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Physical Interaction between the Herpes Simplex Virus Type 1 Immediate-Early Regulatory Proteins ICP0 and ICP4

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The herpes simplex virus type 1 immediate-early protein ICP0 enhances expression of a spectrum of viral genes alone and synergistically with ICP4. To test whether ICP0 and ICP4 interact physically, we performed far-Western blotting analysis of proteins from mock-, wild-type-, and ICP4 mutant virus-infected cells with in vitro-synthesized [^{35}S]Met-labeled ICP0 and ICP4 as probes. The ICP4 and ICP0 polypeptides synthesized in vitro exhibited molecular weights similar to those of their counterparts in herpes simplex virus type 1-infected cells, and the in vitro-synthesized ICP4 was able to bind to a probe containing the ICP4 consensus binding site. Far-Western blotting experiments demonstrated that ICP0 interacts directly and specifically with ICP4 and with itself. To further define the interaction between ICP0 and ICP4, we generated a set of glutathione *S*-transferase (GST)–ICP0 fusion proteins that contain GST and either ICP0 N-terminal amino acids 1 to 244 or 1 to 394 or C-terminal amino acids 395 to 616 or 395 to 775. Using GST-ICP0 fusion protein affinity chromatography and in vitro-synthesized [^{35}S]Met-labeled ICP0 and ICP4, ICP4 was shown to interact preferentially with the fusion protein containing ICP0 C-terminal amino acids 395 to 775, whereas ICP0 interacted efficiently with both the N-terminal GST-ICP0 fusion proteins and the C-terminal GST-ICP0 fusion proteins containing amino acids 395 to 775. Fusion protein affinity chromatography also demonstrated that the C-terminal 235 amino acid residues of ICP4 are important for efficient interaction with ICP0. Collectively, these results reveal a direct and specific physical interaction between ICP0 and ICP4.

Regulation of herpes simplex virus (HSV) gene expression has long been recognized to proceed in a sequential and highly regulated manner. The HSV-1 genome contains at least 70 unique genes (35) which have been divided into three major classes, designated immediate-early, early, and late, based on the temporal order of their expression during productive infection (29, 52). Transcription of the five immediate-early genes is activated by VP16, a constituent of infecting virus particles (4, 10, 44). The immediate-early genes are transcribed by cellular machinery prior to the initiation of viral protein synthesis (7, 30, 48, 63). The protein products of the immediate-early genes, designated infected cell polypeptide 0 (ICP0), ICP4, ICP22, ICP27, and ICP47, are the major regulatory proteins of the virus. ICP0, ICP4, ICP22, and ICP27 are nuclear phosphoproteins that orchestrate the expression of all classes of viral genes (60). ICP0 and ICP4 are the major activators of HSV gene expression. ICP27 plays a role in regulating the expression of both early and late genes at the level of mRNA processing (34, 36, 39, 54, 56). Little is known about the role of ICP22 in productive infection except that it appears to enhance the level of ICP0 in a cell-type-specific manner (49). ICP47 is a cytoplasmic protein of unknown function. ICP4 and ICP27, but not ICP0, ICP22, or ICP47, are essential for virus replication.

Studies of temperature-sensitive and deletion mutants in the gene encoding ICP4 have shown that this protein is the major transcriptional regulatory protein of HSV-1 and that its function is required at very early times in infection (8, 9, 11, 14, 16, 47). With an apparent molecular mass of 175 kDa, ICP4 is a DNA-binding protein that interacts directly with DNA at the consensus sequence, ATCGTCNNNNYCGRC (where R is a purine, Y is pyrimidine, and N is any base). Consensus ICP4 binding sites are found in the promoters of the immediate-early genes encoding ICP0 and ICP4 (15, 24, 25, 40) and in the promoter of the gene specifying the family of joint-spanning transcripts, the L/STs (68). ICP4-DNA interactions at ATCG TCNNNNYCGRC motifs near the transcription initiation sites of the ICP4, ICP0, and L/ST promoters result in the repression of transcription from these promoters (2, 14, 19, 22, 43, 68). ICP4 also binds to a variety of related sequences in the regulatory regions of early and late genes (15, 38). Although poorly understood, the interaction of ICP4 with these related sequences appears to be critical for ICP4's ability to activate the expression of early and late genes (14, 43, 53, 58). Imbalzano et al. (31) have suggested that ICP4 may activate the expression of early and late genes by a mechanism that does not require sequence-specific DNA binding. Recent evidence has indicated that ICP4 exerts both its repressing and its activating activities through a mechanism involving interactions with the general transcription factors, TATA-binding protein (TBP) and TFIIB, as well as with DNA (59). Collectively, available evidence indicates that ICP4 exerts its repressing and activating activities differentially as a function of the location of the ATCGTCNNNNYCGRC motif within the gene, the strength of the interaction of ICP4 with this motif, and the kind and extent of posttranslational modification which ICP4 itself undergoes (e.g., phosphorylation).

Like ICP4, the 110-kDa ICP0 is a nuclear phosphoprotein (19, 32, 45, 64). Although ICP0 is not essential for virus replication, studies of ICP0 mutant viruses have indicated that functional ICP0 significantly enhances the efficiency of virus replication, especially at low multiplicities of infection (3, 20, 55, 61). Among the five HSV immediate-early regulatory proteins, only ICP0 is capable of activating all three kinetic classes of HSV genes, as well as a number of heterologous viral and cellular genes, without apparent DNA sequence specificity

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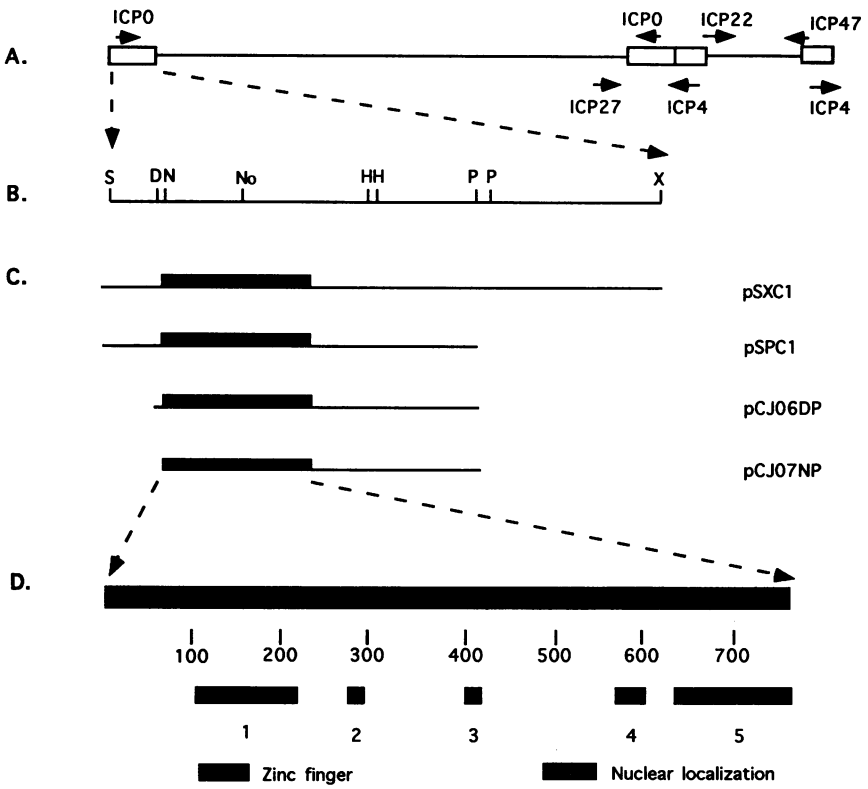

