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Herpes Simplex Virus Type 1 ICP0 Plays a Critical Role in the De Novo Synthesis of Infectious Virus following Transfection of Viral DNA

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As a first step in identifying the functions and intramolecular functional domains of herpes simplex virus type 1 infected cell protein 0 (ICP0) in productive infection and latency, a series of mutant plasmids specifying varying amounts of the ICP0 primary amino acid sequence were constructed. In transient expression assays with mutant and wild-type plasmids, the N-terminal half of the ICP0 molecule was found to be sufficient to transactivate a variety of viral promoters. Although promoters representing the immediate-early, early, and late kinetic classes were transactivated by wild-type ICP0, individual promoters responded to mutant forms of ICP0 in a manner consistent with the possibility that ICP0 transactivates different promoters by different mechanisms. Unlike infection with virus particles, which contain the 65-kilodalton transcriptional transactivator, the initiation of viral replication after transfection of cells with purified viral DNA requires de novo protein synthesis. In order to assess the role of ICP0 in the de novo synthesis of infectious virus, Vero cells were transfected with purified DNA of wild-type virus or an ICP0 null mutant and the production of infectious virus was monitored. In cells transfected with mutant DNA, virus production was delayed by 2 days and the level of virus was reduced by several orders of magnitude relative to Vero cells transfected with wild-type viral DNA, suggesting an important role for ICP0 in the de novo synthesis of infectious particles. In cotransfection experiments with infectious DNA of the ICP0 null mutant and a plasmid specifying wild-type ICP0, titers of infectious virus were significantly enhanced relative to transfection with mutant DNA alone, confirming the role of ICP0 in de novo synthesis. These findings are consistent with the proposed role of ICP0 in reactivation of herpes simplex virus from latency (D. A. Leib, D. M. Coen, C. L. Bogard, K. A. Hicks, D. R. Yager, D. M. Knipe, K. L. Tyler, and P. A. Schaffer, J. Virol. 63:759–768, 1989), a process also thought to require de novo protein synthesis. The complementing activities of ICP0 mutant plasmids for ICP0 null mutant DNA in cotransfection assays correlated well with their transactivating activities for viral promoters in transient assays, indicating that the transactivating function of ICP0 is a critical factor in the de novo synthesis of infectious particles. In order to assess the role of ICP0 and its intramolecular functional domains in infection with virus particles, six nonsense mutations which in plasmid form resulted in varying levels of transactivating activity were introduced into the viral genome. ICP0 polypeptides of the expected sizes were expressed by these mutant viruses. In order to determine the ability of mutant forms of ICP0 to direct the synthesis of infectious virus at very low multiplicities of infection, Vero cells were infected with 0.004 PFU per cell of each mutant and virus production was monitored. It was found that only mutants specifying 90% or more of the N-terminal amino acid sequence of the molecule produced infectious virus above the level produced by the ICP0 null mutant. Together, these observations demonstrate that (i) 90% of the ICP0 molecule is required to produce levels of infectious virus above that of an ICP0 null mutant, (ii) the active site of ICP0 maps to the N-terminal half of the protein, (iii) ICP0 may transactivate different viral promoters by different mechanisms, and (iv) transactivation by ICP0 plays a critical role in the de novo synthesis of infectious virus.

The 152-kilobase (kb) genome of herpes simplex virus type 1 (HSV-1) encodes at least 72 unique proteins (36–38, 43). During productive infection, the synthesis of these proteins is coordinately regulated in a cascade fashion, primarily at the transcriptional level (9, 26, 28). Upon entry into the host cell, a 65-kilodalton (kDa) virion-associated protein activates transcription of the five immediate-early (IE) viral genes encoding infected cell proteins (ICPs) 0, 4, 22, 27, and 47 (2, 7, 33). The IE proteins then act to regulate the expression of viral proteins of the early (E) and late (L) kinetic classes (27). Proteins of the E class are involved in viral DNA replication, whereas those of the L class are primarily virion structural components.

The mechanisms by which the five IE proteins mediate their regulatory activities for E and L genes during productive infection and the manner in which these activities are overridden during latent infection are not yet clear. Much is known, however, about the individual regulatory activities of three of the five (ICPs 0, 4, and 27) during productive infection. In transient expression assays, ICP0 is able to transactivate all classes of viral genes (IE, E, and L) as well as a number of cellular genes—alone or synergistically with ICP4 (17, 18, 21, 22, 34, 40, 41, 45, 51). ICP0 binds nonspecifically to DNA and likely mediates its up-regulatory activity through effects on DNA binding. Alternatively, it may interact with cellular transcription factors or other viral or cellular regulatory proteins. Notably, ICP0 exhibits no detectable up-regulatory activity in cells infected with an ICP4 deletion mutant, in which both ICP0 and ICP27 are expressed (14). ICP4 down regulates expression of IE genes

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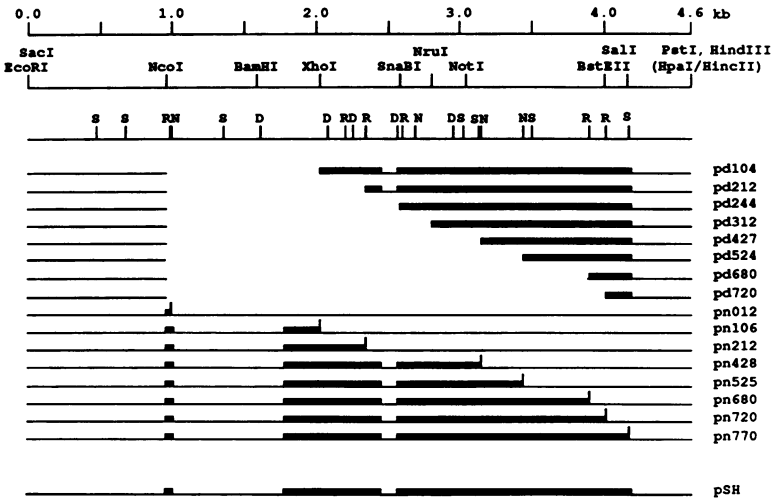

FIG. 1. ICP0 mutant plasmids used in this study. The 4.6-kb *Eco*RI-*Hpa*I fragment from pW3 containing the ICP0 gene (49) was cloned into the *Eco*RI and *Hinc*II sites of pUC9 to yield pSH, which served as parental plasmid (bottom line). Relevant unique (line 2) and multiple (line 3) restriction sites in the 4.6-kb fragment are also shown (*Dpn*I [D], *Nae*I [N], *Rsa*I [R], *Sma*I [S]). ICP0 coding exons in pSH, which read from left (N terminal) to right (C terminal), are shown as heavy bars. Mutant plasmids are shown in the middle of the figure. pd plasmids contain deletion mutations and pn plasmids contain nonsense mutations. Deleted DNA sequences are indicated by the absence of lines or bars. Deletions in plasmids pd104, pd244, and pd312 begin at the *Nco*I site (at the translational initiation codon) and terminate at *Xho*I, *Sna*BI, and *Nru*I sites, respectively. Deletions in plasmids pd212, pd427, pd524, pd680, and pd720 begin at the *Nco*I site and terminate at unique sites generated by linker insertion. The three-digit number in the designation of pd mutants corresponds to the last amino acid residue deleted by the mutation. Nonsense mutations in plasmids pn012, pn680, and pn720 contain a synthetic nonsense *Spe*I linker at *Rsa*I sites. The remaining plasmids contain a synthetic nonsense *Hpa*I linker. The positions of nonsense linkers are shown as short vertical lines. The three-digit number in the designation of pn mutants corresponds to the amino acid residue disrupted by the linker insertion.

and up regulates expression of E and L genes (11–13, 15, 16, 21, 22, 34, 41, 45). Recent evidence indicates that ICP4 exerts these effects, at least in part, by direct binding to *cis*-acting elements in viral promoters (14, 20, 29, 30, 39, 47, 52). ICP27 represses both ICP0-induced IE gene expression and ICP0- and/or ICP4-induced E gene expression, whereas it further enhances ICP0- and/or ICP4-induced L gene expression (16, 35, 46, 48, 51). The manner in which ICP27 mediates its modulatory effects on the activities of ICPs 0 and 4 is not known; however, Rice and Knipe have recently suggested that ICP27 physically modifies ICP4, either directly or indirectly (46).

