The N Terminus and C Terminus of Herpes Simplex Virus 1 ICP4 Cooperate To Activate Viral Gene Expression

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Infected cell polypeptide 4 (ICP4) activates transcription from most viral promoters. Two transactivation domains, one N-terminal and one C terminal, are largely responsible for the activation functions of ICP4. A mutant ICP4 molecule lacking the C-terminal activation domain (n208) efficiently activates many early genes, whereas late genes are poorly activated, and virus growth is severely impaired. The regions within the N terminus of ICP4 (amino acids 1 to 210) that contribute to activation were investigated by analysis of deletion mutants in the presence or absence of the C-terminal activation domain. The mutants were assessed for their abilities to support viral replication and to regulate gene expression. Several deletions in regions conserved in other alphaherpesviruses resulted in impaired activation and viral growth, without affecting DNA binding. The single small deletion that had the greatest effect on activation in the absence of the C terminus corresponded to a highly conserved stretch of amino acids between 81 and 96, rendering the molecule nonfunctional. However, when the C terminus was present, the same deletion had a minimal effect on activity. The amino terminus of ICP4 was predicted to be relatively disordered compared to the DNA-binding domain and the C-terminal 500 amino acids. Moreover, the amino terminus appears to be in a relatively extended conformation as determined by the hydrodynamic properties of several mutants. The data support a model where the amino terminus is an extended and possibly flexible region of the protein, allowing it to efficiently interact with multiple transcription factors at a distance from where it is bound to DNA, thereby enabling ICP4 to function as a general activator of polymerase II transcription. The C terminus of ICP4 can compensate for some of the mutations in the N terminus, suggesting that it either specifies redundant interactions or enables the amino terminus to function more efficiently.

The herpes simplex virus 1 (HSV-1) genome is transcribed in a highly regulated, sequential cascade (25, 26) by the cellular RNA polymerase II machinery (1). VP16 is a potent transactivator that is carried into the cell in the tegument of the virion (3) and activates the transcription of the immediate-early (IE) genes (3, 4, 37). Upon subsequent protein synthesis, the IE protein infected cell polypeptide 4 (ICP4) acts as a transcriptional activator, promoting the expression of early (E) and subsequently late (L) genes (13, 19, 39, 55, 56). In addition to the activation function, ICP4 can also be a repressor of transcription in some contexts (9, 23, 34, 41). As a consequence, temperature-sensitive (*ts*) and deletion mutants of ICP4 overexpress IE proteins but are highly defective for E and L gene expression (8, 13, 38, 55).

ICP4 is a 175-kDa nuclear phosphoprotein (7, 36) that exists as a dimer in an elongated conformation (32, 47). ICP4 is also a sequence specific DNA-binding protein (12, 17). The binding to specific sites is required for the repression of the ICP4, LAT, and OrfP/L/ST promoters (18, 22, 29, 33, 41). In contrast, whereas the DNA-binding activity of ICP4 is required for activation of transcription, individual specific binding sites are not (6, 15, 16, 24, 48).

Alignment of the amino acid sequences from alphaherpesvirus ICP4 homologues reveals two large blocks that are highly conserved among the homologues between amino acids 300 to 500 and the C-terminal 500 amino acids, with respect to HSV-1 ICP4. The extensive homology shared between these homologues is suggestive of the importance of these regions (31). These regions have been shown to specify a DNA-binding domain (35, 46) and a region important for transactivation, respectively (10, 11, 35, 46). There is minimal conservation among ICP4 homologues within the N terminus of the molecule. This apparent lack of homology, however, is not indicative of the importance of this region. This

region has been shown to be important for the ability to activate (47) and repress transcription (22). Although the N terminus of ICP4 shares little homology among alphaherpesviruses, a sequence alignment of the N terminus of ICP4 homologues from HSV-1, HSV-2, and herpes B virus reveals multiple conserved blocks of amino acids between the three homologues (Fig. 1). These conserved regions may confer many of the functions of ICP4 that are attributed to the N terminus, such as the ability to form transcription complexes on early promoters (20, 44) and the ability to repress transcription in a promoter-dependent manner (22). It is also possible that some of these conserved blocks of amino acids function in conjunction with the C terminus, perhaps forming interfaces with many of the same transcription factors.

The N- and C-terminal transactivation domains are necessary to various degrees for the transactivation and repression functions of ICP4. For example, the C-terminal activation domain of ICP4 is necessary for efficient L gene activation but not for E gene activation (10). The N-terminal activation domain contains the site-specific repression functions of the molecule and some activation functions (22, 47). In addition, it appears that the different regions of ICP4 have differing roles during the lytic cycle in epithelial cells as opposed to in neuronal cells (2, 58). Deletion of the conserved polyserine tract within the N terminus of ICP4 results in a mutant that has minor defects in replication in Vero cells and at peripheral

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FIG 1 Sequence alignment of the N terminus of ICP4 from HSV-1 strain KOS, HSV-2, and herpes B virus. The predicted translation products of HSV-1 strain KOS, HSV-2 strain HG42, and herpes B virus strain E2490 ICP4 molecules were aligned using Vector NTI. Amino acids highlighted in black are highly conserved among all three viruses, while those highlighted in red are only conserved among two of the viruses. The black lines above and below the alignment indicate the deletions constructed within the N terminus of ICP4. The deletions were constructed in both the wild-type KOS background (before the backslash) and the C-terminal deletion mutant n208 (after the backslash).

sites of ocular infection in mice but does not replicate within the trigeminal ganglia of mice (2, 58). Thus, various regulatory functions of ICP4 are specified by different parts of the protein, most likely as a result of interactions with different components of the host transcriptional machinery, which in turn may be of varying importance in different cell types or at different times postinfection.