FIG. 1. (A) Diagram of the HSV-1 genome showing the approximate locations and directions of transcription of HSV-1 immediate-early genes. (B) Expanded diagram of the 8.4-kb *Sac*I-*Xba*I fragment from mutant virus 110C1, which contains one copy of an intronless ICP0 gene. Relevant restriction sites: S, *Sac*I; D, *Drd*I; N, *Nco*I; No, *Not*I; H, *Hpa*I; P, *Pst*I; X, *Xba*I. (C) Beneath the diagram of the *Sac*I-*Xba*I fragment are shown the HSV DNA sequences in plasmids pSPC1, pCJ06DP, and pCJ07NP, which contain the intronless ICP0 gene. The filled rectangle represents ICP0 coding sequence. (D) Function of domains of the ICP0 polypeptide identified by Everett (19).

(17, 18, 26, 41, 42, 50). Indeed, evidence to date indicates that ICP0 has the ability to further enhance the expression of any gene that exhibits a basal level of transcription. Because immediate-early genes have a high basal level of transcription, and since ICP4 activates transcription of early and late genes, ICP0 serves to further enhance expression of all three classes of viral genes. Notably, both ICP4 and ICP0 are present within the tegument of purified HSV-1 virions (66, 67), suggesting that upon entering newly infected cells, these proteins may function to initiate viral gene expression. Consistent with its broad transactivating activity, ICP0 plays a major role in enhancing the reactivation of HSV from latency in vivo (33). Moreover, of the five immediate-early proteins, only ICP0 is able to induce reactivation of HSV-2 from latency in an in vitro system (28).

Despite its recognized roles in enhancing viral gene expression during productive infection and in reactivation from latency, the mechanisms by which ICP0 activates gene expression alone and synergistically with ICP4 are unclear. Various insertion and deletion mutations have been introduced into the cloned ICP0 gene, and the resulting mutant forms of ICP0 have been tested for their transactivating activities in transient assays (3, 6, 19). Five functional regions of the protein have been identified to date by using this genetic approach (Fig. 1). Of particular significance are regions 1 and 5 as defined by Everett (19). Region 1 (amino acids 105 to 222) contains two zinc finger binding motifs. Mutations in region 1 result either in complete elimination or in a significant reduction in the ability of ICP0 to activate the HSV-1 gD promoter in the absence of ICP4. Mutations in region 5 (amino acids 633 to 775) markedly reduce the synergistic activation of the gD promoter by ICP0 in the presence of ICP4 (19). The functional significance of region 5 in the synergistic activation of gene expression by ICP0 and ICP4 is further supported by the studies of Chen et al. (6), which showed that deletion of C-terminal amino acid residues 628 to 769 impaired cooperative gene activation by these two proteins. Moreover, in a search for ICP0-responsive *cis*-acting elements in an early gene efficiently activated by ICP0, Ralph and Schaffer demonstrated that deletion of the 5′ untranslated leader sequence of ICP0-responsive HSV-1 genes impaired the ability of these genes to respond to ICP0 (51). Notably, deletion of the leader sequence also impaired responsiveness to ICP4 and to the combined effects of ICP0 and ICP4. Collectively, these observations suggest that ICP0-ICP4 synergy may result from a direct association between the two proteins or an indirect interaction mediated by other viral or cellular proteins.

As part of ongoing studies designed to elucidate the mechanism of ICP0-ICP4 synergy, we have examined the possibility that the two proteins interact physically. Because of difficulties associated with the solubilization of ICP4 from the nuclei of HSV-1-infected cells under nondenaturing conditions (1), coimmunoprecipitation is not the approach of choice to assess potential interactions between ICP0 and ICP4 solubilization. In this report, we describe the results of far-Western blotting analysis and glutathione *S*-transferase (GST)-ICP0 affinity chromatography which indicate that ICP0 is capable of interacting directly and specifically with itself and with ICP4.

MATERIALS AND METHODS

Cells and viruses. Vero cells were grown and maintained as described previously (54). The wild-type virus, HSV-1 strain KOS, and KOS-derived ICP4 deletion mutant viruses d2 and d156 were propagated and assayed as previously described (14). The mutant ICP4 polypeptides specified by d2 and d156 are unable to interact detectably with the consensus sequence ATCGTCNNNNYCGRC in gel shift assays (14). Cells infected with these mutant viruses express only immediate-early proteins (14).

Plasmids. Plasmid pCJ06DP contains an intronless ICP0 gene under control of the SP6 promoter in the vector pGEM-4Z (Promega Biotec, Madison, Wis.). This plasmid was constructed as follows. 110C1 (kindly provided by Roger Everett, MRC Virology Unit, Glasgow, United Kingdom) is an HSV-1 mutant virus in which both introns of the gene encoding ICP0 have been deleted (21). 110C1 DNA was isolated and purified from infected Vero cells as described previously (12). The purified viral DNA was digested first with *Bam*HI and *Eco*RI and then with *Sac*I and *Xba*I (Fig. 1B). The *Sac*I-*Xba*I fragment containing the ICP0 gene was isolated from an agarose gel by electrophoresis onto a DEAE-cellulose column. The isolated fragment was inserted into the *Sac*I and *Xba*I sites in the vector pGEM-4Z to yield pSXC1. Three *Pst*I sites are present in pSXC1, two in viral DNA sequences and one immediately to the right of the *Xba*I site in vector sequences. Plasmid pSXC1 was digested with *Pst*I, religated with T4 DNA ligase, and designated pSPC1 (Fig. 1C). To generate pCJ06DP, pSPC1 was digested with *Drd*I, treated with T4 DNA polymerase, and digested with *Pst*I, and the *Drd*I-*Pst*I fragment was then inserted at the *Sma*I and *Pst*I sites in pGEM-4Z.

To generate pGEX-2TK0, a plasmid in which the intronless ICP0 gene was fused in frame to the GST gene in the vector pGEX-2TK (kindly provided by Bill Kaelin, Dana-Farber Cancer Institute, Boston, Mass.), a plasmid, designated pCJ07NP (Fig. 1C), was first generated by inserting the *Nco*I-*Pst*I ICP0-containing fragment of pSPC1 into the *Bam*HI and *Pst*I sites in the vector pGEM-3Z as follows. Briefly, pSPC1 and pGEM-3Z were digested with *Nco*I and *Bam*HI, respectively. Ends were filled in with the Klenow fragment, and the linearized plasmids were digested with *Pst*I. The *Nco*I-*Pst*I fragment containing the ICP0 gene and the vector DNA were purified and ligated. Blunt-end ligation of the Klenow-treated *Nco*I and *Bam*HI ends regenerates *Bam*HI and *Nco*I sites at the junction. The *Bam*HI-*Hpa*I fragment containing the ICP0 gene from pCJ07NP was then cloned into the *Bam*HI and *Sma*I sites of pGEX-2TK to produce pGEX-2TK0 (see Fig. 5). An *Eco*RI site shown in Fig. 5 is derived from pGEX-2TK, which is located immediately to the right of the *Sma*I site in the vector sequences.