Unlike ICPs 4 and 27, which are essential for virus replication (12, 15, 35, 48), ICP0 is dispensable for virus growth (49, 54), despite its potent and promiscuous transactivating ability. Although ICP0 is not essential for productive infection, it appears to confer a strong selective advantage on virus growth and to play a critical role in reactivation of virus from latency (32). The process of reactivation is thought to involve the de novo synthesis (synthesis initiated in the absence of preexisting viral proteins) of viral proteins from latent viral genomes, since no functional viral proteins or transcripts known to encode viral proteins have been detected in latently infected cells. The studies described herein constitute an initial attempt to elucidate the role(s) of ICP0 during productive infection under conditions that resemble reactivation from latency.

The gene for ICP0 exists in two copies in the inverted-repeat regions of the genome surrounding U_L (3). The gene, which specifies a spliced message consisting of three exons, encodes a polypeptide of 775 amino acid residues (44). The ICP0 protein is a 110- to 120-kDa nuclear phosphoprotein that binds to DNA (2, 24, 42). The protein contains a positively charged series of amino acid residues involved in nuclear localization as well as a zinc finger domain that is probably involved in DNA binding and transactivation (18, 44).

In order to investigate the role of transactivation by ICP0 in the de novo synthesis of infectious virus and to identify the intramolecular functional domain(s) responsible for this activity, a series of ICP0 deletion and nonsense mutations in plasmid form and in the context of the viral genome were isolated and characterized. The properties of these mutants demonstrate that (i) the transactivating activity of ICP0 for viral promoters is associated primarily with the N-terminal half of the molecule, (ii) ICP0 probably activates the expression of various promoters by different mechanisms, and (iii) the transactivating activity of ICP0 plays a critical role in the de novo synthesis of infectious virus.

MATERIALS AND METHODS

Cells and viruses. Vero cells were grown and maintained as previously described (48). The wild-type virus, HSV-1(KOS), and the ICP0 mutant viruses generated in this study were propagated and assayed as described previously (50).

Plasmids. Plasmid pW3 contains the ICP0 gene within a 6.5-kb *Sac*I-*Pst*I fragment derived from the long terminal repeat of the viral genome cloned into pUC13 (49). A 4.6-kb *Eco*RI-*Hpa*I fragment from pW3 was cloned into the *Eco*RI and *Hinc*II sites of pUC8 (55) to produce pSH (Fig. 1, bottom). (The *Eco*RI site in pW3 is adjacent to the *Sac*I site in the multicloning region of pUC13, and the *Hpa*I site is located within HSV-1 sequences.) The 4.6-kb fragment contains the entire 3.2-kb ICP0 coding region (including three exons and two introns) as well as 1.0-kb 5′ and 0.4-kb 3′ flanking sequences.

Restriction maps of plasmid pSH are shown at the top of Fig. 1. This plasmid was used to generate the mutant plasmids shown in this figure. Deletion mutants were con-

structed by digestion of pSH with *Nco*I and an enzyme that recognizes a second unique restriction site within the ICP0 coding region (Fig. 1, line 2); this procedure was followed by recircularization of the vector-containing fragment with ligase. In the case of pd104, the deletion would generate a +1 frameshift mutation, consequently an 8-base pair (bp) *Bgl*II linker was inserted to correct the reading frame. All other deletion mutations were in frame. For construction of nonsense mutants, pSH was partially digested with *Dpn*I, *Nae*I, *Rsa*I, or *Sma*I (Fig. 1, line 3). Singly cut linearized DNA molecules were purified from an agarose gel by using Elutip columns (Schleicher & Schuell, Inc., Keene, N.H.) and were ligated in the presence of a 20-bp *Hpa*I or 14-bp *Spe*I linker containing nonsense codons in all three reading frames. The *Hpa*I site (GTTAAC) in the 20-bp linker (AG ATCTAGTTAACTAGATCT) was flanked by *Bgl*II sites (AGATCT). After transformation of *Escherichia coli*, small batches of plasmids were purified. The locations of linker insertions were verified by restriction digestion and agarose gel electrophoresis. As expected from the restriction map shown in Fig. 1, digestion of pSH with *Dpn*I, *Nae*I, *Rsa*I, or *Sma*I and insertion of a nonsense linker at these sites resulted in the joining of two adjacent fragments to form a new fragment the size of the two fragments combined.

Hybrid plasmids pn012/n106 and pn012/n212 were constructed by replacing the *Bam*HI-*Hin*dIII fragment of pn012 with the nonsense linker containing *Bam*HI-*Hin*dIII fragments from pn106 and pn212, respectively (see Fig. 1 for the location of restriction sites). pn012/dXS was constructed by removal of the *Xho*I-*Sal*I fragment containing the ICP0 coding region from pn012; this procedure was followed by recircularization.

A plasmid containing the *E. coli lacZ* coding region under control of the ICP0 promoter was constructed as follows. First, pn012 (Fig. 1) was digested with *Bgl*II and *Bam*HI. (The *Bgl*II site is contained within the *Hpa*I nonsense linker inserted at the site). The larger vector-containing fragment was ligated to the 3.1-kb *Bam*HI fragment from pMC1871 (8). This fragment, which contains the *E. coli lacZ* coding region beginning at codon 8, was inserted so that the *lacZ* coding region was in the same orientation as the ICP0 gene. The resulting plasmid was designated the first intermediate. The first intermediate was digested with *Bam*HI and *Sal*I, and the larger vector-containing fragment was end filled with Klenow and recircularized. This plasmid was designated the second intermediate. The second intermediate was digested with *Nco*I, end filled with Klenow fragment, and recircularized. The latter operation was performed because in the second intermediate, the *lacZ* coding region was out of frame with respect to the ICP0 initiation codon, which is located within the *Nco*I site. Destruction of the *Nco*I site by end filling introduced four additional base pairs, CATG, and corrected the reading frame. The resulting plasmid was designated p0-lac.

Plasmids pIE3CAT, ptkCAT, and pL42CAT have been described previously (13). pKXXB1CAT was constructed and provided by N. E. Pederson and S. Person (Pennsylvania State University, University Park) (N. E. Pederson, Ph.D. thesis, The Pennsylvania State University, University Park, 1988). pKXXB1CAT contains the HSV-1 gB promoter (−503 to +136) upstream of the gene for chloramphenicol acetyltransferase (CAT).

E. coli RDP 145 (6) was used as the host for all plasmids in transformation (10), and small batches of each plasmid were purified by using the boiling method of Holmes and Quigley (25). Plasmid DNA was isolated by the procedure of Birnboim and Doly (4) and further purified by equilibrium sedimentation in CsCl.