In order to more accurately define regions of importance, their functions, and whether the C terminus can contribute to these functions, several deletion mutants within the N terminus of ICP4 were constructed both in the presence and in the absence of the C-terminal activation domain. Mutants were utilized in transient assays and assessed for their ability to complement an ICP4-null virus. In addition, the mutations were constructed within both ICP4 loci of the viral genome. The viral mutants were analyzed for their ability to replicate, bind to DNA, and activate gene expression. The results indicate that there are multiple regions of importance within the N terminus of ICP4 and that the C terminus of ICP4 may cooperate with the N terminus to regulate viral gene expression.

MATERIALS AND METHODS

Cells and viruses. Vero and E5 cells were maintained as previously described (8). E5 cells express complementing levels of ICP4 (10). Viral

mutants were constructed within the background of HSV-1, strain KOS. The ICP4 mutants n208 (11), d8-10 (58), nd8-10 (43), ΔSER (2), d120 (8), and n12 (11) have been previously described. ΔSERn7 was constructed by coinfecting E5 cells with n208 and ΔSER and screening progeny plaque isolates by PCR and Southern blot hybridization for both copies of the n208 and ΔSER alleles. The mutants d3-8, nd3-8, m20, m20n7, m90, m90n7, d143, d143n7, and nd3-10 were constructed by marker transfer (8). E5 cells were cotransfected with 2 μg of the mutant plasmid DNA digested with EcoRI and 2 μg of viral DNA from either KOS, n12, or n208 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's suggestions. Plaque isolates of the transfection progeny were then screened by PCR and Southern blot hybridization for both copies of the intended alleles. Mutant viruses were plaque purified a minimum of three times.

Recombinant plasmids. The plasmids pK1-2, pn7, d3-8, nd3-8, d8-10, and nd8-10 have been previously described (10, 46). pK1-2 and pn7 encode the promoter and gene for wild-type (wt) (strain KOS) and n208 ICP4, respectively. pK1-2 and pn7 were transformed into *Escherichia coli* GS1783 (a gift from Greg Smith, Northwestern University), and site-directed deletions were constructed by using the RecET recombination system as previously described (51, 52). The primer pairs used for the construction of the corresponding mutants were as follows: m20, CCCGCATCGGCGATGCGTCGGAGAACAGCAGCGCCCCGGC CTGCAGGACCGGACGAGCGGGGTAGGGATAACAGGGTA ATCGATTT and CGTCTCCGCGCCCCACCCGAGGGCCCC

CGCTCGTCGCGGGTCTCTG**CAGG**CGGGCGCTGCTTGTTCGCCAGTGTTACAACCAATTAACC; m90, GCGGGACCGACGCGGCGAGGACACCGGGGACGCCGTCTCGT**GACAG**CATCCCGACGCCGACTAGGGATAACAGGGTAATCGATTT and GGTCCGGGGCGCGAGGCCGCGGGGTCTGGGCGTGGGATCGTCT**CACAG**CGAGACGGCGTCCCGGTGCCAGTGTTACAACCAATTAACC; d143, GC CGGCGACCGGGCCCCGGCCGGGGCGCGAAGCGGAGGCCCT**GACG**CCCGGGCCAGCCGCGCTAGGGATAACAGGGTAATCGATTT and CCACCGCCCGTGACGACGTCTCCGCGCGCGCTGGGCGGCGGCT**GACAGG**CGCTCCCGTTCTCGGCGCGCCAGTGTTACAACCAATTAACC. The six nucleotides in boldface correspond to the PstI sites, which were added at the site of the deletion for diagnostic purposes. The primers were synthesized and gel purified by IDT (Coralville, IA). The kanamycin resistance gene from pEPKan-S (52) was amplified by PCR using a Fail-safe PCR kit from Epicenter Biotechnologies under the following conditions: 50 ng of pEPKan-S, 2 μ M concentrations of the primers, and 0.5 U of Fail-safe enzyme were combined in a 1 $\times$ buffer provided by the manufacturer. The reactions were cycled at 96°C for 5 min, followed by 35 cycles of 96°C for 30 s, 60°C for 1 min, and 72°C for 1.5 min, and then finally a cycle of 72°C for 5 min. The PCR product was gel purified and transformed into GS1783 bacterial cells containing the ICP4 plasmids pK1-2 and pn7 as described previously (52). Transformants containing the appropriate mutant plasmids were selected for by resistance to kanamycin and carbenicillin and screened by restriction endonuclease and agarose gel analysis. Isolates containing the appropriate insert that lacked the wt allele were grown at 30°C for 30 min in Luria broth containing 1% arabinose. The cells were transferred to 42°C for 15 min to induce the SclI enzyme and concomitant removal of the kanamycin cassette. The cells were allowed to replicate for another 2 h at 30°C and plated on agar plates containing 100 μ g of carbenicillin/ml and 1% arabinose. Colonies were screened for their sensitivity to kanamycin and possession of the appropriate allele by restriction endonuclease and agarose gel analysis.

Transient complementation assay. A total of 5×10^5 Vero cells in 35-mm petri dishes were transfected with 2 μ g of plasmid DNA using 5 μ l of Lipofectamine 2000 as described by the manufacturer (Invitrogen). After 24 h, the cells were infected with the ICP4 mutant virus d120 at a multiplicity of infection (MOI) of 1 PFU/ml. At 24 h postinfection (hpi), the cells were scraped into the medium, disrupted by three freeze-thaw cycles, and sonicated, and the viral lysates were clarified by centrifugation at 3,000 rpm for 10 min. The total viral yield was determined by plaque assay on E5 cells.

Single-step growth analysis. A total of 5×10^5 Vero cells in 35-mm petri dishes were infected at an MOI of 5 PFU/cell in 0.1 ml of TBS (137 mM NaCl, 5 mM KCl, 0.5 mM MgCl₂, 0.68 mM CaCl₂, 25 mM Tricine [pH 7.35]) at room temperature for 1 h. After viral adsorption, the cells were washed three times in TBS to remove unadsorbed virus and 37°C medium was added. The infection was allowed to progress for 2, 4, 8, 12, 24, and 36 h at 37°C. At these times, the infected cells were scraped into the medium, disrupted by three freeze-thaw cycles, and sonicated, and viral lysates were clarified via centrifugation at 3,000 rpm for 10 min. The total viral yield was determined by plaque assay on the E5 cells.