To generate pGST0:1-394, a plasmid expressing a hybrid protein containing N-terminal amino acids 1 to 394 of ICP0 fused to the GST gene, pGEX-2TK0 was digested with *Not*I and *Eco*RI and then subjected to treatment with Klenow fragment. The vector-containing fragment was then isolated and recircularized. pGST0:1-244 was generated by digestion of pGEX-2TK0 with *Sna*BI and *Eco*RI, ends were filled in with the Klenow fragment, and the vector-containing fragment was then recircularized. To generate pGST0:395-775, pGEX-2TK0 was digested with *Not*I and *Bam*HI and treated with Klenow fragment, and the vector-containing fragment was isolated and recircularized. pGST0:395-616 was generated as follows. pGEX-2TK0 was linearized with *Sfi*I, treated with T4 DNA polymerase, and then digested with *Eco*RI. After filling in with the Klenow fragment, the vector-containing fragment was purified and ligated with T4 ligase.

Plasmid pCJ4-S6 contains the ICP4 gene under control of the SP6 promoter in the vector pGEM-3Z. This plasmid was constructed by inserting the *Sal*I-*Eco*RI fragment containing the ICP4 gene from pn11 (13) into the *Sal*I and *Eco*RI sites in pGEM-3Z.

In vitro transcription and translation. Capped mRNAs encoding ICP0 and ICP4 were synthesized by in vitro transcription of 6 μg of *Hpa*I-linearized pCJ06DP and pCJ4-S6, respectively, using SP6 polymerase in a final volume of 50 μl of transcription buffer for 30 min at 39°C as instructed by the manufacturer (Promega Biotec). After phenol-chloroform extraction and ethanol precipitation, RNAs were resuspended in 10 μl of 0.1% diethyl pyrocarbonate-treated distilled water. Five microliters of RNA was then used for the in vitro translation reaction with 35 μl of nuclease-treated rabbit reticulocyte lysate (Promega Biotec) containing 40 μCi of [^{35}S] methionine (DuPont, NEN Research Products, Boston, Mass.) in a volume of 50 μl for 1 h at 30°C. A 2.5-μl sample of the translation mixture was then analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Preparation of cell extracts. Vero cells were either mock infected or infected with 20 PFU of either wild-type HSV-1 strain KOS or ICP4 deletion mutant d156 per cell. At 9 h postinfection, cells were washed with cold Tris-buffered saline (TBS; 137 mM NaCl, 5 mM KCl, 25 mM Tris-HCl [pH 7.4]) containing 0.4 mM phenylmethylsulfonyl fluoride (PMSF) and 50 μg of L-1-chlor-3(4-tosylamido)-4-phenyl-2-butanon (TPCK) per ml. Cells were then suspended in 500 μl of lysis buffer containing 150 mM NaCl, 50 mM Tris (pH 8.0), 1% Nonidet P-40 (NP-40), 0.5% deoxycholate, 0.1% SDS, and 0.5% β-mercaptoethanol in the presence of 0.4 mM PMSF, 100 μg of TPCK per ml, and 10 μg of leupeptin per μl for 30 min on ice. Cell debris was removed by centrifugation at 10,000 × *g* for 20 min. The supernatant fluid was stored at −70°C.

DNA binding assays. The 30-bp oligonucleotide used in this study for ICP4 DNA binding assays, 5′-GATCCCGCCCCG ATCGTCCACACGGAGCGG-3′, was synthesized by the Molecular Biology Core Facility, Dana-Farber Cancer Institute. Complementary oligonucleotides in 100 μl of 10 mM Tris (pH 8.0)–1 mM EDTA–100 mM KCl were annealed by heating at 100°C for 5 s, cooling to 68°C, incubation for 1 h, and slow cooling to room temperature. The annealed oligonucleotides were labeled with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. For ICP4 DNA binding assays, 2.5 μl of in vitro-translated ICP4 or unprogrammed reticulocyte lysate was incubated at 25°C for 30 min with the 1 ng of ^{32}P-labeled double-stranded oligonucleotides described above in the presence of 2 μg of poly(dI-dC) · poly(dI-dC) in DNA binding buffer (10% glycerol, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.4], 50 mM NaCl, 1 mM EDTA) in a final volume of 25 μl. For experiments involving antibody supershift assays, after the initial 30-min incubation, reactions were carried out for an additional 10 min in the presence of anti-ICP4 monoclonal antibody 58S as described by DeLuca and Schaffer (14). The protein-DNA complex was analyzed on a native 4% polyacrylamide gel containing 45 mM Tris-borate–1 mM EDTA (pH 8.0). In the DNA binding assays which utilized cell extracts, 2.5 μl (10 μg) of extract from mock-infected cells or cells infected with either KOS or the ICP4 mutant virus d156 was used.

Expression and purification of GST fusion proteins. *Escherichia coli* BL21 cells transformed with plasmids expressing either GST or GST-ICP0 fusion proteins were grown overnight in LB medium containing 50 μg of ampicillin per ml, diluted

1:10 in fresh LB medium in the presence of 50 μg of ampicillin per ml, and grown for an additional 2 h at 37°C with shaking. Protein expression was then induced by addition of 0.1 mM isopropylthio-β-D-galactoside (IPTG) for 2 h at 37°C. For purification of GST or the GST0:1-394 fusion protein, bacterial cultures were centrifuged at 5,000 × *g* for 10 min at 4°C and suspended in 1× TBS containing 0.5% NP-40, 100 μg of TPCK per ml, and 0.4 mM PMSF. Bacteria were lysed by sonication for 1.5 min in three 30-s bursts at 4°C, and the suspension was centrifuged at 25,000 × *g* for 30 min at 4°C. For purification of the GST0:395-775 fusion protein, after centrifugation at 5,000 × *g* for 10 min at 4°C, bacteria were lysed by three cycles of freezing and thawing in 1× TBS containing 0.5% NP-40, 1 mM EDTA, 2 mM dithiothreitol, 100 μg of TPCK per ml, 0.4 mM PMSF, and 4 mg of lysozyme per ml. Cellular debris was removed by centrifugation at 25,000 × *g* for 30 min at 4°C. The supernatant fluids were aliquoted and stored in liquid nitrogen.

For purification and functional analysis of GST and GST-ICP0 fusion proteins, 1-ml aliquots of bacterial supernatant were incubated for 1 h at 4°C with 50 μl of glutathione-Sepharose beads that had been washed three times and resuspended in TBS containing 0.5% NP-40 and 3% bovine serum albumin (BSA). After incubation, the glutathione-Sepharose beads were washed three times with TBS containing 0.5% NP-40. Beads were then suspended in 1× sample buffer (1% SDS, 1% β-mercaptoethanol, 0.5 M urea, and 10% sucrose in 100 mM Tris-HCl [pH 6.7]), and eluted proteins were resolved by SDS-PAGE.

