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Physical and Functional Interactions between Herpes Simplex Virus Immediate-Early Proteins ICP4 and ICP27

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The ordered expression of herpes simplex virus type 1 (HSV-1) genes, during the course of a productive infection, requires the action of the virus immediate-early regulatory proteins. Using a protein interaction assay, we demonstrate specific in vitro protein-protein interactions between ICP4 and ICP27, two immediate-early proteins of HSV-1 that are essential for virus replication. We map multiple points of contact between these proteins. Furthermore, by coimmunoprecipitation experiments, we demonstrate the following. (i) ICP4-ICP27 complexes are present in extracts from HSV-1 infected cells. (ii) ICP27 binds preferentially to less modified forms of ICP4, a protein that is extensively modified posttranslationally. We also demonstrate, by performing electrophoretic mobility shift assays and supershifts with monoclonal antibodies to ICP4 or ICP27, that both proteins are present in a DNA-protein complex with a noncanonical ICP4 binding site present in the HSV thymidine kinase (TK) gene. ICP4, in extracts from cells infected with ICP27-deficient viruses, is impaired in its ability to form complexes with the TK site but not with the canonical site from the α4 gene. However, ICP4 is able to form complexes with the TK probe, in the absence of ICP27, when overproduced in mammalian cells or expressed in bacteria. These data suggest that the inability of ICP4 from infected cell extracts to bind the TK probe in the absence of ICP27 does not reflect a requirement for the physical presence of ICP27 in the complex. Rather, they imply that ICP27 is likely to modulate the DNA binding activity of ICP4 by affecting its posttranslational modification status. Therefore, we propose that ICP27, in addition to its established role as a posttranscriptional regulator of virus gene expression, may also modulate transcription either through direct or indirect interactions with HSV regulatory regions, or through its ability to modulate the DNA binding activity of ICP4.

Infected cell protein 27 (ICP27) is an essential immediate-early protein (43, 69, 75) of herpes simplex virus type 1 (HSV-1). It acts in concert with ICP4 and ICP0, two immediate-early viral transcriptional regulators (18, 19, 23, 25, 48, 55, 56, 67, 69, 80), to modulate the ordered expression of HSV-1 genes (31) during the course of a productive infection. HSV-1 genes are generally classified into three kinetic classes, immediate early (α), early (β), and late (γ), based on their temporal expression during the lytic virus life cycle (31, 32, 74). Genetic experiments have shown that ICP27 is required for the transition from early to late virus gene expression (69, 70, 75). ICP27 is also required for optimal viral DNA replication during infection (43, 69, 72, 75), and recent studies also suggest a role for this protein in the expression of some early genes (76, 92).

ICP27 can act at the posttranscriptional level by inhibiting pre-mRNA splicing (30, 63, 79), which may provide a selective advantage to the virus, whose mRNAs are predominantly unspliced (44, 93). Additionally, ICP27 modulates the processing of mRNAs that contain certain poly(A) sites (45–47, 77, 79).

Evidence from viruses carrying mutant forms of ICP4 and ICP27 suggests a role for ICP27 in the transcriptional regulation of certain early HSV-1 genes (76). This level of regulation may be direct and/or indirect. ICP27 also inhibits the nuclear localization of two HSV-1 transcriptional regulators, ICP0 and ICP4 (54, 103, 104), suggesting that it may affect transcription indirectly. Also, it has been shown that the electrophoretic mobility of ICP4, the major regulator of HSV-1 transcription, is altered in the absence of ICP27 (48, 68, 89). It is not clear, however, whether ICP27 is directly or indirectly involved in the posttranslational modification of the ICP4 molecule. ICP4 is essential throughout virus replication (12, 13, 16, 26, 36, 65, 67, 81, 94, 95). It activates the expression of early and late genes, while down-regulating its own expression and that of ICP0 (12, 25, 38, 55, 65, 73). Because ICP4 undergoes extensive posttranslational modifications (4, 5, 62, 66, 97), the possibility exists that these alterations may affect its regulatory functions.

An interaction between ICP27 and ICP4 was suggested from recent cotransfection experiments which indicated that these proteins can alter each other's intracellular localization (104). Yet the interpretation of results obtained by this experimental approach is complicated by its inability to distinguish between direct protein-protein interactions and indirect effects.

In this report, we provide evidence for a direct physical interaction between ICP4 and ICP27 and map the interacting domains. Furthermore, we provide evidence that ICP27 can modulate the ability of ICP4 to form complexes on DNA. We propose that ICP27, through its interactions with ICP4, may regulate transcription of some HSV genes.

MATERIALS AND METHODS

Cells and viruses. Vero cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, N.Y.) containing 5% bovine calf serum (HyClone Laboratories, Inc., Logan, Utah). 293 cells, a transformed human embryonal kidney cell line expressing the adenovirus type 5 E1A and E1B proteins (27), were grown in DMEM containing 10% fetal bovine serum (HyClone Laboratories, Inc.). The media were supplemented with 100 U of penicillin and 100 μg of streptomycin (Gibco BRL) per ml, unless otherwise noted. HeLa cells were propagated in monolayers as previously described (59).

The wild-type HSV-1 viruses used in this study were the Glasgow strain 17 and KOS 1.1. The ICP27 null virus 27-LacZ (a gift from R. Sandri-Goldin) contains a *lacZ* insertion within the 5′ noncoding, transcribed sequences of the α27 gene (84). The ICP27 deletion virus vBSΔ27 (85) was generated by replacing the α27

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coding sequences of HSV-1 strain KOS, from *Eag*I to *Eco*NI, with those of *lacZ*. The ICP4 null virus *d*120 (a gift from N. DeLuca) has a deletion within the coding sequence of α4 (12). The α0 deletion viruses *dl*1403 (a gift from N. Stow) and *IE-0*:*lacZ* have been described (10, 87). The vCPc0 virus was constructed by introducing the α0 cDNA (101) into *IE-0*:*lacZ* by homologous recombination as described elsewhere (10). This virus exhibits wild-type growth characteristics in the context of a lytic infection. The vCM2/7 virus, with a deletion of α0 sequences coding for amino acids 106 to 212 of ICP0, has been described elsewhere (10). Viruses vC116G, vH126A, and vC116G/C156A (39) were generated by site-directed mutagenesis of α0 cDNA sequences coding for amino acids within the ICP0 C_3HC_4 zinc finger domain.

Transfections. Transfections were performed by the calcium phosphate precipitation method described previously (96). Briefly, 293 cells were seeded at 10^6 cells per 10-cm-diameter plate the day before transfection and each plate was transfected with 15 μg of total plasmid DNA the next day. The transfection mixtures were left on the cells overnight. The following day the medium was replaced, and the cells were harvested 48 h later.