Restriction enzymes, T4 DNA ligase, and the *Spe*I linker (CTAGACTAGTCTAG) were purchased from New England BioLabs, Inc., (Beverly, Mass.). The *Hpa*I linker (AGATC TAGTTAACTAGATCT) was synthesized at the Dana-Farber Cancer Institute (Boston, Mass.).

Transfection. The calcium phosphate method of Graham and Van der Eb (23) was used in transfection assays. Vero cells were seeded in 60-mm or 100-mm petri dishes 24 h prior to transfection. At 3 h before transfection, the medium was changed. For 60-mm dishes, 12 μg of viral and/or plasmid DNA (plus salmon testis carrier DNA) was suspended in 250 μl of transfection buffer (1.6 g of NaCl, 1.18 g of HEPES [*N*-2-hydroxyethyl-piperazine-*N*'-2-ethanesulfonic acid], 0.02 g of Na_2HPO_4 [anhydrous] in 100 ml, pH 7.05). For 100-mm dishes, 25 μg of DNA was suspended in 500 μl of transfection buffer. The DNA solution was mixed with an equal volume of 250 mM $CaCl_2$ for 30 min. The resulting suspension containing the DNA precipitate was added to the petri dish containing cells in medium and was incubated at 37°C for 4 h. The DNA-Ca^{2+}-treated cells were washed with TBS (8.0 g NaCl, 0.38 g KCl, 3.0 g Tris base, H_2O to 1 liter [pH 7.4]) and treated with 15% glycerol (in transfection buffer plus 0.35× TBS) for 2 min. Cells were washed twice with TBS, fresh medium was added, and the culture was incubated at 37°C for the designated times.

CAT assays. CAT assays were carried out as described by DeLuca and Schaffer (13). Two million Vero cells were seeded in 100-mm petri dishes, and 24 h later they were transfected with 25 μg of DNA (2 μg of CAT plasmid, 2 μg of ICP0 plasmid, 21 μg of salmon testis DNA). At 40 h posttransfection, cell extracts were prepared in a volume of 100 μl, the protein concentration was determined, and CAT assays were carried out by addition of 30 μl of 20 mM acetylcoenzyme A (Sigma Chemical Co., St. Louis, Mo.) and 100 nCi of [^{14}C]chloramphenicol (Dupont, NEN Research Products, Boston, Mass.). Samples were incubated at 37°C for 90 min unless otherwise indicated.

Complementation. Vero cells (5×10^5) in 60-mm petri dishes were transfected 24 h postseeding with 12 μg of DNA (including 0.5 μg of viral DNA and varying concentrations of plasmid DNA and carrier DNA). At various times posttransfection, cultures were harvested and viral titers were determined by assay on Vero cell monolayers.

Mutant virus isolation. (i) Isolation of an ICP0$^-$/*lacZ*$^+$ mutant. Vero cells (5×10^5) were seeded in 60-mm dishes and cotransfected after 24 h with 1 μg of KOS DNA and 1 μg of linearized p0-lac DNA. Three days later, when cytopathic effects were generalized, cultures were harvested, frozen and thawed three times, plated on Vero cell monolayers, and overlaid with methylcellulose. After incubation at 37°C for 4 days, 2 ml of 300 μg of X-gal per ml in neutral red was added to each 35-mm petri dish. Blue plaques were picked and purified. Even after several rounds of plaque purification, viral DNA from blue-plaque isolates contained one copy of *lacZ* and one copy of ICP0 and produced wild-type progeny (white plaques). Homozygous mutants containing two copies of the *lacZ* gene but no ICP0 gene likely went undetected because they produced smaller plaques and the blue color was not as obvious as that produced by the heterozygous mutants. To obtain homozygous mutants, approximately 10 plaques were further picked and purified, without X-gal staining. Upon testing in the presence of X-gal, three of these isolates produced uniformly blue plaques and were shown to contain two copies of *lacZ*. One isolate was

designated 7134, and virus stock was prepared for further studies.

(ii) Isolation of nonsense mutants. Six nonsense mutations were transferred to the viral genome by cotransfection of infectious 7134 DNA and ICP0 nonsense mutant plasmid DNA. Progeny virus isolates that produced white plaques in the presence of X-gal were isolated and retested. Southern blot analysis was performed on white-plaque isolates to verify their restriction patterns (53).

IE protein phenotypes. Expression of IE proteins by mutant viruses after cyclohexomide reversal was assessed essentially as described by Honess and Roizman (26). Vero cells (10^6) were seeded in 60-mm petri dishes. One hour before infection, cycloheximide (Sigma Chemical Co.) was added to cultures at a final concentration of 75 μg/ml. Cells were infected with 10^7 PFU of virus (multiplicity of infection, 10 PFU per cell) and incubated at 37°C for 6 h in the presence of 75 μg of cycloheximide per ml. After washing three times with TBS containing 10 μg of dactinomycin, infected cells were pulse-labeled with [^{35}S]methionine (Dupont, NEN Research Products) in 200 μl of TBS containing 10 μg of dactinomycin per ml at 37°C for 20 min. Cell extracts were prepared in sample solution (50 mM Tris hydrochloride [pH 7.0], 2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 5 mg of bromophenol blue per ml), and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (31).

Production of infectious progeny virus. The ability of mutant and wild-type viruses to produce infectious progeny after low-multiplicity infection was determined as follows. Vero cells (2.7×10^5) were infected 24 h postseeding with 1,000 PFU of virus (multiplicity of infection, 0.004 PFU per cell). A low multiplicity was used to ensure that each cell infected had received only one infectious unit and to dilute out defective virus particles present in stocks of ICP0 mutants (54; Sacks and Schaffer, unpublished observations). Two hours after infection, cells were washed with TBS, treated with acid-glycine-saline (5) for 1 min to remove input virus particles that did not enter cells, washed twice with TBS, and incubated in growth medium at 37°C. At 2 and 24 h postinfection, cultures were harvested, and viral titers were determined by standard plaque assays. The number of infectious virus particles per input infectious particle (PFU) within a period of 24 h was calculated by dividing the titer at 24 h by 1,000.

RESULTS

Deletion and nonsense mutant plasmids. To study the functions and functional domains of ICP0, plasmids specifying varying amounts of the ICP0 primary amino acid sequence were constructed. A series of eight deletion and eight nonsense mutant plasmids were isolated, and their mutation sites were confirmed by restriction mapping. These mutants specify progressively shorter ICP0 peptides truncated from both the N terminus (pd plasmids) and the C terminus (pn plasmids) of ICP0, which is 775 amino acid residues in length. These pd and pn plasmids are designated with a three-digit number which represents the boundary codon of the mutated sequence. For example, pn720 contains a nonsense mutation at codon 720. The N-terminal deletions removed from 104 to 720 residues. The nonsense mutations are distributed from codon 12 to codon 770.

Transactivation of viral promoters. The transactivating activities of wild-type and mutant forms of ICP0 were investigated by assessing their abilities to stimulate the expression of chloramphenicoacetyl transferase (CAT) under the control of a preselected target promoter (reporter plasmids). The reporter plasmids used in this study included pIE3CAT, ptkCAT, pKXXB1CAT, and pL42CAT, which contain the CAT gene under control of the promoters for ICP4 (an IE gene), thymidine kinase (an E gene), glycoprotein B (an L_1 gene whose expression is maximal when viral DNA is synthesized), and L42 (an L_2 gene whose expression is stringently dependent upon viral DNA synthesis), respectively. The promoters in reporter plasmids thus represent the major recognized kinetic classes of HSV-1 genes.