Analysis of viral proteins. Viral proteins were analyzed by metabolic labeling and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by Western blot analysis. A total of 5×10^5 Vero cells in 35-mm petri dishes were infected at an MOI of 10 PFU/cell in 0.1 ml of TBS at room temperature for 1 h, after which time the inoculum was removed, 37°C medium was added, and the cells were incubated at 37°C for the indicated times. The infected cells were washed in cold TBS containing 0.1 mM TLCK (*N* α -*p*-tosyl-L-lysine chloromethyl ketone) and scraped into 200 μ l of sample buffer (0.05 M Tris-HCl [pH 7], 2% SDS, 0.01% bromophenol blue, 5% sucrose, 5% β -mercaptoethanol). The infected-cell extracts were denatured at 95°C for 5 min and separated by SDS-PAGE. Polypeptides were separated through a 4% polyacrylamide stacking gel (4% acrylamide, 0.19% N,N-diallyltartardiamide (DATD),

119 mM Tris, 0.1% SDS [pH 7.0]) and a 9% polyacrylamide separating gel (9% acrylamide, 0.4% DATD, 375 mM Tris, 0.1% SDS [pH 8.8]) in Tris-glycine electrode buffer (25 mM Tris, 192 mM glycine, 0.1% SDS [pH 8.5]) at 100 V.

For analysis of metabolically labeled viral polypeptides, the medium (i.e., methionine- and cysteine-free Dulbecco modified Eagle medium) on the infected cells was removed at 3.5, 7.5, and 11.5 hpi and replaced with 1 ml of 37°C TBS containing 25 μ Ci of [³⁵S]methionine (>1,000 Ci/mmol; MP Biomedicals). The cultures were incubated for an additional 30 min at 37°C, after which the labeling medium was removed, and the monolayers were washed with cold TBS containing 0.1 mM TLCK, solubilized, and analyzed by SDS-PAGE as described above. After electrophoresis, the gel was fixed in H₂O-methanol-acetic acid (6:3:1), dried, and exposed to X-ray film (HyBlot CL; Denville).

For Western blot analysis, the SDS-PAGE separated polypeptides were transferred from the polyacrylamide gel to nitrocellulose membranes by electroblotting in 1 $\times$ transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 1.5 h at 250 mA. The membrane was subsequently blocked by incubation at 4°C overnight in 5% dry milk in TBS (50 mM Tris, 150 mM NaCl [pH 7.5]) and then incubated with ICP4-specific antibodies (N15 or 58S) at a 1:500 dilution in TBS plus 0.05% Tween 20 (TBS-T) plus 1% milk for 1 h. The immunoblot was then washed in 1% milk in TBS-T four times for a total of 30 min. It was then incubated with either goat anti-mouse (for 58S) or donkey anti-rabbit (for N15) IRDye-conjugated (Li-Cor) secondary antibodies at a 1:10,000 dilution in TBS-T, subsequently washed four times for a total of 30 min in TBS-T, and finally washed twice for a total of 2 min in TBS. Images were obtained using a Li-Cor Odyssey infrared imager.

Southern blot hybridization. DNA was isolated from infected cells and analyzed by Southern blot hybridization as previously described (42, 50). Infected cell DNA was digested with the restriction endonucleases BamHI and PstI and separated by electrophoresis in a 1.2% agarose gel. A plasmid containing the BamY fragment of the HSV-1 genome was used as the probe DNA and labeled with [³²P]CTP and [³²P]GTP (GE Healthcare) using a Nick translation system (Invitrogen) according to the manufacturer's instructions. The results were visualized by autoradiography.

Northern blot analysis. A total of 5×10^6 Vero cells in 100-mm petri dishes were infected at an MOI of 10 PFU/cell at room temperature for 1 h. The viral inoculum was removed, 37°C medium was added, and the cells were incubated at 37°C for 4 and 8 h. The monolayers were then rinsed once with cold TBS, and 2 ml of TRIzol reagent (Invitrogen) was added. The infected cells were scraped into the TRIzol reagent, and RNA was harvested according to the manufacturer's (Invitrogen) guidelines. RNA was treated with 10 U of RNase-free DNase I at 37°C for 15 min and then ethanol precipitated.

Total RNA was quantified using a NanoDrop 2000 (Thermo Fisher). RNA samples were prepared for electrophoresis by diluting 13 μ g of RNA into 4.5 μ l of water and adding 2 μ l of 10 $\times$ morpholinepropanesulfonic acid (MOPS) running buffer (1 $\times$ MOPS running buffer is composed of 20 mM MOPS, 1 mM EDTA, 5 mM sodium acetate), 3.5 μ l of 37% formaldehyde, and 10 μ l of formamide, and the samples were heated for 15 min at 55°C. Portions (2 μ l) of loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol) were added to each sample, and then the samples were run on a 1.3% agarose-formaldehyde gel (1.3% agarose and 8% formaldehyde in 1 $\times$ MOPS running buffer) at 100 V for 4 h in recirculating 1 $\times$ MOPS running buffer (20 mM MOPS, 1 mM EDTA, 5 mM sodium acetate). The gels were prepared for transfer by washing them for 45 min each in water, 50 mM NaOH, 10 mM NaCl, 100 mM Tris-HCl (pH 7.5), and finally in 10 $\times$ SSC (1.5 M NaCl, 1.5 M sodium citrate). The RNA was transferred to Nytran N (Whatman) overnight in 10 $\times$ SSC, and the RNA was cross-linked to the membrane using a UV-Stratalinker (Stratagene). Membranes were probed with ³²P-nick translated DNA specific to ICP4 (BamY fragment), ICP0 (plasmid pW3 Δ HS8 containing the ICP0 coding sequence), tk (the SacI-Sall fragment corresponding to positions 555 to 1217 relative to the start site),

LAT (positions 5557 to +7559 of the BamB fragment), or gC (plasmid pSXgC containing the gC coding sequence) as previously described (27). The results were visualized via autoradiography.