Plasmid constructions. The bacterial expression plasmid pCPC-X27, expressing HSV-1 (KOS 1.1) ICP27 as a glutathione *S*-transferase (GST) fusion, was constructed as follows. Plasmid pMS13 was constructed by subcloning an end-filled *Bam*HI-*Dde*I fragment from pIGA42 (24) into the *Sma*I site of pIBI31 (International Biotechnologies Inc., Eastman Kodak Co., New Haven, Conn.). pMS13 was digested with *Drd*I, end filled, and then digested with *Eco*RI. The resulting 1.7-kb DNA fragment was cloned into *Sma*I/*Eco*RI-cut pGEX-1 (83) to yield pCPC-X27. pCPC-X27/4-5, expressing the carboxy-terminal region of ICP27 (amino acids 407 to 512) as a GST fusion, was constructed by ligating the *Stu*I/*Eco*RI fragment from pCPC-X27 into *Sma*I/*Eco*RI-cut pGEX-2T (83). Plasmid pCPC-X27/4, expressing a GST fusion with amino acids 1 to 406 of ICP27, was constructed by digesting pCPC-X27 with *Eco*RI, end filling, cutting with *Stu*I, and religating. Plasmid pCPC-X27/2, expressing amino acids 1 to 262 of ICP27 as a GST fusion, was constructed by digesting pCPC-X27 with *Sal*I and *Eco*RI, end filling, and religating. Plasmids pCPC-X27/1.5 and pCPC-X27/1, expressing GST fusions with amino acids 1 to 153 and 1 to 103 of ICP27, respectively, were constructed by digesting pCPC-X27 with either *Sma*I or *Dra*III and *Eco*RI, end filling and religating. Plasmid pCPC-X27/0.3, expressing amino acids 1 to 27 of ICP27, was made by digesting pCPC-X27/4 with *Eco*RI, end filling, digesting with *Nae*I, and religating. Plasmid pCPC-X27/1-4, expressing a GST fusion with amino acids 104 to 406 of ICP27, was constructed by ligating the *Dra*III (filled)/*Eco*RI fragment from pCPC-X27/4 into *Sma*I/*Eco*RI-cut pALEX (58). Plasmid pCPC-X27/2-4, expressing amino acids 178 to 406 of ICP27 as a GST fusion, was made by cloning the *Sma*I/*Eco*RI fragment from pCPC-X27/4 into *Sma*I/*Eco*RI-digested pALEX.

Plasmid pCPC-$XE7_{51}$, expressing a GST fusion with the E7 oncoprotein from human papillomavirus type 51 (40), was generated by ligating a *Bam*HI/*Eco*RI-digested PCR amplimer of the E7 open reading frame in plasmid pGEX-3X (83).

Plasmid pCPC-X03, expressing a GST fusion with amino acids 1 to 385 of ICP0, was constructed by cloning a 1.2-kb *Bgl*II/*Sma*I fragment from plasmid pDS18, which contains an ICP0 cDNA, into a *Bam*HI/*Sma*I-digested pGEX-1 expression vector. pDS18 is a pDS16 (101) derivative whose 5′ *Nco*I site was converted to *Bgl*II. Plasmids pCPC-X02 and pCPC-X01, expressing GST fusions with amino acids 1 to 214 and 1 to 103 of ICP0, respectively, were generated by digesting pCPC-X03 with either *Asp*718 or *Xho*I and *Sma*I, end filling, and religating. Plasmid pDS32, expressing amino acids 312 to 400 of ICP0, was constructed by inserting an *Nru*I/*Eco*RI fragment from pCM6 (10) into *Sma*I/*Eco*RI-digested pGEX-3X.

Plasmid pCPC-X4(2-5), expressing the ICP4 DNA binding domain (amino acids 245 to 523) as a GST fusion, was constructed by cloning an 840-bp *Eco*NI (filled)/*Xho*I fragment from plasmid pCPC-CMV4-5 (see below) into *Eco*RI (filled)/*Xho*I-cut pALEX (58). Plasmid pCPC-X4(5-6), expressing amino acids 573 to 686 of ICP4, was generated by cloning a 335-bp *Bam*HI/*Not*I fragment from the α4 coding sequence into *Bam*HI/*Not*I-cut pALEX.

The 4.6-kb *Hin*dIII fragment from plasmid pTEG-2 (102), containing α4 coding and noncoding sequences from HSV-1 strain 17, was cloned in the *Hin*dIII site of pcDNAI.neo (Invitrogen, San Diego, Calif.). The resulting plasmid, pCPC-CMV4, directs ICP4 expression from a cytomegalovirus promoter when transfected in mammalian cells or from a T7 promoter when used for in vitro transcription-translation. Plasmid pCPC-CMV4-10 was generated by subcloning a 3.2-kb *Hin*dIII/*Hin*cII DNA fragment from pTEG-2, containing 5′ noncoding and coding sequences of the α4 gene, into the *Hin*dIII/*Eco*RV sites of pcDNAI.neo. This construct directs the synthesis of an ICP4 peptide containing amino acids 1 to 1029. Plasmid pCPC-CMV4-5, directing the synthesis of amino acids 1 to 523 of ICP4, was made by digesting pCPC-CMV4 with *Bam*HI and religating. Plasmid pCPC-CMV4-4, expressing amino acids 1 to 450 of ICP4, was generated by digesting pCPC-CMV4-5 with *Not*I and religating. Constructs pCPC-CMV4-2 and pCPC-CMV4-1, expressing amino acids 1 to 243 and 1 to 83 of ICP4, respectively, were created by cloning the 820-bp *Hin*dIII/*Eco*NI (filled) or the 335-bp *Hin*dIII/*Pvu*I fragment from pCPC-CMV4-4 into *Hin*dIII/*Eco*RV-cut pcDNAI.neo. Plasmid pCPC-CMV4(6-13), expressing amino acids 1 to 12 and 575 to 1298 of ICP4, was generated in a two-step process. First, plasmid pCPC-CMV4Δ5 was derived by cloning a 2.6-kb *Bam*HI fragment from pTEG-2, containing α4 sequences coding for amino acids 575 to 1298 and 3′ noncoding sequences, in the *Bam*HI site of pcDNAI.neo. To insert an initiator ATG as well as α4 5′ noncoding sequences, and thus generate pCPC-CMV4(6-13), a 120-bp *Hin*dIII/*Sma*I fragment from pCPC-CMV4-1 was inserted in pCPC-CMV4Δ5 that was partially digested with *Bam*HI, end filled, and digested with *Hin*dIII. Plasmid pCPC-T7-4(6-13), which expresses amino acids 1 to 12 and 575 to 1298 of ICP4 under the control of the T7 promoter, was generated by subcloning a *Hin*dIII/*Eco*RV fragment from pCPC-CMV4(6-13) into *Hin*dIII/*Hin*cII-cut pIBI31. Plasmid pCPC-T7-4(6-10), expressing amino acids 1 to 12 and 575 to 1029 of ICP4, was constructed by cloning a *Hin*dIII/*Hin*cII fragment from pCPC-CMV4(6-13) in pIBI31. Plasmid pCPC-CMV27 was constructed by cloning a 1.7-kb *Bam*HI/*Eco*RI (filled) DNA fragment from pCPC-X27 into *Bam*HI/*Eco*RV-cut pcDNAI.neo. Plasmid pBST7-27 (85), expressing full-length ICP27 under the control of the T7 promoter, was also used in some experiments.