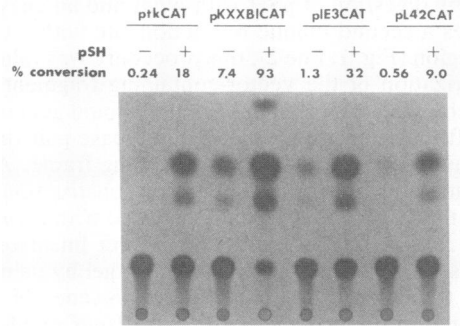

FIG. 2. Transactivation of HSV-1 promoters by wild-type ICP0. Vero cells were transfected with ptkCAT, pKXXB1CAT, pIE3CAT, or pL42CAT, with or without pSH. Cells were harvested, and cell extracts were assayed for CAT activity. Values above the figure are the ratios of acetylated chloramphenicol to its unacetylated form, expressed as percents.

Prior to testing the transactivating activities of mutant forms of ICP0, the activity of wild-type ICP0 for these promoters was determined. Vero cells were transfected with a reporter CAT plasmid with or without pSH, and CAT activity was assessed. To compare individual promoters, the four reporter CAT plasmids were always tested in the same experiment (Fig. 2). In the absence of ICP0, the basal level of expression was strongest from the gB(L_1) promoter, followed by expression from the ICP4 (IE), L42 (L_2), and tk (E) promoters, respectively. Although the basal levels of expression among the four promoters varied markedly, expression from all four was greatly elevated when pSH was included in the transfection mix (Fig. 2).

Individual ICP0 mutant plasmids were then used in place of pSH in CAT assays (Table 1). With the exception of pd104, mutant plasmids containing deletions beginning at the N terminus of ICP0 (pd plasmids) exhibited no transactivating activity. Mutant pd104 contains the smallest deletion (i.e., 104 amino acid residues) and reproducibly exhibited low but significant transactivating activity for the ICP4 promoter in pIE3CAT. In contrast, the nonsense mutants (pn plasmids) induced widely varying levels of CAT activity. Truncation of six amino acid residues from the C terminus of the ICP0 molecule (pn770) did not affect transactivating activity. Truncation of 56 residues from the C terminus (pn720) reduced activity for the tk and ICP4 promoters by approximately twofold relative to wild-type ICP0. By contrast, pn720 activity for the L42 promoter was enhanced approximately twofold relative to wild-type pSH, whereas activity for the gB promoter was only slightly reduced. Truncation of 96, 251, or 358 residues from the C terminus (pn680, pn525, and pn428, respectively) further reduced ICP0 transactivating activity for tk (E) promoters to levels just above the basal level. These truncated mutants exhibited only four- to eightfold stimulation of the L42 (L_2) and gB

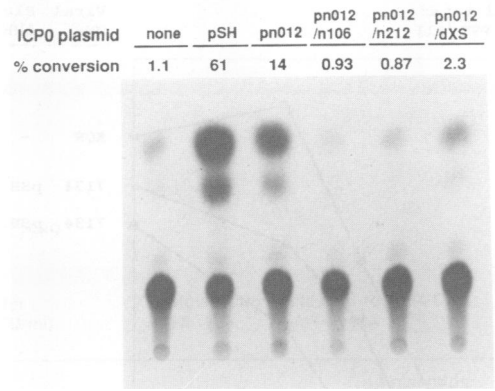

FIG. 3. Transactivation of the ICP4 promoter by pn012 and its derivatives. Vero cells were cotransfected with pIE3CAT and an ICP0 plasmid. CAT activities were assayed and are presented as described in the legend to Fig. 2.

(L_1) promoters. Truncation of 564 or 670 residues from the C terminus (pn212 and pn106, respectively) abolished transactivating activity for all four promoters. On the basis of these observations, we conclude that the amino acid sequences absolutely required for ICP0 transactivating activity are located within the N-terminal 427 residues of the molecule, most likely between residues 105 and 427, as determined by the activities of plasmids pd104 and pn428. Clearly, however, other more C-terminal residues contribute to full activity.

Unexpectedly, pn012, containing a nonsense mutation after codon 11, exhibited significant transactivating activity for all four promoters (Table 1). To test the unlikely possibility that the first 11 amino acid residues were responsible for this activity, sequences downstream of the nonsense mutation in pn012 were further mutated. The nonsense mutations in plasmids pn106 and pn212 (Fig. 1) were transferred to the background of pn012 to generate plasmids pn012/n106 and pn012/n212, respectively. In addition, the *Xho*I-*Sal*I (Fig. 1) fragment was deleted from pn012 to yield plasmid pn012/dXS. As shown in Fig. 3, pSH and pn012 exhibited significant transactivating activities, but the three mutant plasmids pn012/106, pn012/n212, and pn012/dXS exhibited little or no activity. Therefore, sequences downstream of codon 11 are required for the activity of pn012. On the basis of this observation and on DNA sequence information (44), it is likely that alternative methionine codons located at codons 135 and 143 function in reinitiation of translation in pn012. To date, however, efforts to detect the pn012-encoded polypeptide have failed.

TABLE 1. Transactivating activities of ICP0 mutant plasmids

ICPO plasmid	CAT activity (fold stimulation over the basal level[a])			
	pIE3CAT	ptkCAT	pKXXB1CAT	pL42CAT
pSH	20.5 ± 2.44	47.6 ± 39.0	57.4 ± 46.6	18.0 ± 8.98
pd104	3.59 ± 0.08	1.51 ± 0.49	1.49 ± 0.59	0.62 ± 0.08
pd212	1.49 ± 0.52	1.44 ± 0.66	0.78 ± 0.63	0.66 ± 0.01
pd244	0.89 ± 0.23	0.92 ± 0.12	0.38 ± 0.25	0.57 ± 0.06
pd312	1.35 ± 0.04	0.92 ± 0.02	0.84 ± 0.24	0.74 ± 0.06
pd427	1.37 ± 0.41	0.88 ± 0.03	0.79 ± 0.16	0.69 ± 0.02
pd524	1.43 ± 0.53	0.94 ± 0.05	1.73 ± 1.35	0.73 ± 0.08
pd680	1.14 ± 0.05	0.92 ± 0.16	0.65 ± 0.03	0.65 ± 0.10
pd720	1.56 ± 1.22	0.78 ± 0.07	0.67 ± 0.01	0.70 ± 0.04
pn012	17.2 ± 8.84	9.55 ± 10.3	22.6 ± 2.12	11.9 ± 1.76
pn106	1.74 ± 1.69	1.46 ± 0.77	1.05 ± 0.12	1.03 ± 0.04
pn212	1.39 ± 0.59	0.91 ± 0.12	0.67 ± 0.01	0.78 ± 0.08
pn428	1.59 ± 0.01	2.04 ± 1.15	7.76 ± 6.99	4.73 ± 0.81
pn525	0.84 ± 0.11	2.17 ± 0.06	4.46 ± 2.96	4.29 ± 0.65
pn680	1.82 ± 1.04	2.43 ± 0.40	8.11 ± 6.78	8.76 ± 2.75
pn720	8.12 ± 2.43	18.3 ± 2.05	46.8 ± 37.2	32.6 ± 4.45
pn770	27.1 ± 8.45	34.7 ± 21.8	74.4 ± 65.9	15.5 ± 5.16

[a] CAT assays were performed as described in the legend to Fig. 2. CAT activity for a particular plasmid is expressed as the ratio of the amount of labeled chloramphenicol converted in the presence of the plasmid to the amount converted in the absence of the plasmid (basal level) (Refer to Fig. 2 for actual basal levels of activity; basal levels were given the value of 1 in all CAT assays). Values represent the average of two to four experiments; standard deviations are also included.