SDS-PAGE and Western blot analysis. The procedures used for SDS-PAGE have been described previously (46). Proteins were electrophoresed on 7 or 9% polyacrylamide gels cross-linked with *N,N*-methylenebisacrylamide. Proteins on gels were either visualized directly by Coomassie blue staining or transferred to hydrophobic polyvinylidene difluoride (PVDF) membranes and immunoblotted with anti-ICP0 monoclonal antibody H1083 (kindly provided by Lenore Pereira, University of California, San Francisco) or anti-ICP0 polyclonal antibody J17 as described previously (67) except that the antigen-antibody complexes were either reacted with chemiluminescence reagent as described by the manufacturer (DuPont, NEN) and exposed on Kodak X-AR5 film (Fig. 4) or visualized by an alkaline phosphatase assay (Bio-Rad, Hercules, Calif.) (Fig. 6).

Far-Western blot analysis and protein binding assays using GST fusion proteins. For far-Western blot analysis, proteins separated on 7% gels by SDS-PAGE were electrotransferred to PVDF membranes in 1× Tris-glycine buffer (25 mM Tris base, 192 mM glycine) and renatured as follows. Blots were washed once for 10 min with 1× protein binding buffer (20 mM HEPES [pH 7.4], 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM $ZnCl_2$, 1 mM dithiothreitol, 10% glycerol) and incubated at 4°C with fresh 1× protein binding buffer sequentially for 1 h, overnight, and for 3 h. The blots were then reacted with 1× protein binding buffer containing 5% BSA for 3 h at 4°C and probed with in vitro-translated [^{35}S]methionine-labeled ICP0 or ICP4 or equivalent amounts of unprogrammed reticulocyte lysate in 10 ml of 1× protein binding buffer in the presence of 3% BSA overnight at 4°C. Excess probe was removed by washing the blot with 10 20-ml volumes of 1× protein binding buffer containing 0.1% NP-40 for 20 min at room temperature. The blot was then dried and exposed to Kodak X-AR5 film.

For protein binding assays using GST fusion proteins, equal amounts of GST and GST-ICP0 fusion proteins were purified on glutathione-Sepharose beads as described above. The purified GST or GST-ICP0 fusion proteins bound to beads were allowed to react overnight at 4°C with 25 μl of in vitro-translated [^{35}S]Met-labeled ICP0 or ICP4 in 225 μl of 1× protein binding buffer containing 3% BSA. Beads were then washed three times with 1× protein binding buffer containing 0.1% NP-40, suspended in sample buffer (2% SDS, 1 M urea, 2% mercaptoethanol, 10% sucrose, 125 mM Tris [pH 6.7]), and resolved by SDS-PAGE. The gels were dried and exposed to Kodak X-AR5 film.

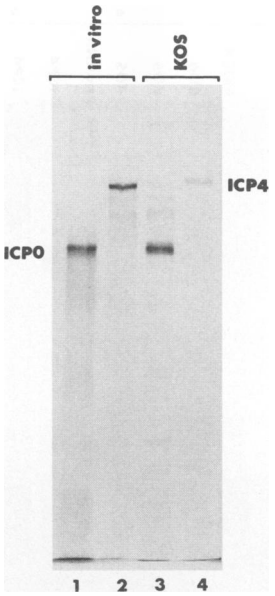

FIG. 2. Molecular weights of in vitro-translated ICP0 and ICP4. An intronless ICP0 gene (derived from mutant virus 110C1) and the gene encoding ICP4 were cloned into an in vitro transcription vector and expressed in rabbit reticulocyte lysate in the presence of [^{35}S]Met, and the resulting proteins were resolved by SDS-PAGE (lanes 1 and 2). Lanes 3 and 4 show ICP0 and ICP4 synthesized in KOS-infected cells and immunoprecipitated with anti-ICP0 (H1083) or anti-ICP4 (58S) monoclonal antibody.

RESULTS

Properties of ICP4 and ICP0 synthesized by in vitro transcription-translation. The ICP4 gene and an intronless ICP0 gene were cloned individually into an in vitro transcription vector and expressed in a rabbit reticulocyte lysate as [^{35}S]Met-labeled proteins. Although the genes encoding ICP4 and ICP0 specify polypeptides of 1,298 and 775 amino acids, respectively, with predicted molecular masses of 132,835 Da (for ICP4) and 78,452 Da (for ICP0), the ICP4 and ICP0 proteins synthesized in HSV-1-infected cells migrate as proteins with apparent molecular masses of 175 and 110 kDa, respectively, by SDS-PAGE. To determine if the molecular weights of the ICP4 and ICP0 species translated in vitro were similar to those of their counterparts expressed in HSV-1-infected cells, proteins from the two sources were compared by SDS-PAGE. As shown in Fig. 2, the ICP4 and ICP0 molecules synthesized in vitro were stable and exhibited molecular weights similar to those their counterparts synthesized in KOS-infected cells. These findings suggest that the apparent differences observed in the predicted molecular weights and the molecular weights seen on SDS-PAGE are due primarily to inherent properties of the two proteins, such as high proline content.

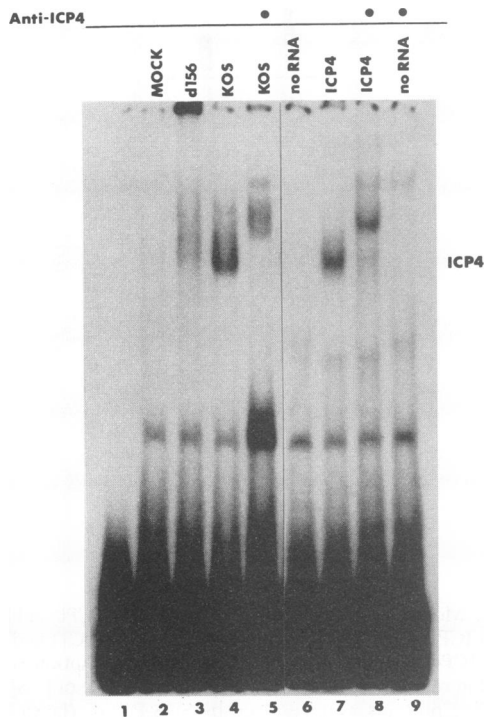

FIG. 3. Gel shift assay with in vitro-translated ICP4. Extracts from mock-infected cells (lane 2), cells infected with wild-type HSV-1 strain KOS (lanes 4 and 5) or the ICP4 deletion mutant virus d156 (lane 3), in vitro-translated ICP4 (lanes 7 and 8), and unprogrammed reticulocyte lysate (lanes 6 and 9) were incubated with a ^{32}P-labeled double-stranded oligonucleotide containing the ICP4 binding site, ATCGTC. Lane 1 contains probe alone. In supershift experiments (designated by dots above lanes 5, 8, and 9), after the initial 30-min incubation of extract with probe, reactions were carried out for an additional 10 min in the presence of anti-ICP4 monoclonal antibody 58S.