The cloning junctions of the above constructs were verified by DNA sequencing. Their abilities to express the desired proteins were determined by Western blot analysis (91) or coupled in vitro transcription-translation (see below).

Protein expression and purification. The GST fusion proteins were expressed in bacteria and purified as previously described (3, 83). Following purification, proteins were dialyzed overnight at 4°C against 200 volumes of a solution containing 10 mM HEPES-KOH (pH 7.9), 50 mM NaCl, 1 mM EDTA, 4 mM dithiothreitol, and 20% (vol/vol) glycerol, with two changes. Protein concentrations were determined by the Bradford method (8), and protein purity was ascertained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (37). Proteins were stored in aliquots at −80°C.

Coupled in vitro transcription-translation reactions were performed with the TNT system (Promega, Madison, Wis.) according to the manufacturer's instructions. Each reaction was programmed with 2 μg of the appropriate supercoiled plasmid, and proteins were ^{35}S labeled with translation-grade [^{35}S]methionine (DuPont, NEN, Boston, Mass.). The in vitro-translated proteins were immediately used in the protein binding reactions.

GST fusion protein capture assays. Protein binding assays were performed as follows: 130 μl of binding buffer (10 mM Tris-HCl [pH 7.9], 50 mM NaCl, 5% [vol/vol] glycerol, 1 mM EDTA, 4 mM dithiothreitol, 0.2 mg of ovalbumin per ml) was mixed on ice with 20 μl of in vitro transcription-translation reaction mixture containing the appropriate ^{35}S-labeled polypeptide. Fifty microliters of a 50% (vol/vol) suspension of glutathione-agarose beads containing 20 to 50 μg of bound GST fusion protein was added to the above mixture, and binding was allowed to proceed for 4 h at 4°C with constant mixing. The beads were washed four times (5 min each time) at 4°C with 0.5 ml of binding buffer, and fusion proteins were eluted with 15 mM glutathione in 50 mM Tris-HCl (pH 8.0). The bound ^{35}S-polypeptides were detected by fluorography after separation by SDS-PAGE (37).

Nuclear extract preparation and electrophoretic mobility shift analysis (EMSA). Nuclear extracts were prepared from HSV-1 infected HeLa cells at 6 h postinfection according to the method of Dignam et al. (15), with some modifications (60). Total cell extracts from transfected 293 cells were prepared by combining cells from six transfected 10-cm-diameter plates; suspending them in 1 ml of lysis buffer, containing 20 mM HEPES-KOH (pH 7.9), 150 mM NaCl, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone (TPCK), 0.1 mM L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone (TLCK) (Boehringer Mannheim, Indianapolis, Ind.), and 5% (vol/vol) glycerol; and sonicating (three 15-s pulses). The lysates were clarified by centrifugation at 20,000 × *g* for 30 min, and the extracts were stored frozen in aliquots at −80°C.

EMSA of ICP4 complexes was performed by incubating 4 μg of nuclear or total cell extracts with ^{32}P-end-labeled restriction fragments, followed by native PAGE (59). The DNA binding reactions were performed for 20 min at room temperature in a solution containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5% (vol/vol) glycerol, 1 mM EDTA, 4 mM dithiothreitol, and 0.2 mg of bovine serum albumin per ml in a final volume of 20 μl. Sonicated salmon sperm DNA (3 μg) was included as a nonspecific competitor. When supershift analyses were performed, 1 μl of the appropriate monoclonal antibody (ascites fluid), dialyzed against binding buffer, was added to each binding reaction and incubation was continued for 30 min. Following separation by native PAGE, the DNA-protein complexes were visualized by autoradiography. The same conditions were used when the bacterially expressed ICP4 DNA binding domain (50 ng) was used for EMSA analysis. The DNA probes included a 49-bp *Ava*I/*Bam*HI fragment containing the high-affinity ICP4 binding site from the HSV-1 α4 gene cap site (−17/+32) and a *Hin*dIII/*Eco*RI fragment, from plasmid TKB (61), containing the thymidine kinase (TK) gene cap site (−16/+56).

Coimmunoprecipitations and Western blot analyses. Five microliters of monoclonal antibody (ascites fluid), dialyzed against binding buffer, was mixed with 200 μg of nuclear extract proteins and incubated at 4°C for 2 h in a final volume of 100 μl. Thirty microliters of a 50% (vol/vol) suspension of Gamma-Bind Plus Sepharose (Pharmacia Biotech AB, Uppsala, Sweden) in binding buffer was added, and the incubation was continued for 2 h at 4°C with constant mixing. The beads were washed four times, for 5 min each time, at 4°C with 0.5 ml of binding buffer, resuspended in 40 μl of 1.5× SDS-PAGE sample buffer (37), and boiled for 10 min. The proteins were separated by SDS-PAGE (37) and electrophoretically transferred to nitrocellulose membranes (91). ICP4 was detected as described elsewhere (57), with a mixture of mouse monoclonal antibodies (58S and H640). Immunoblots were developed by using the chemiluminescent substrate LumiGlo (Kirkegaard and Perry, Gaithersburg, Md.). Two

antibodies against ICP4, 58S (82) and H640 (1), and one, H1113 (1), which recognizes ICP27, were used for the immunoprecipitations. To eliminate the possibility of nonspecific protein binding or protein precipitation to the beads, control precipitations were performed in the absence of antibody.