Interestingly, the responses of the four promoters in reporter CAT plasmids to individual mutant forms of ICP0 were not identical (Table 1). First, the transactivating activity of pd104 for the ICP4 (IE) promoter was reproducibly three- to fourfold higher than the basal level, although this was not observed for the other three promoters tested. Second, pn680, pn525, and pn428 exhibited activities that were four- to eightfold higher than basal levels for the gB and L42 promoters (the two L promoters), yet their activities for the tk (E) promoter were only twofold above the basal level; no increase in activity was detected for the ICP4 (IE) promoter. Finally, truncation of 66 residues from the C terminus of the protein (pn720) resulted in transactivating activity for the L42 (L_2) promoter that was reproducibly twofold higher than that of wild-type ICP0 (pSH), whereas activity for the other promoters was lower than that of wild-type ICP0 for the remaining three promoters. Together, these observed variations among the responses of various HSV-1 promoters to ICP0 suggest that the ICP0 protein contains regions of its primary amino acid sequence that affect various viral promoters differently.

Construction and properties of an ICP0$^-$/*lacZ*$^+$ null mutant. Both the functional analysis of mutant forms of ICP0 in complementation tests and the introduction of selected ICP0 mutations into the viral genome necessitated the use of an ICP0 null mutant. Moreover, the availability of a mutant in which both copies of the ICP0 gene were replaced by the *lacZ* gene would afford a screening marker for transfer of ICP0 mutations into the viral genome. As the first step in the isolation of the ICP0$^-$/*lacZ*$^+$ virus, the 3.1-kb *Bam*HI fragment containing the *lacZ* coding sequence from pMC1871 (8) was inserted in place of the ICP0-containing *Bgl*II-*Sal*I fragment in pn012 to yield p0-lac (Fig. 4C). In this construct, the *lacZ* coding region was flanked by HSV-1 sequences (1 kb to the right and 0.4 kb to the left) and was under control of the ICP0 promoter. The *lacZ* region in p0-lac was transferred to the viral genome by homologous recombination after cotransfection of p0-lac and infectious KOS DNA to yield the recombinant virus, 7134. This virus produced uniformly blue plaques, whereas wild-type KOS produced white plaques. To verify that the *lacZ* sequence was inserted at the correct position, 7134 DNA was isolated, digested with appropriate restriction enzymes, and subjected to Southern blot analysis (Fig. 4A). As expected from the predicted genome structure (Fig. 4B), digestion with both *Sac*I and *Pst*I generated a single 6.5-kb fragment from KOS DNA but two fragments of 3.45 kb and 2.95 kb from 7134 DNA when pSH was used as probe. This difference is due to the presence of an additional *Sac*I site within the *lacZ* sequence. The absence of the 6.5-kb fragment in 7134 DNA indicates that both copies of the ICP0 coding region had been replaced by the *lacZ* coding region. The insertion of *lacZ* into both copies of ICP0 was further confirmed when 7134 was shown to express a β-galactosidase-specific polypeptide

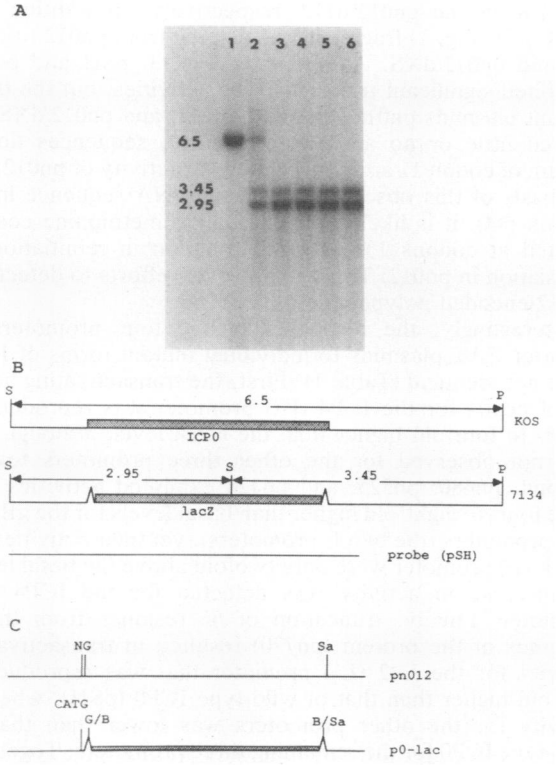

FIG. 4. Construction of the ICP0$^-$/*lacZ*$^+$ mutant, 7134. (A) Southern blot analysis of 7134 DNA. Viral DNA was digested with *Sac*I and *Pst*I, separated by agarose gel electrophoresis, and analyzed by Southern blot hybridization by using the probe shown in panel B. Lane 1, Wild-type viral DNA; lanes 2 to 6, DNA of ICP0$^-$/*lacZ*$^+$ insertion mutants. Partial digestion of the sample is evident in lane 2. The virus whose DNA is shown in lanes 6 (designated 7134) was plaque purified and picked for subsequent studies. (B) Predicted genomic structure of ICP0$^-$/*lacZ*$^+$ mutants. The diagram shows a *Sac*I (S)-*Pst*I (P) restriction map of the sequences containing the ICP0 gene with flanking sequences. The numbers are sizes (in kilobases) of relevant restriction fragments. (C) Structure of p0-lac. Plasmid p0-lac was derived by replacing the 3.2-kb *Bgl*II (G)-*Sal*I (Sa) fragment of pn012 (Fig. 1) with the 3.1-kb *lacZ*-coding *Bam*HI (B) fragment. The *Nco*I site at the initiation codon of ICP0 was digested with *Nco*I, end filled with Klenow fragment, and circularized by ligase. This generated a 4-bp (CATG) insertion at the *Nco*I site and provided an in-frame initiation codon for the inserted *lacZ* coding region. This plasmid was used to cotransfect Vero cells with KOS DNA to yield 7134.

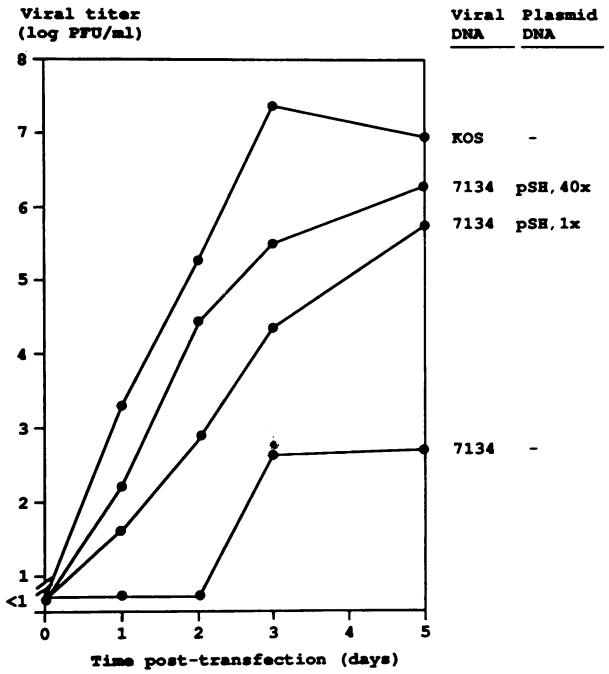

FIG. 5. Complementation of the DNA of the ICP0 null mutant, 7134, by plasmid pSH containing wild-type ICP0. Vero cells were cotransfected with 0.5 μg of infectious KOS or 7134 DNA, with or without pSH. The molar ratio of pSH DNA to viral DNA was either 1 (0.05 μg) or 40 (1 μg). Cultures were harvested on days 1, 2, 3, and 5 posttransfection, and viral titers were determined by standard plaque assays.

but no ICP0-specific polypeptide, as judged by immunoprecipitation with a monoclonal antibody to β-gal and a polyclonal rabbit anti-ICP0 serum (not shown). In order to demonstrate that 7134 DNA contained no secondary mutations, both copies of *lacZ* were rescued by using pSH to yield 7134R. The recombinant virus appeared to be identical to KOS, both genotypically and phenotypically (see below).