Interaction of in vitro-translated ICP4 with a probe containing the ICP4 consensus binding site, ATCGTCNNNNYCGRC. To determine whether the ICP4 synthesized in vitro was able to interact with its consensus DNA binding site, gel shift experiments were performed. As shown in Fig. 3, the ICP4 translated in vitro bound to a synthetic probe containing the consensus sequence ATCGTCNNNNYCGRC (lane 7), and the ICP4/DNA complex was supershifted by addition of anti-ICP4 monoclonal antibody (lane 8) but not anti-ICP0 monoclonal antibody (data not shown). Moreover, the ICP4-DNA complex formed when KOS-infected cell extract was used as a source of ICP4 (lane 4) exhibited a mobility on native polyacrylamide gels similar to that of the ICP4-DNA complex formed by in vitro-translated ICP4 with the same probe (lane 7). The supershifted bands were also of similar mobilities (compare lanes 5 and 8). As anticipated, the mutant form of ICP4 specified by d156 bound poorly to the probe (lane 3). These observations demonstrate that in vitro-translated ICP4 is able to bind to a probe containing a consensus ICP4 binding site. Moreover, because of the similarities in the migration patterns of the complexes shown in lanes 4 and 7 and lanes 5 and 8, other viral proteins are likely not associated with the ICP4-DNA complex formed with infected cell extracts.

Physical interaction of ICP0 with ICP4 and with itself. In an effort to investigate the possibility that ICP0 and ICP4 interact physically, far-Western blotting analysis was performed. The ICP4 deletion mutant d2 was used in these tests as a negative control because the d2 polypeptide has been shown to be incapable of down-regulating the expression of immediate-early genes or activating the transcription of early and late genes. Thus, in d2-infected cells, the synthesis of immediate-early proteins is significantly enhanced and the synthesis of early and late proteins is undetectable (14).

Figure 4A shows the results of Western blot analysis of the protein blot shown in Fig. 4C, using anti-ICP0 monoclonal antibody. Consistent with the previous observation of DeLuca and Schaffer (14), the level of ICP0 expression at 9 h postinfection was significantly higher in d2-infected cells than in KOS-infected cells. The far-Western blots shown in Fig. 4B and C demonstrate that a protein expressed in d2-infected cells, with a molecular weight similar to that of ICP0, interacts with in vitro-translated ICP0 and ICP4 (designated by dots in the right-hand margins) but not with proteins in an unprogrammed reticulocyte lysate (Fig. 4D). Although Western blot analysis in conjunction with Coomassie blue staining indicated that the mutant ICP4 peptide specified by d2 (with an apparent molecular mass of ~160 kDa) (data not shown and reference 14), ICP22, and ICP27 (14) were present at high levels in d2-infected cells, these peptides did not interact with in vitro-translated ICP0 or ICP4 (Fig. 4B and C). When in vitro-translated [^{35}S]Met-labeled HSV-1 UL8.5 protein was used as a probe, far-Western blot analysis of d2-infected cell extracts revealed little to no interaction between UL8.5 protein and ICP0 (data not shown). It should be noted that the heavy band migrating below the ICP0 band is nonspecific, as it is present in Fig. 4B to D as well as in mock-infected and infected cell extracts.

Collectively, these observations indicate that ICP0 interacts specifically with ICP4 and with itself. Notably, in addition to interacting with ICP0, the in vitro-translated ICP4 also interacted specifically with a cellular protein with an apparent molecular mass of 130 kDa (Fig. 4B). We have designated this cellular protein p130. Little or no interaction was detected between p130 and ICP0 (Fig. 4C).

Interaction of ICP0 and ICP4 with GST-ICP0 fusion proteins. Having shown that ICP0 interacts with ICP4 and with itself by far-Western blotting analysis, we next attempted to confirm and further define these interactions by using GST-ICP0 fusion proteins. Initially, an intronless ICP0 gene was fused in frame to the GST gene (pGEX-2TK0; Fig. 5), and the fusion protein was expressed in several different strains of *E. coli*. The full-length GST-ICP0 fusion protein proved to be highly unstable when expressed in bacteria. To overcome this problem, we generated two GST-ICP0 fusion proteins that contain GST and either N-terminal amino acids 1 to 394 or C-terminal amino acids 395 to 775 of ICP0 (Fig. 5). These fusion proteins were designated GST0:1-394 and GST0:395-775, respectively. GST and the two GST-ICP0 fusion proteins were expressed in *E. coli* BL21 and purified on glutathione-Sepharose 4B beads. The purified proteins were resolved by SDS-PAGE and visualized directly by Coomassie blue staining (Fig. 6A) or transferred to PVDF membranes and subjected to Western blot analysis with anti-ICP0 polyclonal antibody J17 (Fig. 6B) or monoclonal antibody H1083 (Fig. 6C). On the basis of their migration patterns in gels and their reactivity with anti-ICP0 antibodies (Fig. 6B and C), the bands marked by dots in lanes 1 and 2 of Fig. 6A represent full-length fusion proteins GST0:1-394 and GST0:395-775, respectively. Faster-migrating bands below the dots most likely represent degradation products of GST0:1-394 and GST0:395-775. The polyclonal antibody J17 reacted with both GST0:1-394 and GST0:395-775 and their degradation products (Fig. 6B); however, monoclonal antibody H1083 reacted only with the GST0:395-775 fusion protein (Fig. 6C). This observation is consistent with

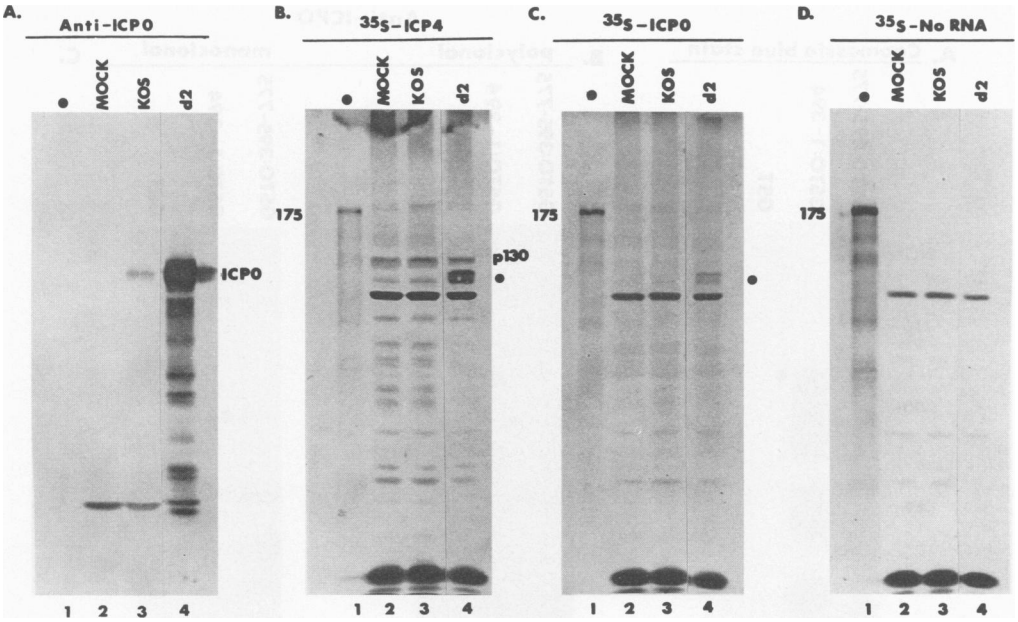

FIG. 4. ICP0 interacts directly with ICP4 and with itself. Vero cells were either mock infected (lanes 2), infected with wild-type HSV-1 strain KOS (lanes 3), or infected with ICP4 mutant d2 (lanes 4) at a multiplicity of 20 PFU per cell. At 9 h postinfection, cells were harvested and proteins were resolved by SDS-PAGE and transferred to PVDF membranes. Lanes 1 (dots) contain in vitro-translated ICP4 as a marker. After renaturation, blots were probed with in vitro-translated [^{35}S]Met-labeled ICP4 (B), in vitro-translated [^{35}S]Met-labeled ICP0 (C), or [^{35}S]Met-labeled unprogrammed reticulocyte lysate (D). The dots on the right of panel B and C indicate the locations of proteins that interact with ^{35}S-labeled ICP4 and ICP0, respectively. Panel A shows a Western blot analysis of the blot shown in panel C, using the anti-ICP0 monoclonal antibody H1083 to detect ICP0-specific peptides. Sizes are indicated in kilodaltons.

previous studies demonstrating that H1083 recognizes a C-terminal epitope of ICP0 (65).