Immunoprecipitations of ^{35}S-labeled viral proteins were performed as follows. Vero cell monolayers (3×10^6 cells/60-mm-diameter plate) were infected with HSV-1 at a multiplicity of infection of 1. Eight hours postinfection the cells were washed three times with methionine-free DMEM, and 100 μCi of Tran35S-Label (1,214 Ci/mmol; ICN Pharmaceuticals Inc., Costa Mesa, Calif.) in 500 μl of methionine-free DMEM was added. After 45 min, the cells were washed with ice-cold phosphate-buffered saline containing 1 mM PMSF, 0.1 mM TPCK, and 0.1 mM TLCK and then were lysed for 10 min in 600 μl of ice-cold radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl [pH 8.0], 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing 1 mM PMSF, 0.1 mM TPCK, and 0.1 mM TLCK. The cells were harvested and pipetted vigorously to ensure lysis, and 50 μl of a 10% (vol/vol) suspension of IgGsorb (Enzyme Center, Malden, Mass.) in RIPA buffer was added to each suspension. Following mixing, the suspensions were clarified by centrifugation in a microfuge for 15 min at 4°C. One hundred microliters from each supernatant was mixed with 2.5 μl of antibody and incubated at 4°C for 1 h prior to the addition of 20 μl of GammaBind Plus Sepharose (50% [vol/vol] suspension in RIPA buffer), and incubation continued for another hour at 4°C with constant mixing. The beads were washed three times, for 15 min each time, at 4°C with RIPA buffer, resuspended in 25 μl of 1.5× SDS-PAGE sample buffer, and boiled for 5 min. The immunoprecipitated proteins were analyzed by SDS-PAGE on 7.5% gels, followed by either fluorography or Western blot analysis. The immunoprecipitations were performed with an anti-ICP27 rabbit polyclonal antibody (CLU38), raised against bacterially expressed GST-ICP27 fusion protein, and a mouse monoclonal antibody (H1114; Goodwin Institute, Plantation, Fla.) against ICP4.

RESULTS

Protein-protein interactions between ICP27 and ICP4. Interactions between ICP4 and ICP27, the two essential immediate-early regulatory proteins of HSV-1, have been suggested from previous studies. These include observations that the presence of ICP27 can alter the electrophoretic mobility of ICP4 (48, 68, 89) and affect its intracellular localization (104). It has not been clear, however, whether ICP27 interacts directly or indirectly with ICP4.

In the present study we examine whether there are direct protein-protein interactions between ICP4 and ICP27. To explore this possibility and to identify the interacting domains, we used an assay that involves binding of in vitro-synthesized, ^{35}S-labeled ICP4 polypeptides to various regions of ICP27 that have been expressed in bacteria as GST fusions. The GST-ICP27 fusions are immobilized on glutathione-agarose beads, and binding is assessed by the ability of ICP4 to remain bound after rigorous washing (see Materials and Methods). To establish the specificity of the assay, we used several positive and negative controls.

The positive controls include a GST fusion with the amino-terminal 385 amino acids of the HSV-1 regulatory protein ICP0 (Fig. 1). This ICP0 domain was previously shown to interact directly with ICP4 (100), and it binds ICP4 in our experiments as well (Fig. 1). We further delimited the ICP4 interaction domain of ICP0 to amino acids 312 to 385, as a GST fusion with amino acids 312 to 400 of ICP0 also binds ICP4 (Fig. 1). In contrast, neither the amino-terminal 103 nor 214 amino acids of ICP0 interact with ICP4 to any significant extent (Fig. 1). The specificity of this protein interaction assay system was further demonstrated by the failure of either GST alone or a GST fusion protein with the E7 oncoprotein, from human papillomavirus type 51, to bind ICP4 (Fig. 1). The E7 oncoprotein provided a valuable negative control, as it binds the retinoblastoma gene product (17), transcription factors of the AP-1 family (2), cyclins (90), and the nuclear matrix (28).

Having established the specificity of this assay, we used it to ask if ICP27 interacts with ICP4. Our results (Fig. 1) demonstrate that these proteins do interact, as more than 10% of the input ICP4 binds to immobilized GST-ICP27. Furthermore, more ICP4 binds to ICP27 than to the ICP0 positive control.

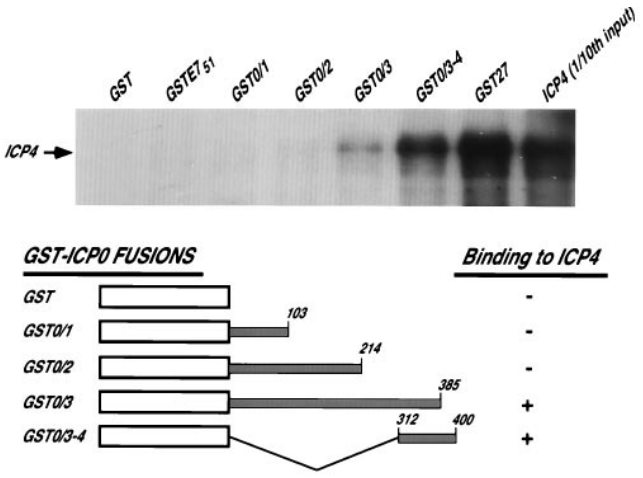

FIG. 1. ICP4 interactions with ICP0 and ICP27 in vitro. In vitro-transcribed-translated ^{35}S-labeled ICP4 was incubated with GST or with approximately 50 μg of a protein formed by the fusion of GST with the E7 oncoprotein from human papillomavirus type 51 ($GSTE7_{51}$), ICP0 (GST0/1 through GST0/3-4), or ICP27 (GST27), bound to glutathione-agarose beads as described in Materials and Methods. The beads were washed four times, and the bound ^{35}S-ICP4 was eluted with the fusion proteins with 15 mM glutathione in 50 mM Tris-HCl (pH 8.0). After separation by SDS-PAGE, ^{35}S-ICP4 was detected by fluorography. To assess the efficiency of the binding, 1/10 of the ^{35}S-ICP4 protein used in each binding reaction (ICP4, 1/10 input) was loaded in the far right lane. The maps of the GST-ICP0 fusion proteins used in this experiment and a summary of their ICP4 binding activities are given.

These results establish that ICP4 and ICP27 can directly interact in vitro.

Stability of the ICP27-ICP4 complexes. To eliminate the possibility that the ICP27-ICP4 interaction represents a relatively nonspecific ionic interaction, we studied the salt sensitivity of the ICP27-ICP4 complex. Following binding of ^{35}S-labeled ICP4 to immobilized GST-ICP27 in 50 mM NaCl, the immobilized complexes were washed four times with buffers containing varying concentrations of NaCl. The ^{35}S-labeled ICP4 that remained bound was visualized by fluorography after SDS-PAGE. The ICP4-ICP27 complex is stable at NaCl concentrations up to 150 to 250 mM (Fig. 2). Fifty percent of the complexes dissociate at NaCl concentrations between 250 and 800 mM, and almost complete dissociation takes place at 1,500 mM (Fig. 2). These data demonstrate that the ICP27-ICP4 complex is stable at physiological salt concentrations and argue against the possibility of the interaction being weak or nonspecific.