DNA binding assays. Protein extracts were prepared by scraping 10^6 infected (MOI = 10 PFU/cell for 6 h) Vero cells in 60-mm petri dishes into TBS plus 0.1 mM TLCK and then pelleting the cells at 12.5K for 20 s. The pellet was then resuspended in 30 μ l of TE (Tris-HCl [pH 8.0], 1 mM EDTA), 30 μ l of 2 $\times$ lysis buffer (100 mM Tris [pH 8.0], 1 M KCl, 4% NP-40), and 0.1 mM TLCK. The samples were incubated on ice for 45 min. Cell debris was pelleted at 12.5K for 10 min, and the supernatant was used for the binding reaction. Binding reactions were prepared with 3 μ l of the protein extract, 1 ng of 32 P-end-labeled probe DNA, and 2 μ g of the nonspecific inhibitor dI-dC (polydeoxyinosinate-polydeoxycytidylate; Midland Certified Reagent Company) in binding buffer (10 mM Tris [pH 7.5], 1 mM EDTA, 5% glycerol, 0.1% NP-40, 50 mM KCl, 0.1 mM TLCK). The probe DNA utilized in this assay was a fragment of the ICP4 promoter termed P4, which covers bp -108 to +27 relative to the transcription initiation site of ICP4. The binding reactions were incubated at room temperature for 30 min and then run on a 4% polyacrylamide gel in 0.5 $\times$ TBE (1 $\times$ TBE; 88 mM Tris, 88 mM boric acid, 1.25 mM EDTA) at 200 V for 1 h 45 min. The gel was then dried, and the results were visualized by autoradiography.

Size-exclusion chromatography. Infected cell extracts were prepared as described above for the DNA-binding assay. The extracts were run on a calibrated Superose 6 10/300 column (GE Healthcare), and the fractions were analyzed for ICP4 as previously described (45, 47). LMW and HMW gel filtration calibration kits (GE Healthcare) were used to calibrate the columns. The Stokes radii were determined as previously described (45, 47) (see Fig. 5).

Immunofluorescence. A total of 2×10^5 Vero cells were seeded onto glass coverslips in a 12-well plate. Cells were infected at an MOI of 10 PFU/cell for 1 h with the indicated viruses at room temperature. The inoculum was replaced with 37°C media, and the infections were allowed to progress at 37°C for 2 h. The monolayers were then fixed for 10 min in a 4% paraformaldehyde solution and subsequently washed three times for 5 min each time in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 5.3 mM Na_2HPO_4 , 1.7 mM KH_2PO_4 [pH 7.4]), followed by permeabilization in PBS plus 0.4% Triton X-100 plus 1% bovine serum albumin (BSA) for 30 min. The permeabilized cells were incubated with a primary antibody for ICP4 (N15; rabbit polyclonal serum) at a 1:500 dilution in PBS plus 1% BSA. The cells were then washed in PBS plus 1% BSA three times for a total of 30 min and twice in PBS for 20 min before being incubated in Alexa Fluor-conjugated secondary antibodies (Santa Cruz) at a 1:500 dilution in PBS. The cells were then washed six times for a total of 1 h in PBS, and the coverslips were mounted onto a glass slide using Immumount (Electron Microscopy Services). Images were obtained by using a Olympus Fluoview FV1000 confocal microscope.

RESULTS

A mutant ICP4 molecule expressed from the virus n208 lacks the C-terminal activation 520 amino acids due to truncation. The n208 molecule retains the ability to repress transcription from the ICP4 promoter and efficiently activates early promoters, but true late gene expression is substantially impaired, resulting in relatively poor viral growth (11). Deletion of amino acids 30 to 274 from the n208 molecule results in a dimeric protein that can bind to DNA, and yet it cannot activate or repress transcription (21, 22, 47), suggesting a role for the N-terminal region in both repression and activation. Although this region may sufficiently specify interactions involved in repression and activation, it is also possible that N-terminal activation domain and the C-terminal activation domain function together to constitute the crucial functions of ICP4. To investigate the functions of the N-terminal and C-terminal activation domains with respect to the regulatory functions

of ICP4, deletions within the N terminus of ICP4 were constructed in the context of the entire 1294-amino-acid protein and in the background of the truncated n208 molecule.

Sequence alignments of ICP4 homologues between multiple members of *Alphaherpesviridae* showed very little homology within the N terminus of ICP4. However, a sequence alignment between the primate alphaherpesviruses, HSV-1, HSV-2, and herpes B virus revealed several relatively conserved regions within the N terminus of ICP4 (Fig. 1). The sequence shown for HSV-1 is the N terminus of the KOS strain. Differences between the KOS amino acid sequence and strain 17 (31) are highlighted in green with the strain 17 residue given above the main sequence. The sequences highlighted in black are conserved in all three viruses, while those highlighted in red are conserved in two out of three.

Deletions were constructed as indicated in Fig. 1 such that a PstI site was also inserted into the site of the deletion. The nomenclature for the newly constructed mutants, m90/m90n7 for example, represents the indicated deletion in the full-length molecule, and the C-terminal truncation mutant n208 (n7). The Δ ser mutants were previously constructed (2). The d3-8 and d8-10 mutants were also previously constructed (46), with the “nd” version representing the deletion within the n208 molecule. The resulting clones containing the deletions of interest, were verified by restriction digestion and agarose gel electrophoresis.