To test their abilities to interact with ICP4 and ICP0, GST, GST0:1-394, and GST0:395-775 were purified on glutathione-Sepharose beads and allowed to react with in vitro-translated full-length [^{35}S]Met-labeled ICP4 (Fig. 7A) or ICP0 (Fig. 7B). The bound proteins were analyzed by SDS-PAGE and visualized by autoradiography. The intensities of the [^{35}S]Met-labeled bands in each lane were quantified by PhosphorImager scanning (model 400 E PhosphorImager; Molecular Dynamics, Sunnyvale, Calif.). These data indicated that the interaction of

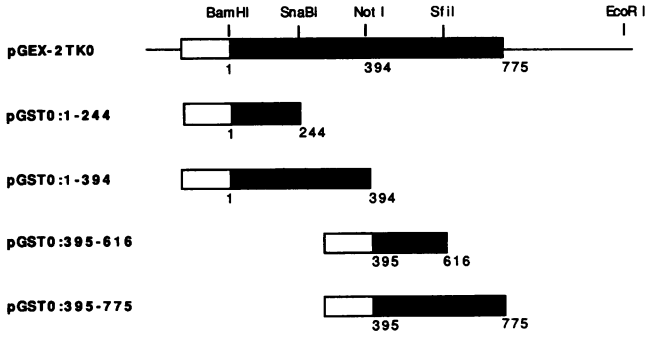

FIG. 5. Compositions of GST-ICP0 fusion proteins. Plasmid pGEX-2TK0 contains an intronless ICP0 gene was fused in frame to the GST gene in vector pGEX2TK. The GST portion of the hybrid protein product is shown as an open box, and the ICP0 amino acid sequence is shown as a black box. The GST-ICP0 fusion proteins GST0:1-244, GST0:1-394, GST0:395-616, and GST0:395-775 were generated as described in Materials and Methods.

ICP4 with GST0:395-775 was 85-fold greater than that of ICP4 with GST alone and 13-fold greater than that of ICP4 with GST0:1-394. As shown in Fig. 7A, in vitro-translated ICP4 interacted strongly with the fusion protein containing ICP0 C-terminal amino acids 395 to 775. In these tests, about 15 to 30% of input ICP4 was retained on the GST0:395-775 column. Notably, ICP4 interacted significantly less strongly with the fusion protein containing ICP0 N-terminal amino acids 1 to 394. In Fig. 7B, ICP0 interacted equally well with both the N-terminal and C-terminal GST-ICP0 fusion proteins.

In an effort to map the domains of ICP0 required for its interaction with ICP4 and with itself more precisely, two additional GST-ICP0 fusion proteins were generated. These proteins contain GST and either N-terminal ICP0 amino acids 1 to 244 (GST0:1-244) or C-terminal amino acids 395 to 616 (GST0:395-616) (Fig. 5). To determine the effect of deletion of amino acids 245 to 394 from fusion protein GST0:1-394 or amino acids 617 to 775 from GST0:395-775 on the ability of these proteins to interact with ICP4 and ICP0, equal amounts of GST and GST-ICP0 fusion proteins GST0:1-244, GST0:1-394, GST0:395-616, and GST0:395-775 were purified on glutathione-Sepharose beads and reacted with either in vitro-translated ^{35}S-labeled ICP4 (Fig. 8A) or ICP0 (Fig. 8B). As demonstrated in Fig. 8A, deletion of amino acids 617 to 775 from fusion protein GST0:395-775 significantly impaired its ability to interact with ICP4, whereas, as with GST0:1-394, a weak interaction was detected between GST0:1-244 and ICP4. As shown in Fig. 8B, deletion of amino acids 245 to 394 from GST0:1-394 had no effect on the ability of the protein to associate with in vitro-translated ICP0, whereas little interaction was detected between the in vitro-translated ICP0 and fusion protein GST0:395-616, demonstrating a role for amino

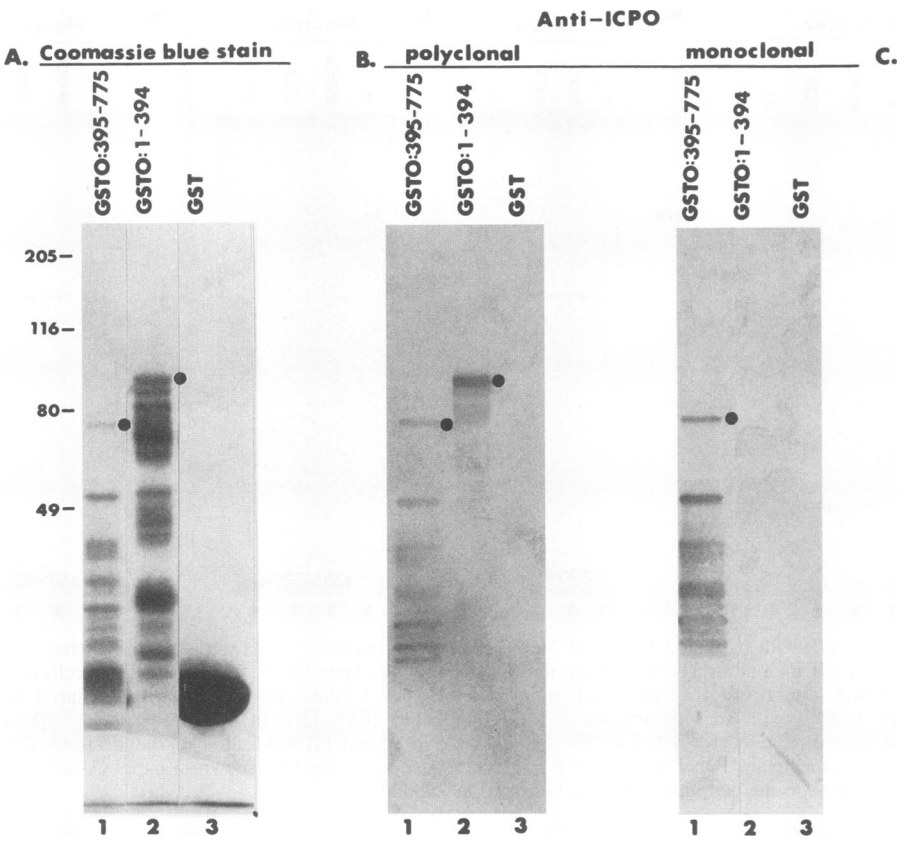

FIG. 6. Expression and purification of GST-ICP0 fusion proteins GST0:1-394 and GST0:395-775. GST-ICP0 fusion proteins GST0:395-775 (lanes 1) and GST0:1-394 (lanes 2), as well as GST (lanes 3), were expressed in *E. coli* and purified by using glutathione-Sepharose beads. The purified proteins were resolved by SDS-PAGE and either visualized directly by Coomassie blue staining (A) or transferred to PVDF membranes and subjected to Western blot analysis with anti-ICP0 polyclonal antibody J17 (B) or monoclonal antibody H1083 (C). Sizes are indicated in kilodaltons.

acids 616 to 775, but not 245 to 394, in the interaction of ICP0 with itself.