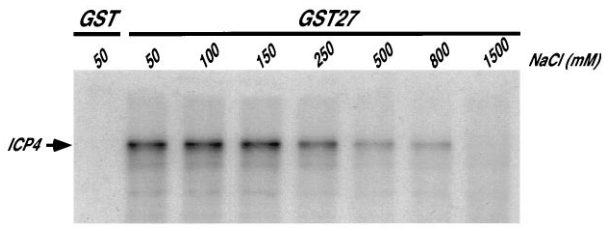

FIG. 2. Salt sensitivity of the ICP4-ICP27 complexes. Following binding of ^{35}S-labeled ICP4 to immobilized GST-ICP27 (GST27; 30 μg), the beads were washed rigorously with binding buffer containing the indicated concentrations of NaCl (see Materials and Methods). Fusion proteins, together with bound ICP4, were eluted with 15 mM glutathione in 50 mM Tris-HCl (pH 8.0) and analyzed by SDS-PAGE on 7.5% gels, and ^{35}S-labeled ICP4 was visualized by fluorography.

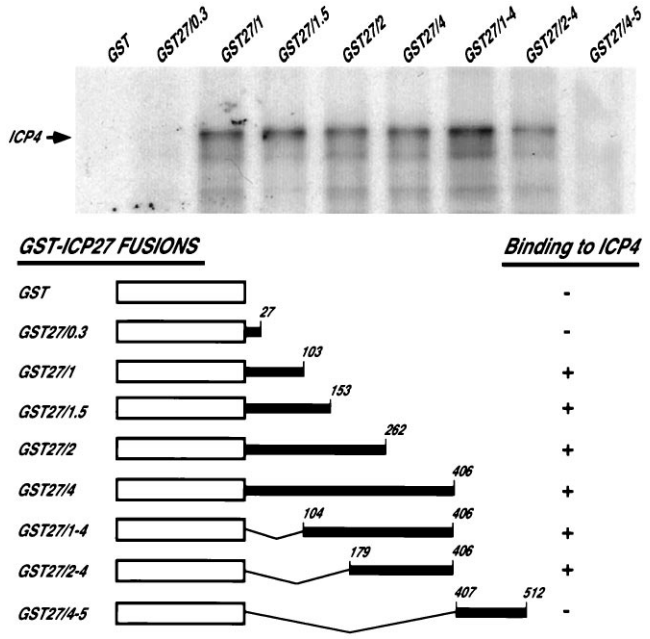

FIG. 3. Mapping of the ICP27 domains that interact with ICP4. In vitro-synthesized ^{35}S-labeled ICP4 was incubated with the indicated GST-ICP27 fusions (20 μg), which were bound to glutathione-agarose beads. Binding, washes, and detection of bound ^{35}S-ICP4 were performed as for the experiment represented in Fig. 1. A summary of the binding results, together with the maps of the various GST-ICP27 fusion proteins, is also given.

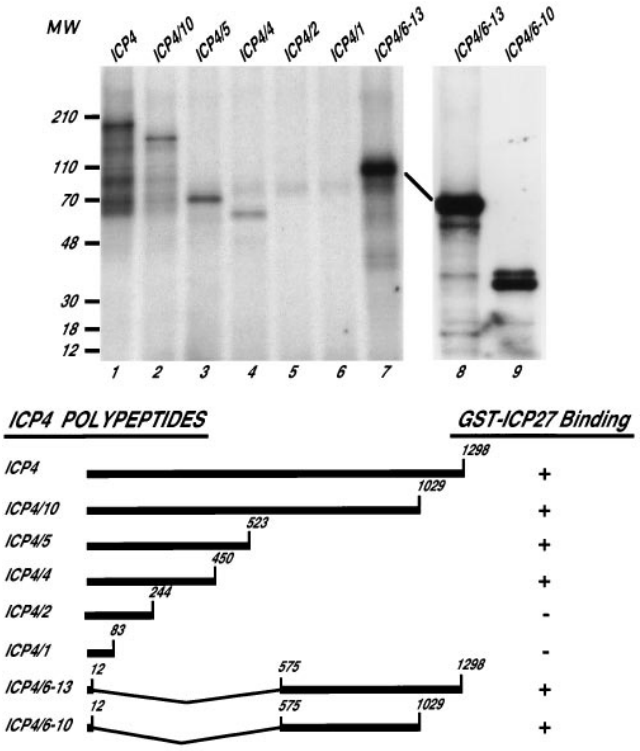

FIG. 4. Mapping of the ICP4 domains that interact with ICP27. Plasmids directing the expression of ICP4 polypeptides of varying sizes were constructed (see Materials and Methods). In vitro-transcribed-translated ^{35}S-labeled ICP4 polypeptides were used in binding experiments as described in Materials and Methods. GST-ICP27 (30 μg) bound to glutathione-agarose beads was used to capture interacting ICP4 polypeptides, which were then analyzed on a 7 to 15% linear gradient SDS-PAGE gel (lanes 1 through 7). Lanes 8 and 9 represent a separate experiment in which the proteins were analyzed by SDS-PAGE on a 7.5% gel. The positions and molecular sizes (MW; in kilodaltons) of prestained molecular markers are indicated. A map of the in vitro-synthesized ICP4 polypeptides and a summary of the binding results are also given.

Mapping of the ICP4 and ICP27 interacting domains. To determine which domain(s) of ICP27 interacts with ICP4, we expressed various regions of ICP27 in bacteria as GST fusions (Fig. 3). Following purification, the recombinant proteins were used as "bait" in protein-protein interaction assays. The demonstration that the carboxy-terminal region of ICP27 (amino acids 407 to 512) does not interact with ICP4 (Fig. 3) suggests that the ICP4 binding domain(s) of ICP27 is located within the first 407 amino acids. Because the minimal ICP27 amino-terminal region that binds ICP4 consists of amino acids 1 to 103, and those between 1 and 27 fail to do so (Fig. 3), we conclude that amino acids 27 to 103 of ICP27 represent an ICP4 binding domain. The interaction appears to be specific and not the result of nonspecific ionic interactions between the acidic amino terminus of ICP27 and basic regions of ICP4, as we have shown that the acidic amino-terminal region of ICP0 fails to bind ICP4 (Fig. 1).

Further dissection of ICP27 revealed that amino acids 179 to 406 represent another ICP4 binding domain, as fusion proteins including this region (GST27/1-4 and GST27/2-4) also bind ICP4 (Fig. 3).

To determine which region(s) of ICP4 interacts with ICP27, we synthesized various ^{35}S-labeled ICP4 polypeptides (Fig. 4), using a coupled in vitro transcription-translation system, and tested their ability to bind GST-ICP27. Carboxy-terminal truncations of ICP4 demonstrated that amino acids 1 to 450 constitute one ICP27 binding region (Fig. 4). This binding domain is likely to include amino acids 245 to 450, as a polypeptide consisting of amino acids 1 to 244 of ICP4 does not bind ICP27 (Fig. 4). To eliminate the possibility that a portion of the ICP27 binding domain of ICP4 is located at or near amino acid 245, and that our truncations disrupt it, we performed a binding experiment using a bacterially expressed GST fusion with amino acids 245 to 523 of ICP4 as bait to capture ^{35}S-labeled ICP27. This fusion protein was found to interact with ICP27 (data not shown). Therefore, we conclude that amino acids 245 to 450 of ICP4, which include the ICP4 dimerization domain and part of its DNA binding domain (22, 98), compose an ICP27 binding domain.

