for the repressor activity of ICP4 (22), the reduction in the ability of *d*8-10 to activate transcription may be a consequence of its reduced ability to interact with the general transcriptional machinery. Therefore, this region may be directly involved in the protein-protein interactions or may regulate the affinity of the interactions. Recently, it has been shown that a 15-kDa cellular coactivator, termed p15 (31) or PC4 (17), has striking amino acid similarity to the serine tract of ICP4 and is involved with the formation of tripartite complexes with TBP and, in this case, TFIIA (31). It was found that the coactivator function of p15 was regulated by cellular kinases (31). It may be that ICP4 is an activator and coactivator in one and that the function of ICP4 and the requirement for the serine tract may also depend on the abundance or state of cellular p15 in different cellular environments or as a consequence of external stimuli. These possibilities are currently being examined.

TABLE 2. Phosphorylation of ICP4tide

Kinase	Peptide	Phosphorylation (cpm)	γ-^{32}P incorporated (%)	Ratio[a]	K_m (μM)[b]
PKA	Kemptide	768,687	26.1	88.5	9
	ICP4tide	182,104	6.2	26.2	38.96
None	Kemptide	8,682	0.3		
	ICP4tide	6,946	0.24		

[a] Ratio of phosphorylation with PKA to that without PKA.
[b] Determined by linear regression analysis of the Lineweaver-Burk representation for the dependence of the reaction velocity on substrate concentration.

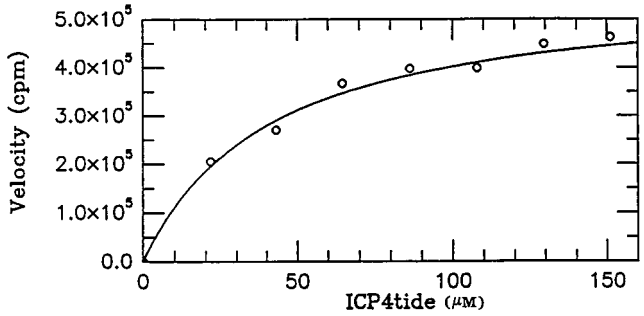


FIG. 9. Kinetic analysis of ICP4tide phosphorylation. Various amounts of ICP4tide were incubated with 60 U of PKA and 60 μM ATP, and the incorporation of ^{32}P into ICP4tide was determined as described in the text. The plot shows the best fit for the data according to the Michaelis-Menton equation. The apparent K_m for ICP4tide was calculated to be 39 μM.

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