Interactions of ICP4 and truncated ICP4 peptides with GST0:1-394 and GST0:395-775. ICP4 contains multiple functional domains that are required for autoregulation, activation of early and late gene expression, nuclear localization, DNA binding, intranuclear distribution, and virion association (14, 65). To determine whether a particular domain or primary amino acid sequence within the ICP4 protein is critical for the interaction of ICP4 with ICP0, three truncated ICP4 polypeptides specifying amino acids 1 to 522, 1 to 681, and 1 to 1063 were synthesized in vitro and labeled with [^{35}S]Met. Approximately equal amounts of [^{35}S]Met-labeled full-length ICP4 and truncated ICP4 peptides were then allowed to interact with GST or the GST-ICP0 fusion proteins bound to glutathione-Sepharose beads, and bound proteins were analyzed by SDS-PAGE (Fig. 9).

In this experiment, the wild-type form of ICP4 interacted more efficiently with GST0:395-775 than did the mutant forms of ICP4 (Fig. 9; compare lane 6 with lanes 7 to 9). Moreover, just as in Fig. 7A and 8A, the wild-type form of ICP4 again interacted more efficiently with the C-terminal GST-ICP0 fusion protein than with the N-terminal fusion protein (Fig. 8A; compare lanes 2 and 6). The 1,298-amino-acid wild-type ICP4 protein interacted strongly with the ICP0 C-terminal fusion protein GST0:395-775, yet scanning analysis revealed that the truncated ICP4 protein (amino acids 1 1063) lacking the 235 C-terminal amino acid residues interacted ~10-fold less strongly, suggesting that the ICP4 domain that interacts with ICP0 may include C-terminal amino acid residues 1064 to 1296. Alternatively, the intact ICP4 protein may be required for optimal interaction with ICP0.

DISCUSSION

Several lines of evidence suggest that functional interactions may occur among HSV-1 immediate-early proteins. First, ICP0 and ICP4 activate gene expression synergistically, and ICP27 functions as either a *trans* activator of late gene expression or a *trans* repressor of early gene expression induced by ICP4, ICP0, or a combination of ICP4 and ICP0 (57, 62). Second, ICP27 has been shown to play a role in the posttranslational modification of ICP4 during productive infection (37, 62). Third, the intranuclear localization of ICP0 has been reported to be affected by ICP4 (27, 32). Lastly, recent evidence has demonstrated that ICP4 facilitates, whereas ICP27 inhibits, the nuclear localization of ICP0 (69). To elucidate the molecular mechanism underlying the cooperative regulation of HSV-1 gene expression mediated by immediate-early proteins, we have investigated the possibility that direct physical interactions occur among the HSV-1 immediate-early proteins. Using far-Western blotting analysis, GST-ICP0 fusion protein affinity chromatography, and in vitro-translated ICP0 and ICP4, we have shown that ICP0 is able to interact specifically with ICP4

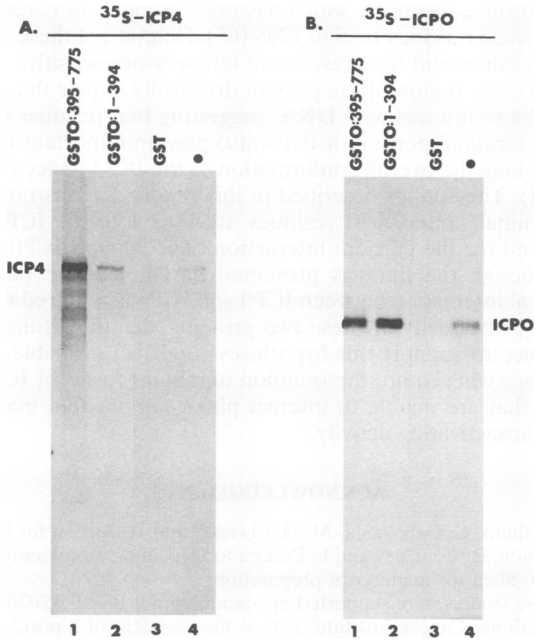

FIG. 7. Interactions of ICP0 and ICP4 with GST-ICP0 fusion proteins. GST (lanes 3) and the GST-ICP0 fusion proteins GST0:1-394 (lanes 2) and GST0:395-775 (lanes 1) were expressed in *E. coli* and purified on glutathione-Sepharose beads. The purified GST and GST-ICP0 fusion proteins bound to beads were allowed to react with in vitro-translated [^{35}S]Met-labeled ICP4 (A) or ICP0 (B) overnight at 4°C. The bound proteins were resolved by SDS-PAGE and visualized by autoradiography. In vitro-translated [^{35}S]Met-labeled ICP4 or ICP0 (lanes 4) was used as a marker and was not reacted with the affinity column.

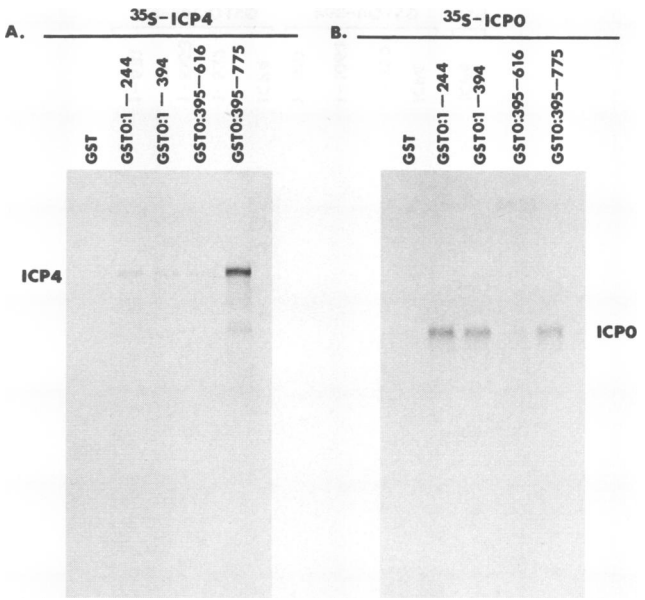

FIG. 8. Effects of C-terminal truncations of GST0:1-394 and GST0:395-775 on their abilities to interact with ICP4 and ICP0. GST and the GST-ICP0 fusion proteins GST0:1-244, GST0:1-394, GST0:395-616, and GST0:395-775 were purified on glutathione-Sepharose beads and reacted with either in vitro-translated [^{35}S]Met-labeled ICP4 (A) or ICP0 (B). After several washes with 1× protein binding buffer containing 0.1% NP-40, the bound proteins were analyzed by SDS-PAGE and visualized by autoradiography.

and with itself. Furthermore, the C-terminal portions of the two proteins appear to play important roles in mediating these interactions.