An additional ICP27 binding domain(s) exists within the carboxy-terminal half of ICP4, as ^{35}S-labeled ICP4 polypeptides containing amino acids 575 to 1298 and 575 to 1029 bind to ICP27 (Fig. 4). The existence of these binding domains was verified by protein binding experiments in which GST-ICP4 fusions were used as bait to capture ^{35}S-labeled ICP27. These experiments provided evidence for at least two ICP27 binding domains (amino acids 573 to 686 and 875 to 1298) within the carboxy-terminal half of ICP4 (data not shown).

Having shown that ICP4 and ICP27 make multiple contacts, we then probed the interactions between discrete regions of these proteins. GST fusion proteins with the minimal domains of ICP27 that interact with ICP4 (amino acids 1 to 103 and 179 to 406) were used as bait in protein capture assays. Our results (Fig. 5) demonstrate that amino acids 1 to 103 of ICP27 (GST27/1) interact strongly with the carboxy terminus of ICP4 (amino acids 575 to 1298) but weakly with amino acids 1 to 523. In contrast, a second ICP4-interacting domain of ICP27, i.e., amino acids 179 to 406 (GST27/2-4), interacts with both the carboxy-terminal and the amino-terminal domains of ICP4 (Fig. 5). We attribute the differences in the intensities of the

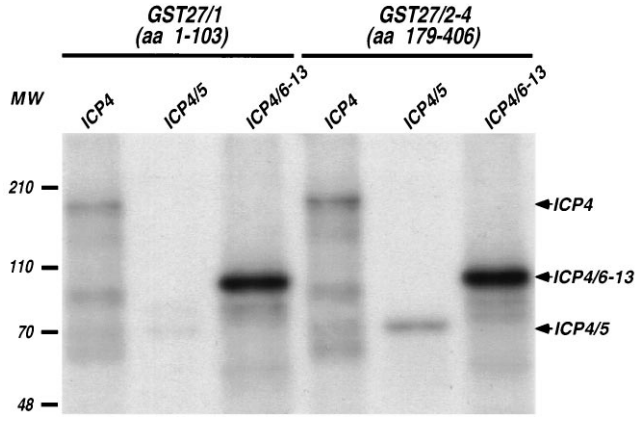

FIG. 5. Interactions between discrete domains of ICP4 and ICP27. ^{35}S-labeled full-length ICP4, ICP4/5 (amino acids 1 to 523), and ICP4/6-13 (amino acids 1 to 12 fused in frame to amino acids 575 to 1298) were synthesized in vitro by transcription-translation. Binding was performed by incubating these ICP4 polypeptides with 20 μg of ICP27 amino acids 1 to 103 fused to GST (GST27/1) or ICP27 amino acids 179 to 406 fused to GST (GST27/2-4), immobilized by binding to glutathione-agarose beads, under standard binding conditions (see Materials and Methods). The bound ^{35}S-labeled polypeptides were detected by fluorography, following analysis by SDS-PAGE on a 7.5% gel. The individual ICP4 polypeptides are indicated by arrows, and positions of prestained molecular-size markers (MW; in kilodaltons) are given.

^{35}S-labeled ICP4 polypeptides that bind GST27/1, or GST27/2-4 (Fig. 5), to variable ^{35}S-labeled ICP4 polypeptide inputs rather than to differences in their binding activities. The input variability is due to different efficiencies of the in vitro transcription-translation reactions. These data are consistent with the existence of multiple ICP27 binding sites within the carboxy-terminal half of ICP4, and they also argue for the existence of more than one binding site within amino acids 179 to 406 of ICP27.

Coimmunoprecipitation of ICP4 with ICP27 from HSV-1-infected cell nuclear extracts. The preceding results demonstrate that ICP4 and ICP27 interact in vitro. We next asked if an ICP4-ICP27 complex can be detected in nuclear extracts from HSV-1-infected cells. To this end, ICP4 and ICP27 were immunoprecipitated from HSV-1-infected HeLa cell nuclear extracts by using monoclonal antibodies, H640 and 58S (1, 82), that recognize different regions of ICP4, and the monoclonal antibody H1113 (1) to ICP27. The presence of ICP4 in the immunoprecipitates was monitored by Western blot analysis with a mixture of monoclonal anti-ICP4 antibodies. These analyses revealed that a fast-migrating form of ICP4 preferentially coimmunoprecipitates with ICP27 (Fig. 6A). This form of ICP4, with an apparent molecular mass of 145 kDa, is likely to represent a partially modified species, although the possibility exists that it is a degradation product. Furthermore, the coimmunoprecipitation is specific, as ICP4 is absent in control precipitates lacking antibody and in immunoprecipitates from cells infected with vBSΔ27 (85), an ICP27 deletion virus (Fig. 6A). The amounts of ICP4 in extracts from cells infected with viruses expressing or lacking α27 are equivalent, as anti-ICP4 antibodies precipitated comparable amounts of ICP4 from both extracts (Fig. 6A).

To ensure the specificity of the interaction between ICP4 and ICP27, we performed immunoprecipitations on ^{35}S-labeled infected cell extracts under stringent conditions. These analyses confirmed that ICP4 and ICP27 coimmunoprecipitate (Fig. 6B) and that ICP27 associates primarily with faster-migrating forms of ICP4 (Fig. 6A and B). Of the six electrophoretic ICP4 isoforms that precipitate with the anti-ICP4 antibody, only the five faster-migrating forms coimmunoprecipitate with ICP27 (Fig. 6B). This is consistent with the concept that ICP27 interacts primarily with less-modified ICP4, as posttranslational modifications are expected to result in decreased electrophoretic mobilities. However, the possibility that these forms represent degradation products, rather than partially modified nascent polypeptides, cannot be formally excluded. The identities of the coimmunoprecipitated proteins as ICP4 and ICP27 were verified by Western blot analysis (data not shown). Furthermore, immunoprecipitations were performed with extracts from cells infected with a battery of viruses with mutations in the α0 gene. Viruses carrying mutations in this gene synthesize lower levels of ICP27 when they infect cells at low multiplicities of infection (39). Therefore, they allow us to assess the specificity of the immunoprecipitation results by providing a correlation between ICP27 levels and the amount of coimmunoprecipitated ICP4, whose synthesis is only modestly affected by these mutations. Our results demonstrate that the amount of coimmunoprecipitated ICP4 correlates well with the levels of ICP27 (Fig. 6B).