Complementation of an ICP0 null mutant by mutant forms of ICP0. As in the proposed hypothesis regarding the reactivation of HSV from latency, the initiation of new virus synthesis after transfection of infectious viral DNA requires de novo protein synthesis. In order to examine the role of wild-type ICP0 and the roles of specific intramolecular functional domains of ICP0 in the de novo synthesis of infectious virus, an assay in which individual mutant plasmids were cotransfected with DNA from the ICP0 null mutant 7134 was used. The ability of a mutant plasmid to complement 7134 was assessed by comparing the titers of progeny virus resulting from cotransfection with the titer resulting from transfection of 7134 DNA alone.

Prior to testing mutant plasmids for complementing ability, the baseline of infectious virus production by 7134 DNA was established. For this purpose, the kinetics of virus production after transfection of KOS and 7134 DNAs was compared. In these tests, purified viral DNAs from the two viruses were transfected separately into Vero cells and the number of infectious units (PFU) produced was determined by standard plaque assays at various times after transfection (Fig. 5). Infectious virus was detected one day after transfection in the case of KOS DNA, and the viral titer continued to increase through days 2 and 3 posttransfection. In contrast, 7134 DNA produced infectious virus two days later than KOS, resulting in viral titers on days 3 and 5 that were 4 to 5 orders of magnitude lower than that of KOS. To verify that this effect was due to the absence of ICP0 in 7134 DNA, Vero cells were cotransfected with infectious 7134 DNA and pSH, which specifies wild-type ICP0. In these tests, infectious 7134 virus was first detected on day 1 posttransfection, indicating that the presence of ICP0 obviated the delay in virus production seen in its absence. An increase in 7134 PFU of 2 to 3 orders of magnitude was observed over the 5-day test period. Moreover, this increase was dependent upon the relative amount of pSH used in the cotransfection mixture (compare 1× pSH and 40× pSH). The increase in virus titer observed could be due either to complementation by pSH or to recombination between 7134 and pSH DNA. It has been observed that the plaque size of 7134 (like that of other mutants lacking ICP0; 49) is significantly smaller than that of KOS. The size of the majority of the plaques (>90%)

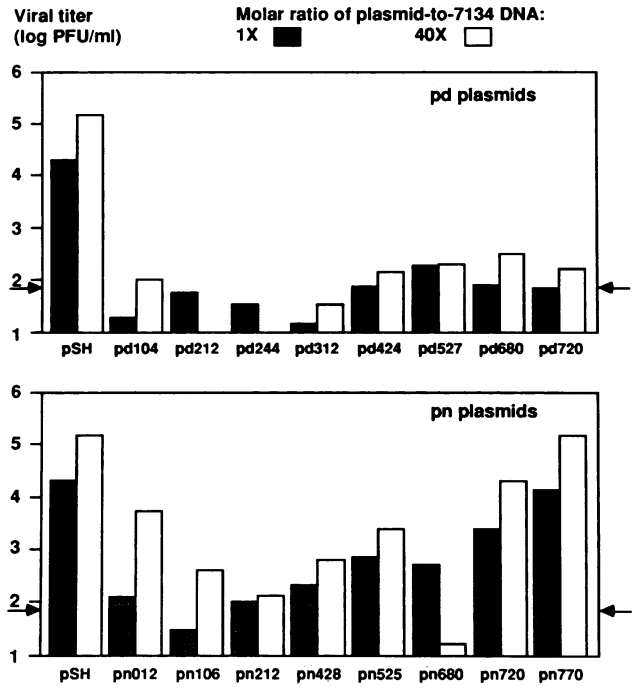

FIG. 6. Complementation of the DNA of the ICP0 null mutant, 7134, by ICP0 mutant plasmids. Vero cells were transfected with 0.5 μg of 7134 DNA and an ICP0 mutant plasmid at a molar ratio of plasmid-to-viral DNA of either 1 or 40. Cultures were harvested on day 2 posttransfection, and the titers of infectious virus were determined by plaque assay. Complementation of 7134 DNA by pd plasmids (upper panel) and pn plasmids (lower panel) are shown.

resulting from cotransfections with 7134 and pSH DNA was typically small. Therefore, the increase in viral titer was due largely to complementation by wild-type ICP0 encoded by pSH. Taken together, these observations indicate an important role for ICP0 in the de novo synthesis of infectious virus.

Individual mutant plasmids were next used in cotransfections with infectious 7134 DNA to test their ability to provide ICP0 function by complementation (Fig. 6). The data shown in Fig. 6 are yields on day 2 posttransfection. As described above, pSH increased the titer of 7134 by 2 to 3 orders of magnitude at molar ratios of 1:1 and 40:1. No significant increase in the titer of 7134 above basal level (arrows) was observed with any of the deletion (pd) plasmids (upper panel) at either ratio of plasmid DNA-to-infectious KOS DNA tested. The nonsense mutant plasmids, however, exhibited varying levels of complementing activity (lower panel). pn770 expressed the same level of activity as pSH did. pn720 activity was lower than that of pSH and pn770 by approximately 1 order of magnitude at both molar ratios tested. pn525 activity was lower than that of pSH by approximately 2 orders of magnitude, while pn428 activity was barely detectable above background. Notably, pn680 exhibited minor complementing activity when the plasmid DNA-to-viral DNA ratio was 1 but not when the molar ratio was 40. The basis for this unexpected observation is currently being investigated. pn106 and pn212 expressed little or no activity at a molar ratio of plasmid DNA-to-viral DNA of 1 and minor activity at a ratio of 40:1. As in CAT assays, pn012 expressed an intermediate level of activity, especially at the higher ratio tested.

In general, the complementing activities of mutant plasmids correlated well with their transactivating activities in CAT assays (Table 1), suggesting that the critical function of ICP0 in viral replication after transfection involves stimulation of viral gene expression.

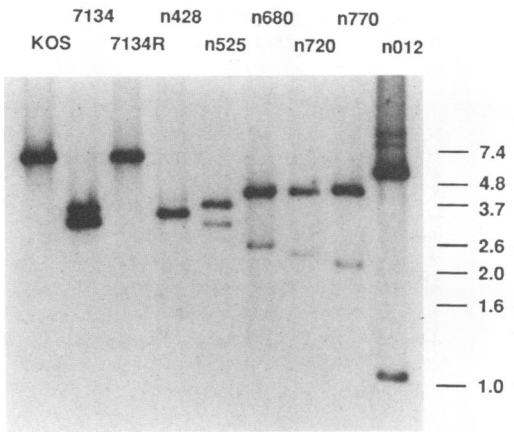

FIG. 7. Southern blot analysis of ICP0 mutant viral DNAs. Viral DNA was digested with *Sac*I and *Pst*I and the following enzymes: *Bgl*II (for KOS, 7134, 7134R, *n*428, *n*525, *n*770, and *n*012) and *Spe*I for (KOS, 7134, 7134R, *n*680, and *n*720). Refer to Fig. 1 for relevant restriction sites. The DNA fragments generated were then separated by agarose gel electrophoresis, followed by Southern blot analysis. Molecular weight markers (in kilobases) are indicated on the right. The two faint bands above the major band in the lane containing *n*012 DNA are due to partial digestion and were not evident in other gels.