The mutant plasmids were assessed for their ability to complement an ICP4-null virus, d120, as any defects in complementation ability may be predictive of defects in viral transcription. Briefly, ICP4 mutant plasmids were transfected into Vero cells. The transfected cells were used as hosts for infection with d120. Transfected wt ICP4 expressed from the plasmid pK1-2 resulted in an $\sim 10^5$ -fold increase in the yield of d120 over mock-transfected cells (not shown). Figure 2 displays the results of the complementation assay for each mutant as a percentage of the yield resulting from the pK1-2 transfection. Of the mutants containing the C-terminal 500 amino acids, m90, d8-10, and d3-8 were partially impaired, while the mutant that deleted the whole region d3-10 was highly impaired. Truncation of the C terminus (n208) resulted in a 300-fold decrease in the yield of d120. The deletions within the N terminus of the n208 molecule had a similar effect to the deletions within the intact molecule, with m90n7, nd3-8, nd8-10, and nd3-10 yielding very little to no d120 in excess of mock transfections. These defects in complementation ability are most likely reflective of reduced transcription of essential viral genes and predictive of the possible phenotypes of viruses bearing these mutations. In addition, transient transfections of the plasmids followed by Western blot analysis revealed that each mutant plasmid specified a protein of the approximate expected molecular weight, demonstrating that defects in complementation were not the result of stability issues or unintended gross rearrangements (data not shown).

To more thoroughly assess the effects of the mutations on the viral life cycle, the deletions were constructed in both ICP4 alleles within the wild-type and n208 genomes, as described in Materials and Methods. The d3-10 construct proved to be transdominant-negative, and therefore we were unable to construct a virus with this mutation. The PstI insertion at the deletion in all of the mutants was used as a diagnostic for the intended mutations. Mutant viral DNA was digested with BamHI and PstI and then separated via agarose gel electrophoresis. Southern blot analysis was performed using the BamY fragment of the genome as a probe. Figure 3 shows the results from the Southern blot analyses of the viral

FIG 2 Complementation activity of mutant plasmids. ICP4 mutant expressing plasmids were transfected into Vero cells and their ability to complement the ICP4-null virus, d120, was determined as described in Materials and Methods. The activity is expressed as the percent yield relative to the transfection with the plasmid expressing wt ICP4 (pK1-2).

deletion mutants. As expected, the wild-type and n208 genomes, which do not have a PstI site, yielded a fragment of 1.84 kb. PstI cleaved this 1.84-kb fragment from the deletion mutants into two smaller fragments (Fig. 3), indicating that the genotypes of the mutant viruses were as intended. Once it was established that each viral mutant had the correct genotype, we wanted to ascertain (i) that each mutant virus was expressing an ICP4 protein of the correct size, (ii) whether each mutant molecule localized to the nucleus of infected cells, and (iii) whether mutant molecules retained their ability to

bind to DNA. To investigate whether mutant ICP4 molecules were expressed and whether the molecules were the expected molecular weight, SDS-PAGE, followed by Western blot analyses were performed. The analysis was performed using the ICP4 N-terminal N15 polyclonal rabbit serum as a probe. Figure 4A shows the results of the Western blot analyses. The mutants, nd3-8 and nd3-10, appear as though they are not expressed within the context of viral infection. However, since these mutations consist of large deletions within the N terminus and the antibody N15 recognizes regions within the N

FIG 3 Structure of mutant virus genomes. (A) Diagram of deletions relative to the ICP4 gene. The location (PstI site) and size of the deletions are given by the vertical arrowhead. The sizes (in kb) of the predicted BamHI/PstI fragments for each mutant are also given. (B) Southern blot of BamHI/PstI-digested viral DNA. The 1.84-kb BamHI “Y” fragment (in panel A) was used as a probe.

FIG 4 ICP4 mutant viruses produce proteins of the expected molecular weight and subcellular localization. (A) Whole-cell extracts were collected from wild-type and mutant-infected Vero cells at 6 hpi, and proteins were separated on a 9% polyacrylamide gel. ICP4 proteins were detected with either the ICP4 N-terminal N15 antibody (top and bottom panels) or the ICP4 C-terminal antibody 58S (middle panel). (B) Immunofluorescence of ICP4 in virus-infected cells.

terminus, we hypothesized that the N15 antibody primarily recognized the regions deleted in d3-8 and nd3-8. To address this concern in part, Western blot analyses were also performed with the ICP4 C-terminal monoclonal antibody, 58S. As also

displayed in Fig. 4A, the d3-8 mutant is expressed to a level similar to that of wild-type ICP4. Although the antibodies at our disposal cannot detect nd3-8 and nd3-10, we presume that these truncated proteins are being produced. In addition, all

FIG 5 EMSA on ICP4 mutants. Infected Vero cell extracts were prepared and incubated with ^{32}P -end-labeled DNA corresponding to the mRNA start site region of ICP4 as described in the text. The protein-DNA complexes were separated by native PAGE and visualized by autoradiography. Each lane is labeled with the corresponding virus from which the protein extracts were collected.

deletion mutants produced ICP4 molecules of the anticipated molecular weight relative to wild-type ICP4 (Fig. 4A).

Immunofluorescence assays were performed to determine whether the mutant ICP4 molecules localized to the nucleus of the infected cells. Vero cells grown on glass coverslips were infected at an MOI of 10 for 2 h before being fixed and stained with the ICP4 N-terminal antibody N15 and an Alexa Fluor-conjugated secondary antibody. Staining was imaged on a confocal microscope at $\times 102$ magnification. Figure 4B shows the images collected from the immunofluorescence assay. Images show ICP4 staining within the nucleus of infected cells, indicating that all mutant ICP4 molecules localize to the nucleus during infection (Fig. 4B). Again, the d3-8, nd3-8, and nd3-10 are not strongly recognized by this antibody. Some faint nuclear staining can be visualized with the d3-8 and nd3-8 mutants; however, the staining is weaker than that of the wild-type virus.