Our results also reveal the following about the ICP4-ICP27 interaction. This interaction is quantitative, as a significant portion (approximately 10%) of the nascently synthesized ^{35}S-labeled ICP4 associates with ICP27 (data not shown). Furthermore, the ICP4-ICP27 complexes are very stable; they can withstand the ionic and nonionic detergents present in the RIPA buffer.

ICP27 is present in an ICP4-DNA complex. ICP4 is a dimeric sequence-specific DNA binding protein (51, 53) that binds to high-affinity sites containing the consensus DNA sequence ATCGTCNNNNYCGRC (14, 20, 21). In addition to these canonical, high-affinity sites, ICP4 also binds, with lower affinity, to noncanonical binding sites lacking the above motif. Such low-affinity ICP4 binding sites, which do not have any apparent sequence homology, have been found in the promoters and in the transcribed regions of several HSV-1 genes (52, 53).

We, and others (33, 59–61), have demonstrated by EMSA the formation of an ICP4-containing nucleoprotein complex with the −16/+56 region of the HSV-1 TK gene (TKB). This sequence lacks a canonical ICP4 binding site. The presence of ICP4 is demonstrated by the ability of an anti-ICP4 antibody (H640) to supershift this protein-DNA complex (Fig. 7). The presence of ICP27 in this complex is demonstrated by the ability of H1113, an anti-ICP27 antibody, to supershift as well (Fig. 7). An amount of anti-ICP27 antibody producing only a partial supershift was used in the experiment shown (Fig. 7), as addition of higher concentrations of antibody results in complex dissociation rather than a supershift (data not shown). In contrast, an antibody against ICP0 fails to supershift (Fig. 7), thus providing a control for specificity. The anti-ICP27-induced supershift is not the result of H1113 antibody cross-reactivity with ICP4. This antibody does not recognize ICP4 in immunoprecipitation experiments (Fig. 6) or in Western blots (data not shown). It also fails to supershift or dissociate an ICP4-containing protein-DNA complex with a high-affinity binding site from the α4 promoter (see below). These data demonstrate that ICP27 participates in an ICP4-containing nucleoprotein complex that forms on the DNA sequences contained within the TKB site.

ICP27 requirement for the ICP4-TKB interaction. We next asked if ICP27 affects the ability of ICP4 to form complexes on the TKB site. Nuclear extracts were prepared from HeLa cells infected with wild-type HSV-1 or 27-LacZ, a virus containing a *lacZ* insertion in the gene encoding ICP27 (84), and their

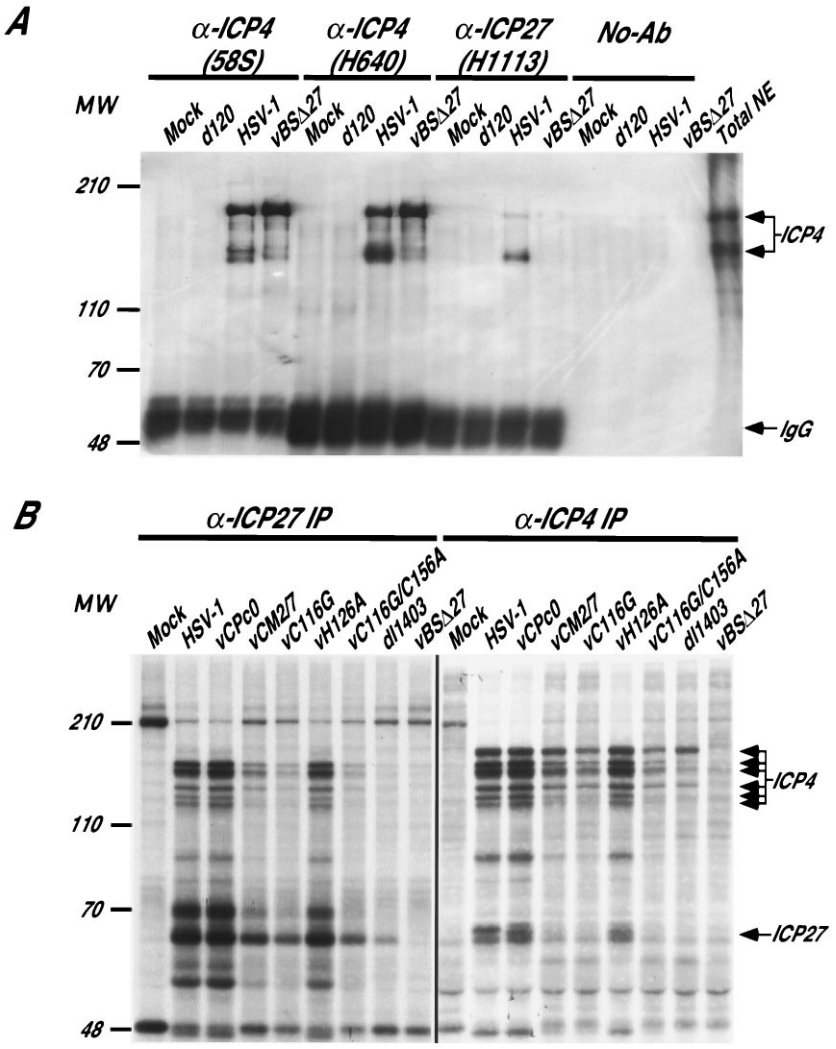

FIG. 6. Coimmunoprecipitation of ICP27 and ICP4. (A) Immunoprecipitations from nuclear extracts. Nuclear extracts were prepared from HeLa cells that were either mock-infected or infected with HSV-1 strain KOS, an ICP4-deficient virus (*d*120), or an ICP27-deficient virus (vBSΔ27). Immunoprecipitations were performed with 200 μg of nuclear extract and 5 μl of each monoclonal antibody as described in Materials and Methods. Control precipitations were performed with protein G-Sepharose beads in the absence of antibody (No-Ab). Two monoclonal antibodies directed against different ICP4 epitopes (H640 and 58S) and one against ICP27 (H1113) were used. Complexes were analyzed by SDS-PAGE and Western blot with a mixture of anti-ICP4 monoclonal antibodies (see Materials and Methods). A total nuclear extract from HSV-1 (KOS)-infected HeLa cells (Total NE) was also loaded on the gel as a marker. The positions of ICP4 and immunoglobulin G (IgG) heavy chains are indicated by arrows, and the electrophoretic mobilities of prestained molecular-size markers (MW; in kilodaltons) are shown. (B) Immunoprecipitations (IP) from lysates of ^{35}S-labeled cells. Vero cells were infected with the indicated viruses and pulse-labeled with ^{35}S-labeled amino acids for 45 min at 8 h postinfection. Total cell lysates were prepared, and immunoprecipitations were performed as described in Materials and Methods. The immunoprecipitated ^{35}S-labeled proteins were visualized by fluorography after SDS-PAGE analysis on 7.5% gels. ICP27 was immunoprecipitated with a rabbit polyclonal antibody (CLU38), whereas a mouse monoclonal antibody (H1114) was used for the immunoprecipitation of ICP4. The viruses used in this experiment were HSV-1 type 17; vCPc0, an ICP0 cDNA virus; *dl*1403, an α0 deletion virus; vBSΔ27, an α27 deletion virus; vCM2/7, a virus with a deletion within the α0 gene coding region that removes amino acids 106 to 212 of ICP0; vC116G and vC116G/C156A, two viruses that carry one and two mutations, respectively, in residues that are critical for the structure of the C_3HC_4 zinc finger in ICP0; and vH126A, a virus with a silent mutation in the C_3HC_4 zinc finger domain of ICP0. The positions of ICP27 and the multiple electrophoretic ICP4 forms are indicated by arrows. The positions of the molecular-size markers in the gel are also indicated.