Isolation of nonsense mutant viruses. In order to characterize ICP0 function in productive and latent infections, as well as to induce expression of sufficient levels of mutant ICP0 polypeptides for biochemical analysis, phenotypically informative mutations as described above were transferred to the viral genome. Transfer was achieved by homologous recombination between infectious viral DNA and individual mutant plasmid DNAs after transfection. Because ICP0 is not essential for virus replication (i.e., ICP0 deletion mutants grow and plaque in Vero cells), recombinant progeny could not be readily distinguished from parental virus. To facilitate the isolation of recombinant mutants, the ICP0 null mutant, 7134, which produces blue plaques in the presence of X-gal, proved especially useful in these studies. Cotransfection of Vero cells with ICP0$^-$/*lacZ*$^+$ 7134 DNA and mutant plasmid DNA resulted in the production of heterozygous recombinants containing one copy of *lacZ* and one copy of ICP0. These viruses would produce blue plaques upon addition of X-gal, whereas recombinant viruses in which both copies of *lacZ* were replaced by mutant ICP0 alleles would produce white plaques in the presence of X-gal.

The nonsense mutations in pn428, pn525, pn680, pn720, pn770, and pn012 were then transferred to the viral genome by cotransfection of each linearized plasmid with infectious 7134 DNA. The resulting recombinant viruses, designated *n*428, *n*525, *n*680, *n*720, *n*770, and *n*012, respectively, produced white plaques in the presence of X-gal. To verify the locations of the mutations, mutant viral DNAs were digested with appropriate restriction enzymes and subjected to Southern blot analysis (Fig. 7). After *Sac*I and *Pst*I digestion, a 6.5-kb fragment was evident in KOS DNA and two fragments, a 3.45-kb and a 2.95-kb fragment, were detected in 7134 DNA, due to the presence of a *Sac*I site within *lacZ* sequences. A 6.5-kb fragment was also detected in 7134R DNA (lane 3) and in the DNAs of the six nonsense mutants

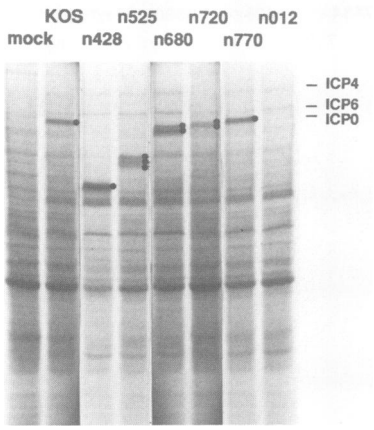

FIG. 8. ICP0 peptides specified by KOS and ICP0 mutant viruses. Monolayers of Vero cells were infected with 10 PFU per cell of each virus in the presence of cycloheximide. After 6 h, cycloheximide-containing medium was removed, medium containing 10 μg of dactinomycin per ml was added, and cultures were pulse labeled with [^{35}S]methionine for 30 min. Labeled polypeptides were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The major viral polypeptides expressed under these conditions (ICP4, ICP6, and ICP0) are indicated. Wild-type and mutant forms of ICP0 are indicated by dots.

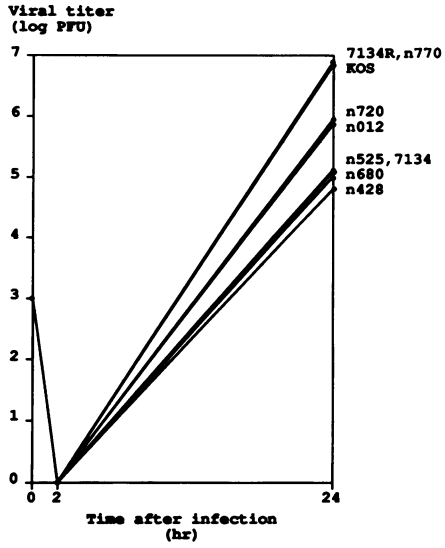

FIG. 9. Production of progeny virus after low-multiplicity infection with wild-type virus and ICP0 mutants. Subconfluent Vero cell monolayers in 35-mm petri dishes were infected with approximately 1,000 PFU of virus. Virions that did not enter cells were removed by low pH treatment after a 2-h incubation at 37°C. Cultures were harvested for virus assay at 2 h postinfection or after a 24-h incubation at 37°C. Inocula were assayed at the time of infection, and the actual multiplicity was used to normalize the 24-h titer to yield total plaque-forming units at 24 h per 1,000-PFU inoculum.

(data not shown). Codigestion with a third enzyme, *Bgl*II or *Spe*I, did not alter the restriction pattern within the 6.5-kb region for KOS, 7134, and 7134R. However, codigestion with *Bgl*II cleaved the 6.5-kb fragment into two subfragments in four mutant DNAs: two 3.25-kb fragments for *n*428 DNA, a 3.45- and a 3.05-kb fragment for *n*525 DNA, a 4.2- and a 2.3-kb fragment for *n*770 DNA, and a 5.5- and a 1.0-kb fragment for *n*012 DNA. Similarly, codigestion with *Spe*I converted the 6.5-kb fragment into two such fragments for two mutants: a 3.9- and a 2.6-kb fragment for *n*680 DNA and a 4.0- and a 2.5-kb fragment for *n*720. These alterations in restriction pattern were precisely those anticipated on the basis of the positions of linker insertions in the ICP0 gene (see Fig. 1).

Characterization of mutant viruses. (i) ICP0 mutant peptide expression. Mutant viruses were first characterized with regard to the expression of ICP0 peptides in cycloheximide reversal experiments. Wild-type strain KOS produced a peptide with a mobility corresponding to its reported size, 110 to 120 kDa (Fig. 8). For mutants *n*428, *n*525, *n*680, and *n*720, no protein which migrated as wild-type ICP0 was detected. Instead, IE peptides of faster mobilities were detected. The mobilities of these peptides were those expected due to C-terminal truncation of ICP0 caused by insertion of nonsense codons into the sites shown in Fig. 1. Interestingly, multiple forms of mutant peptides were evident in extracts of *n*525-, *n*680-, and *n*720-infected cells (Fig. 8), as well as in KOS-infected cells, when resolution was improved (not shown). For *n*770, which lacks the C-terminal 6 codons of ICP0, a peptide with a mobility indistinguishable from that of wild-type ICP0 was detected. No peptide was detected in extracts of *n*012-infected cells in this experiment, despite the likelihood that a functionally active mutant peptide is generated by translational restart at either residue 135 or 143. By using rabbit polyclonal antibodies to sodium dodecyl sulfate-gel-purified ICP0 in Western blot (immunoblot) analysis, antibody-reactive peptides were detected in extracts of KOS- and *n*720-infected cells (not shown). Again no ICP0-specific peptides were detected in extracts of *n*012-infected cells. Thus, with the exception of the *n*012 peptide, the sizes of mutant peptides confirmed the locations of nonsense mutations within ICP0 coding sequences.