To determine whether the mutant ICP4 molecules were capable of binding DNA, electromobility shift assays (EMSA) were performed. Protein extracts were collected from infected Vero cells and incubated with ^{32}P -end-labeled DNA corresponding to the region from bp -108 to $+27$ relative to the transcription initiation site of ICP4 in the presence of competitor DNA. Protein-DNA complexes were then separated on a 4% native polyacrylamide gel, and the protein complexes bound to the radiolabeled probe were visualized by autoradiography. The results of the electromobility shift assay are shown in Fig. 5. Bands were seen in the mock-infected lane, indicating that some cellular proteins are capable of binding the ICP4 promoter region. An additional band is visible within the KOS-infected extract band, indicating that wild-type ICP4 can bind the probe under these conditions. In addition, all of the ICP4 deletion mutants, with the exception of n12, which is missing the DNA-binding domain, retained the abil-

ity to bind to the probe DNA, as revealed by an additional band of the anticipated mobility present that is not present in the mock-infected cell extracts (Fig. 5). Of importance, both nd3-8 and nd3-10, which were not detectable by Western blot, also bound DNA, indicating that these mutant proteins are in fact produced in the context of viral infection. In addition, all of the mutants that possess the C-terminal 520 amino acids produced gel shifts of lower mobility and intensity relative to the main gel shift, whereas the viruses lacking the C terminus only produced a single gel shift. This is due to the previously shown property of the C terminus, which results in the multimerization of ICP4 on DNA (28).

An additional property of ICP4 is revealed by the mobility of the shifts in Fig. 5. Deletions within the N terminus significantly increased the mobility of the ICP4/DNA complex, whereas deletion of the entire C-terminal 520 amino acids (n208) had a relatively small effect by comparison. Size-exclusion chromatography on Superose 6 was performed on the full-length protein (KOS), n208, d3-8, d8-10, and nd3-8 ICP4 molecules to determine their Stokes radii. Relatively small deletions from the primary sequence at the amino terminus (d3-8 and d8-10) resulted in greater reductions in the Stokes radius than deletion of the 520 amino acids at the C terminus. The reduction in Stokes radii roughly corresponded to the increase in the mobility of the protein-DNA complex. These data suggest that the amino terminus more greatly contributes to the elongated nature of the molecule.

The facts that all ICP4 mutant molecules produced the expected size protein, retained the ability to bind to DNA, and localized to the nucleus indicates that any defects in the regulation of viral gene expression are a direct effect of the deletions within the transactivation domain(s). To determine how the deletions affect the virus life cycle, single-step growth and viral gene expression analyses were performed on mutant virus-infected cells. For sin-

FIG 6 Single-step growth curve analysis of mutant viruses. (A) Growth curve analyses of ICP4 mutants that contain the C terminus. (B) Growth curve analyses of ICP4 mutants that do not contain the C-terminal activation domain.

gle-step growth analysis, Vero cells were infected at an MOI of 5 for 2, 4, 8, 12, 24, and 36 h. Cells were harvested in their media, subjected to three freeze-thaw cycles, and sonicated, and viral lysates were clarified via centrifugation. Viral yields were determined by plaque assay on the E5 complementing cell line and plotted versus time postinfection (Fig. 6).

At 24 hpi, wild-type KOS-infected cells yielded $\sim 10^9$ PFU, which is $\sim 1,000$ PFU per cell. The DNA-binding-deficient virus n12, which contains only the first 251 amino acids of the ICP4 protein, did not produce a viral yield higher than the initial inoculum and was reduced by more than 4 orders of magnitude with respect to the wild type. The viral growth profiles of m20, m90, d143, and ΔSER did not differ significantly from the wild type. These mutants all contain small deletions (~ 10 amino acids) within the N terminus of ICP4. However, the mutants d3-8 and d8-10, which have deletions of 112 and 68 amino acids, respectively, were reduced in viral yield by ~ 1.5 orders of magnitude (Fig. 6A). The yield of the C-terminal deletion mutant n208 was reduced by ~ 2.5 orders of magnitude relative to the wild-type virus. In the absence of the C terminus, the m20n7 and d143n7 mutants have growth profiles similar to n208, while the m90n7, Δsern7, nd3-8, nd8-10, and nd3-10 mutants had growth profiles similar to that of the n12 virus (Fig. 6B). Of these, m90n7 and Δsern7 are perhaps the most interesting since in the presence of the C terminus they have growth profiles similar to that of the wild type but in the absence of the C terminus they appear to have a relatively greater effect, underscoring the importance of these regions when the C terminus is not present. Furthermore, the relative growth properties of the mutant viruses closely correspond to the ability of the mutant plasmids from which they were derived to complement d120, strongly supporting that the defects of the viruses are due to the mutations in ICP4.

Given that the deletion mutants displayed various defects in viral growth (Fig. 6), it may be anticipated that the spectrum of viral genes expressed as a function of the mutant ICP4 molecules will also vary and differ considerably from that of wt virus. To investigate this, the kinetics of viral polypeptide synthesis and the abundance of representative viral mRNAs were determined for the mutants and wt virus. To investigate viral protein synthesis, Vero cells were infected at an MOI of 10 PFU/cell with the indicated viruses, and the proteins were metabolically labeled with

[³⁵S]methionine at 3.5 to 4, 7.5 to 8, and 11.5 to 12 hpi. Laemmli extracts of the infected cells were prepared and subsequently separated on 9% SDS-polyacrylamide gels. Figure 7 is a collage of SDS-gel profiles of mock-infected cells and wt, n208, m90n7, nd3-10, and n12 virus-infected cells. The phenotypes of these viruses are representative of five general classes (I to V). The phenotypes of the remainder of the viruses are categorized into one of the five classes and are indicated below the representative member of the class.