ICP4 is a sequence-specific DNA-binding protein. Relative to its high-affinity consensus DNA binding site, ATCGTCN NNNYGRC, other sequences to which ICP4 binds are highly degenerate such that the binding affinity of ICP4 to these sites can vary dramatically depending on the sequence of the site (15, 38). High-affinity binding sites are present within the promoters of the genes specifying ICP4, ICP0, and the L/STs and are absent from the promoter regions of most early and late genes. Because the expression of early and late genes is induced by ICP4, and mutant forms of ICP4 impaired in the ability to interact with DNA exhibit little or no transactivating activity (14, 43, 58), it is likely that under certain circumstances, the interaction of ICP4 with its low-affinity binding sites in the promoters and/or 5′ untranslated regions of early and late genes plays an important role in mediating the transactivating effect of ICP4 on these genes (15, 53, 58). Given its synergy with ICP0 in activating gene expression and the recent observation that ICP4 can form tripartite complexes with the basic transcription factors, TBP and TFIIB, on a template containing both a TATA sequence and the ICP4 binding site, it is possible that the interaction of ICP0 with ICP4 facilitates the interaction of ICP4 with its low-affinity DNA binding site and with factors in the basic transcription complex such as TBP and TFIIB (59).

ICP0 is a potent transactivator of all three classes of HSV genes and is associated with chromatin in infected cells in a manner that is not sequence specific (23, 45). Moreover, purified, baculovirus-expressed ICP0 is able to associate with DNA-cellulose columns, although it fails to form stable protein-DNA complexes in solution (23). These observations suggest that stable ICP0-DNA complex formation may require both a nonspecific, low-affinity interaction of ICP0 with DNA as well as an interaction with DNA-bound protein(s). Preliminary studies from this laboratory suggest that like ICP4, ICP0 can activate gene expression through a mechanism involving an interaction with TBP and association with DNA (unpublished observations). From the observation reported herein, that ICP0 and ICP4 can interact directly with each other, it is reasonable to speculate that the interaction of ICP4 with ICP0 may serve to stabilize the association of ICP0 with DNA, which in turn leads to synergistic gene activation. We are currently investigating this possibility.

During the past 5 years, considerable evidence has accumulated to indicate that the C-terminal half of ICP0 is critical in mediating the several functions of this protein. Thus, this half of the ICP0 molecule contains multiple functional domains, including (i) a signal required for nuclear localization mapped to amino acids 474 to 509 (19); (ii) a region required for efficient transactivation mapped to amino acid residues 526 to 720 (3); and (iii) a region within amino acid residues 720 to 770 required for the intranuclear localization of ICP0 (19, 69), its association with the HSV-1 virion (65), and responsiveness to the inhibitory effect of ICP27 on the nuclear localization of ICP0 (69) (Fig. 1). Using GST-ICP0 fusion protein affinity chromatography, we have shown that ICP4 interacts preferentially with C-terminal amino acid residues 395 to 775 of ICP0. The observation that deletion of amino acids 617 to 775 from fusion protein GST0:395-775 significantly impaired its ability to interact with ICP4 suggests that amino acids 617 to 775 of ICP0 may represent the major domain for the physical inter-

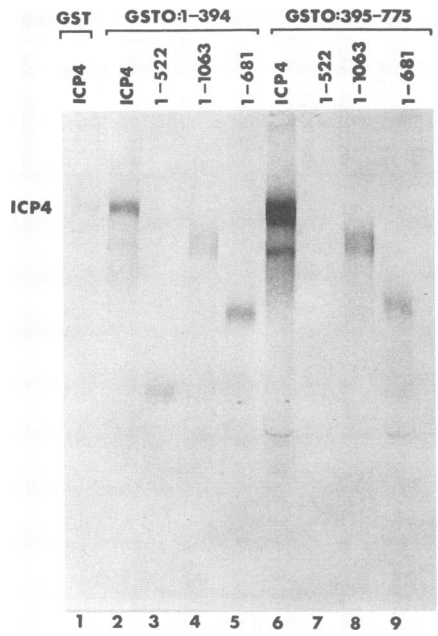

FIG. 9. Effects of C-terminal deletions on the ability of ICP4 to interact with ICP0. GST (lane 1) and the GST-ICP0 fusion proteins GST0:1-394 (lanes 2 to 5) and GST0:395-775 (lanes 6 to 9) were purified on glutathione-Sepharose beads and reacted at 4°C overnight with in vitro-translated [^{35}S]Met-labeled wild-type ICP4 (lanes 2 and 6) or with truncated ICP4 polypeptides specifying amino acids 1 to 522 (lanes 3 and 7), 1 to 1063 (lanes 4 and 8), and 1 to 681 (lanes 5 and 9). The beads were washed, and bound proteins were resolved by SDS-PAGE and visualized by autoradiography.

action of ICP0 with ICP4. Notably, this region of ICP0 also contains the domain involved in the synergistic activation of gene expression with ICP4 (19). Together, these observations strongly support the hypothesis that the synergistic effects of ICP0 and ICP4 result from a direct interaction between these two proteins.

By using glycerol gradient centrifugation (23) and Sephacryl chromatography elution techniques (5), ICP0 has been shown to oligomerize in solution. The observation that a mutant ICP0 polypeptide specifying only N-terminal amino acid residues 1 to 525 and lacking the virion association domain (residues 720 to 770) is able to inhibit association of wild-type ICP0 with HSV-1 virions also suggests that ICP0 can dimerize and that the dimerization domain lies within the N-terminal portion of the molecule (65). Using far-Western blotting analysis and GST-ICP0 affinity chromatography, in this report we provide direct evidence that ICP0 can interact with itself. Furthermore, we have shown that in contrast to ICP4, ICP0 is capable of interacting both with N-terminal GST-ICP0 fusion proteins GST0:1-244 and GST0:1-394 and with C-terminal GST-ICP0 fusion protein GST0:395-775, whereas little interaction was detected between ICP0 and GST-ICP0 fusion protein GST0:395-616. These results indicate that ICP0 is able to oligomerize and that both N-terminal amino acids 1 to 244 and C-terminal amino acids 617 to 775 of ICP0 are involved in this process.

Studies of temperature-sensitive, deletion, and nonsense mutants have clearly demonstrated that the C-terminal portion of ICP4 (amino acid residues 775 to 1298) plays a critical role in the multiple functions of this protein. This region of the protein specifies (i) autoregulation and activation of early and late gene expression; (ii) intranuclear localization (14); and (iii) virion association, which has been mapped to C-terminal amino acid residues 1029 to 1298 (65). Deletion of these amino acid residues and the presence of temperature-sensitive mutations in this region of the protein drastically impair the ability of ICP4 to interact with DNA, suggesting that residues within the C-terminal portion of ICP4 also play an important role in controlling the overall conformation of the ICP4 molecule (11, 12, 14). The studies described in this report demonstrate that C-terminal amino acid residues 1064 to 1298 of ICP4 are required for the efficient interaction of ICP4 with ICP0.

Although the findings presented herein indicate that the physical interaction between ICP0 and ICP4 is required for the synergistic activity of these two proteins, definitive functional evidence to support this hypothesis is not yet available. Such evidence will require the isolation of mutant forms of ICP4 or ICP0 that are unable to interact physically but that maintain full transactivating activity.

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