(ii) Replication efficiencies of ICP0 mutants. As a measure of the ability of mutant forms of ICP0 to up regulate viral gene expression in the viral replicative cycle, the ability of the mutants to generate infectious progeny virus after low-multiplicity infection was tested. In these tests, Vero cells in 35-mm petri dishes were infected with 1,000 PFU per plate (2.7×10^5 cells) of each virus and viral titers were determined after 24 h (Fig. 9). This low multiplicity of infection (~0.004 PFU per cell) was used to assure that infected cells had received only a single plaque-forming unit each. During the 24-h test period, replication of wild-type strain KOS yielded more than 7,000 PFU per infectious unit of inoculum. In contrast, the ICP0 null mutant, 7134, produced only slightly more than 100 PFU per infectious unit of inoculum, a reduction of more than 50-fold relative to KOS. The recombinant virus, 7134R, produced a titer nearly identical to that of KOS. We conclude from these observations that the reduction in the yield of progeny 7134 virus was caused by the absence of ICP0.

Like 7134R, nonsense mutant *n*770 also produced a titer indistinguishable from that of KOS, indicating that the C-terminal 6 amino acid residues are not required to attain wild-type levels of virus replication. Mutants *n*720 and *n*012 produced titers lower than that of KOS but higher than that of 7134, indicating a partial contribution of these mutant forms of ICP0 to viral growth. The roles of the mutant forms of ICP0 specified by *n*770, *n*720, and *n*012 in generating infectious virus after low-multiplicity infection correlate well with their transactivating and complementing activities in transient expression assays. Mutants *n*680, *n*525, and *n*428, on the other hand, produced titers similar to that of 7134. Although the mutant forms of ICP0 specified by these three

viruses exhibited partial transactivating and complementating activities in transient expression assays, these activities were not sufficient to facilitate viral growth in the context of the viral genome. Consistent with this observation, the plaque sizes of KOS, 7134R, and *n*770 are larger than those of *n*012 and *n*720, which in turn are larger than those of 7134, *n*480, *n*525, and *n*608.

DISCUSSION

ICP0 is a potent and promiscuous transcriptional regulatory protein. Its regulatory activity, however, has been demonstrable only in transient expression assays by using the isolated gene in which plasmids carrying the ICP0 gene are able to transactivate all classes of viral promoters. In the context of the viral genome, ICP0 is unable to exert its regulatory activity in the absence of ICP4 (14). In this study, we have demonstrated a close correlation between the transactivating activities, complementing activities, and progeny-producing capabilities of mutant forms of ICP0. Reductions in transactivating activity correlate well with reductions in complementing activity and with lower titers of progeny virus in growth tests at low multiplicities of infection. These lines of evidence indicate that a major function of ICP0 in viral replication—both in de novo infection and infection with virions—is to stimulate viral gene expression.

As an IE regulatory protein, ICP0 is thought to stimulate viral gene expression during the initial stages of productive infection. The mechanism of initiation of viral gene expression operative during productive infection differs significantly from the mechanism operative during reactivation of virus from latency. During the initial stages of infection with virions, a 65-kDa transcriptional regulatory protein from the virus particle stimulates expression of IE genes, whereas initial gene expression in reactivation is not thought to be mediated by preexisting viral proteins. Transfection with viral DNA is similar to reactivation but different from infection with virions in that no preexisting viral regulatory proteins are thought to exist before the initiation of the reactivation process. In this study, ICP0 was shown to play a critical role in the de novo synthesis of infectious virus after transfection of viral DNA. The absence of ICP0 in these tests caused both a significant delay in the synthesis of new infectious virus and a decrease in viral titer of several orders of magnitude. If, as we suspect, this situation also pertains during reactivation in vivo, a critical role for ICP0 in the reactivation process is predicted. In support of this concept are the observations that deletion of ICP0 greatly affects the frequency of reactivation of virus from latency (32). We propose that the major function of ICP0 is to stimulate expression of viral genes at a very early stage in productive infection and reactivation so that sufficient levels of proteins are available simultaneously within a relatively short time. This function would be especially critical for reactivation from latency to ensure that the switch to productive infection occurs in a timely fashion within metabolically quiescent neurons.

It is apparent that there is a difference in the requirement for ICP0 in de novo infection with viral DNA as opposed to infection with virions. For example, the mutant form of ICP0 specified by pn525 (and perhaps the forms specified by pn428 and pn680), which exhibited limited transactivating activity in transient assays, exhibited partial complementing activity for viral replication in de novo infection after transfection with viral DNA. The same mutation in the context of the viral genome, on the other hand, appeared to have no effect on progeny virus production in infection with virions. This apparent discrepancy is likely due to the presence of the 65-kDa virion component which functions to up regulate IE gene expression in infection with virions. With respect to stimulation of IE gene expression, limited activity of mutant forms of ICP0 would likely be overshadowed by this strong up regulatory protein.

Interestingly, mutant pn525 exhibited stronger transactivating activity for two late promoters (L42 and gB) than for an IE promoter (ICP4) in transient expression assays. If this pattern holds for late promoters in the context of the viral genome, the pn525 protein would also transactivate the 65-kDa regulatory protein, a late protein. This would serve to boost gene expression in de novo viral replication. The role of the 65-kDa protein in de novo infection is suggested because a mutation in the gene specifying this protein greatly impairs viral plating efficiency after infection, which in the absence of the functional 65-kDa protein resembles de novo infection (1). Therefore, although ICP0 transactivates all three kinetic classes of HSV-1 genes, its transactivation of the 65-kDa regulatory gene may—in addition to up regulation of IE genes—prove to be especially important for de novo infection. If the 65-kDa protein is considered the earliest regulatory protein active during productive infection, ICP0 may be considered the earliest regulatory protein active in de novo infection. Currently the effects of mutations in ICP0 on the establishment and reactivation of HSV-1 from latency, as well as on gene expression during productive infection, are under investigation.

We have mapped the active domain of the ICP0 molecule to the N-terminal 427 amino acids residues of the molecule, perhaps between residues 105 and 427. The C-terminal portion of the protein is dispensable but important for full ICP0 activity. The six C-terminal-most residues of the ICP0 molecule appear to be completely dispensable for wild-type levels of virus growth. These observations are consistent with the observations of Everett (18, 19) that the predicted zinc finger domain (amino acid residues 99 to 156) is susceptible, and the C-terminal portion of the molecule is less susceptible, to mutagenic inactivation when activity is measured by transactivation of the gD promoter. Our results, using other late promoters (L42 and gB) and nonsense and deletion mutations in the N terminus of the protein, support his observations.

Because of the promiscuity of its transactivating capabilities, the mechanism of ICP0 transactivation is thought to be without promoter specificity. This notion is likely incorrect on the basis of results obtained with mutant forms of ICP0. We have observed that different mutant forms of ICP0 were able to distinguish between promoter types. Some mutant forms of the protein transactivated some promoters but not others or transactivated certain promoters better than others (Table 1). These differentially acting mutant forms of ICP0 lack portions of the C terminus of the protein and, in one case (pd104), a portion of the N terminus. On the basis of these observations, we propose that the ICP0 protein contains both an active domain, which is located between N-terminal amino acid residues 105 and 427 and includes the zinc finger domain, as well as one or more supporting domains. The active domain is required for transactivating activity. The supporting domains have at least two functions: (i) they contribute to the full transactivating capability of the protein, and (ii) they increase the range of promoter utilization. Thus, ICP0 likely possesses multiple mechanisms for transactivation of different kinds of HSV-1 promoters. Individual supporting domains may be involved in

one or more of the distinct transactivating mechanisms of ICP0 in promoter recognition. Alternatively, if ICP0 achieves promiscuous transactivation by activating various components of the cellular transcriptional machinery (e.g., cellular transcription factors), different supporting domains may be involved in activating different factors. Because of the wide variation in the responses of individual HSV-1 promoters to ICP0 transactivation, it is necessary to include a spectrum of promoters (e.g., promoters representing kinetic classes) in future efforts to further define the functional domains of ICP0.

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