Class I is represented by wt virus (strain KOS). The classic progression from IE (ICP4 and ICP27) to true L (ICP1/2 and ICP19/20) is evident. The ΔSER, d8-10, m20, m90, and d143 viruses share this phenotype, suggesting that these mutations alone do not greatly affect the ability of ICP4 to function. In contrast, class V, which is represented by the virus n12, was completely defective. The proteins ICP0, ICP6, and ICP27 were highly overexpressed, and later proteins were not easily detected, as previously shown (11). None of the mutants in the present study precisely displayed the phenotype of n12.

Class II is represented by n208. n208 has nonsense mutations in all three reading frames such that the molecule is truncated at amino acid 774. Despite lacking the C-terminal 520 amino acids, readily detectable quantities of ICP5, ICP8, gB, and ICP25 were detected, whereas the expression of the mutant ICP4 protein and ICP27 diminished over time. Importantly true late proteins were not easily detectable. This is consistent with previous findings (11). Like their counterparts in the context of the otherwise wild-type background, the mutations in ΔSERn7, m20n7, and d143n7 had little effect on the n208 phenotype. Interestingly, the 112 amino acids in the N terminus of the d3-8 ICP4 (Fig. 1) had similar effects on the activity of the ICP4 molecule as the lack of the C-terminal 520 in n208, as determined by this assay.

Class III is represented by m90n7. This 15-amino-acid deletion (Fig. 1) had a profound effect on the activity of the n208 molecule, greatly reducing the abundance of ICP5, ICP8, ICP25, and gB. The region deleted by this mutation represents the most conserved stretch of amino acids in the N terminus of ICP4. Interestingly, this deletion had a relatively small effect in the presence of the C-terminal region of ICP4 (m90). nd3-8 had a similar phenotype to m90n7, an observation consistent with the fact that the region deleted in nd3-8 contains the region deleted in m90n7. The dele-

FIG 7 Protein expression profiles from ICP4 mutant viruses. Vero cells infected with the indicated viruses were labeled for 30 min at 4, 8, and 12 hpi. SDS-PAGE was performed as described in the text. Viruses with similar phenotypes are listed below the examples shown. Where possible, the ICP numbers are given. M, mock-infected cells.

tion in nd8-10 consists of 68 amino acids just C-terminal to the d3-8 deletion residues and has a similar effect on the activity of the n208 molecule. Thus, two nonoverlapping regions of the N terminus have a similar effect on the activity of the ICP4 molecule.

Class IV is represented by the virus nd3-10. This is the only member of this class. The deletion in this virus combines the two deletions that independently resulted in a marked reduction in viral later gene expression in the n208 background. As a consequence, gene expression beyond the IE class of virus genes was very difficult to detect and was similar to that of class V (n12). The phenotypes nd3-10 and n12 subtly differ in that ICP6, ICP0, and ICP27 appear to be more abundantly expressed in n12-infected cells.

To more directly assess the activities of the mutant ICP4 molecules synthesized during infection, the abundance of mRNAs for genes representative of different kinetic classes was determined by Northern blot analyses (Fig. 8). The abundances of ICP4 and ICP0 (IE), tk (E), and gC and LAT (L) mRNAs were determined at 4 and 8 hpi. The IE transcripts for ICP4 and ICP0 accumulate differently during infection. ICP4 accumulates early and then is reduced in abundance, whereas ICP0 continues to accumulate throughout infection (57). The decreased accumulation of ICP4 is due to the ability of ICP4 to repress transcription as a function of an ICP4 binding site at the start site of ICP4 transcription. All of the mutants show less ICP4 mRNA at 8 h relative to n12, suggesting that

they all may retain the ability to repress transcription. Another observation that supports this is that none of the mutants that express LAT demonstrate accumulation at early times. It has been shown that the LAT promoter is converted to an early promoter by mutating a repressive ICP4 binding site at the start site of LAT transcription (40). Interestingly, in the absence of the C terminus of ICP4, ICP0 is still highly expressed at 8 hpi but is not as highly expressed as the wild type. This is true of all mutants lacking the C terminus (Fig. 8, right panel), perhaps reflecting the defect in activation.

tk (early) mRNA was not detected at this level of exposure in n12-infected (Fig. 8). At 4 hpi, the abundance of tk mRNA in n12-infected cells is ca. 2% that of wt (strain KOS) virus (27). Although tk RNA was expressed in all of the backgrounds containing an intact C terminus, its abundance was significantly reduced in m90- and d3-8-infected cells. The larger growth defect (Fig. 6) and more restrictive SDS-gel profile (Fig. 7) of d3-8 relative to m90 is probably because of defects in later gene expression. The abundance of the late RNAs (gC and LAT) in d3-8-infected cells were considerably less than in m90-infected cells (Fig. 8). In the absence of the C terminus, the abundance of tk RNA in nd3-10-infected cells was as low as in n12-infected cells. tk mRNA was also significantly reduced in m90n7- and nd3-8-infected cells. n208 was defective in late gene expression as previously shown (11), as were all of the viruses lacking the C-terminal 520 amino acids.

FIG 8 mRNA expression from ICP4 mutant viruses. RNA was isolated from cells infected at an MOI of 10 PFU/cell for 4 and 8 h. Northern blot analysis was performed as described in Materials and Methods, probing for ICP0, ICP4, tk, gC, and LAT.

Some of the other deletions had a more modest effect on tk and gC mRNA abundance. The deletion of amino acids 142 to 210 in d8-10 resulted in a modest reduction in tk, gC, and LAT accumulation. Lastly, the deletion in d143 affected the kinetics IE (ICP4 and ICP0) and tk expression. Therefore, while the conserved block of amino acids deleted in m90 (i.e., amino acids 81 to 96) (Fig. 1) had the greatest effect on the accumulation of the analyzed transcripts that ICP4 activates, determinants flanking this block also affect activation as inferred by the greater defect in d3-8 relative to m90. The region deleted in d8-10 further contributes to activation, as inferred by the highly defective phenotype of nd8-10.

DISCUSSION

This study addresses the extent to which the conserved regions of the N terminus that contribute to the regulatory functions of the molecule and whether the N and the C termini of ICP4 have any redundant or overlapping functions. To address these questions, directed deletions were constructed within the N terminus of ICP4, both in the presence and in the absence of the C-terminal regulatory region, and the resulting mutant viruses were compared with respect to viral gene expression. We have previously shown that the region N terminal to the DNA-binding domain of ICP4 is important for activation and repression (5, 22, 46). In the present study we found that multiple regions within the N terminus contribute to activation. To put these findings into perspective, it is helpful to consider some general properties of the ICP4 molecule and the N terminus in particular.

ICP4 exists in cells as an obligate dimer possessing hydrodynamic properties suggestive of an elongated protein with a Stokes radius of about 95 to 100 Å (32, 47). The N terminus contributes greatly to the elongated character of ICP4 relative to the C terminus (Fig. 5). Deletions in the 50- to 100-amino-acid range within the N terminus more substantially reduced the Stokes radius of ICP4 compared to the deletion of the entire 500 amino acids of the

C terminus. The N terminus also has a greater effect on the magnitude of the mobility shift of an ICP4/protein DNA complex (Fig. 5). This suggests that an ICP4 molecule bound at a fixed ICP4 binding site may be able to act at a considerable distance engaging in interactions between cellular molecules and domains within the N terminus.

Another property to consider is the predicted degree of order versus disorder in the ICP4 molecule. Disordered regions are generally of low complexity and are flexible. The probability of disorder can be predicted (54) with the use of an online server (<http://bioinf.cs.ucl.ac.uk/disopred> [53]). Figure 9A shows this prediction for the entire ICP4 (strain KOS) protein. There are two regions that have a fairly low probability of disorder. The first is the domain between amino acids 300 and 600. This constitutes the DNA binding/dimerization domain. The second is the C terminus of the molecule roughly from amino acids 900 to 1294. This is largely the region deleted from the n208 molecule. It is also the region that when deleted had a relatively small effect on the Stokes radius. Together, these observations suggest that this is a relatively ordered, more globular part of the ICP4 molecule. In between the two regions of relative predicted order is a region from amino acids 700 to 850 that is predicted to be highly disordered. This region is poorly conserved among the ICP4 homologs in other alphaherpesviruses.

The amino terminus is a heterogeneous mixture of ordered and disordered regions, with the conserved regions not necessarily corresponding to the degree of predicted disorder (Fig. 9). A conserved disordered region may fold upon binding to a functional target, such as a cellular transcription factor. Other disordered regions may serve as flexible linker regions between interacting regions (14). One such region may be that corresponding to amino acids 82 to 97, which are deleted in m90 (Fig. 9C). In the absence of the C terminus, this region had a relatively large effect on the activity of the molecule. It is also the most conserved region within the region within the N terminus surveyed by this analysis

FIG 9 Disorder prediction for the ICP4 protein. (A) The probability of disorder for ICP4 (strain KOS) was determined as described in the text. (B) Expansion of panel A for the amino terminus of ICP4. The degree of conservation between HSV-1, HSV-2, and herpes B virus from Fig. 1 is also shown along the axis depicting the residue number. (C) Relative locations of the deletions examined in these studies.

(Fig. 1). It is possible that this region is important for making contacts with key transcription factors such as TFIID and mediator, which are known to form a complex with ICP4 in infected cells (30). However, the participation of any region within the N terminus

in contacts with other molecules cannot be ruled out. It is likely that there are multiple regions within the N terminus that participate in protein-protein interactions that are connected by flexible linker regions, enabling ICP4 to make multiple contacts

with the cellular transcription machinery, possibly at a distance from where it is bound to DNA.

The presence of the C terminus almost completely masked the deleterious effects of the m90 deletion. Therefore, the C terminus independently substitutes for the activity of the deleted amino acids, or it augments the activity of the N terminus that remains when these amino acids are deleted. The n208 molecule interacts with TBP and TFIID on DNA (20, 49), and the contributing protein-protein interactions are specified by amino acids 30 to 274 (20, 22). However, the C terminus of ICP4 specifies an interaction with TAF1 enabling ICP4 to interact with TFIID in solution (5). Therefore, there are regions within both the carboxyl- and amino-terminal regions that specify interactions with TFIID that could potentially synergize to recruit TFIID. The regions of ICP4 involved in interactions with mediator are unknown at present. However, since mediator is ubiquitously involved in polymerase II transcription, as is TFIID, regions interacting with these two central complexes will be operationally redundant to some extent.

The region deleted in d8-10 (amino acids 142 to 210) had a considerable effect on activation, particularly in the absence of the C terminus. Part of this is due to contribution of the region deleted in d143. Because d8-10 still retains sequences deleted in m90, it likely functions in gene activation by a distinct mechanism. At present, it is not clear whether this contribution is as a spacer or due to interactions with different transcription factors, or both.

It is likely that the entire region between comprising the N-terminal 200 amino acids is involved in interactions with multiple transcription factors. The hydrodynamic properties of mutants in this region suggest that it extends out from the DNA-binding domain and is potentially flexible, allowing it to contact multiple transcription factors and act at a relative distance from where ICP4 is bound to DNA. This region, along with the DNA-binding domain, functions as an activator, resulting in activities characteristic of n208. It is important to note that while n208 is relatively defective for viral growth compared to wt virus, some viral growth does occur (Fig. 6). Perhaps the more globular and conserved C-terminal region (amino acids 774 to 1294) “augments” the activities of the N terminus either by specifying redundant interactions with the N terminus or interactions affecting the location of ICP4. One example of the latter type of interaction is that the C-terminal 500 amino acids allow ICP4 to multimerize on DNA, increasing its affinity for DNA (28). This would enhance the ability of the interactions specified by the N terminus to contribute to activation. Further studies are under way to test this model.

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