abilities to form DNA-protein complexes with TKB DNA were compared. The ICP4-containing TKB complex was barely detectable when nuclear extracts from cells infected with the 27-LacZ virus were used (Fig. 8A). Western blot analysis showed that this diminution does not result from decreased ICP4 protein levels, as ICP4 is more abundant in the ICP27-deficient extract than in the extract from cells infected with wild-type HSV-1 (data not shown), nor is the ICP4 protein in the ICP27-deficient extract inactive; it is competent to form protein-DNA complexes with a high-affinity site (Fig. 8B). Furthermore, the ICP4-DNA complexes formed with the high-affinity ICP4 binding site from the α4 promoter are supershifted with the anti-ICP4 antibody H640 but not with H1113, an antibody specific for ICP27 (Fig. 8B). This eliminates the possibility that the ICP27 antibody cross-reacts with ICP4 under the conditions of the supershift experiment. The decreased mobility of the ICP4-DNA complex from the 27-LacZ-infected cell extract is consistent with previous studies showing that the electrophoretic mobility of ICP4 from cells infected with ICP27-deficient viruses is altered (48, 68, 89). These results indicate that, in HSV-1-infected cell extracts, the presence of ICP27 may modulate the ability of ICP4 to form nucleoprotein

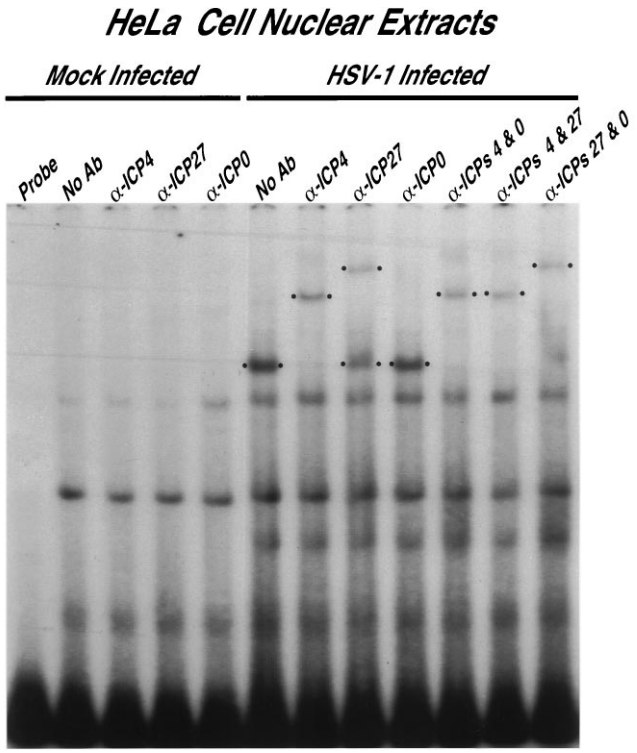

FIG. 7. ICP27 participates in an ICP4 complex with TKB. DNA-protein complex formation, between the TKB DNA and nuclear extracts from mock-infected or HSV-1-infected (6 h) HeLa cells, was measured by EMSA with 4% native polyacrylamide gels, as described in Materials and Methods. Supershift analyses were performed by adding 1 μl of monoclonal antibody directed against ICP4 (α-ICP4) (H640), ICP27 (α-ICP27) (H1113), or ICP0 (α-ICP0) (H1083). No Ab, no antibody. The infected-cell-extract-specific complexes are marked with black dots.

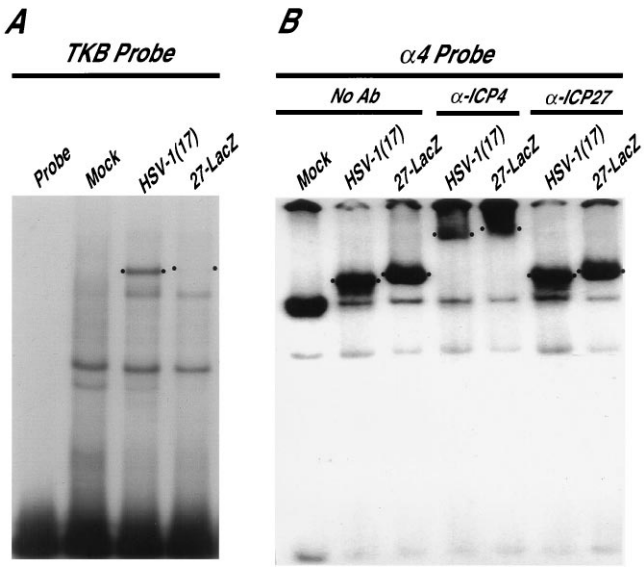

FIG. 8. ICP27 is required for ICP4-TKB complex formation by HSV-1-infected cell extracts. Nuclear extracts (4 μg) from HeLa cells infected for 6 h with either wild-type HSV-1 or the ICP27-deficient 27-LacZ virus were tested for their abilities to form DNA-protein complexes with the noncanonical TKB site (A) or the high-affinity site from the α4 promoter (B). The conditions for binding and EMSA are described in Materials and Methods. Infected-cell-specific nucleoprotein complexes are indicated by dots. Supershift analysis (B) was performed with 1 μl of ICP4-specific (α-ICP4) (H640) or ICP27-specific (α-ICP27) (H1113) monoclonal antibodies, as described in Materials and Methods. No Ab, no antibody.

complexes with TKB but has no effect on ICP4's ability to bind a high-affinity site. Similar results are also produced with cells infected with vBSΔ27, an ICP27 deletion virus (85).

The inability of ICP4 from infected cell extracts to form complexes with TKB in the absence of ICP27 could either reflect a direct requirement for ICP27 in complex formation or an indirect effect on ICP4 resulting from changes in phosphorylation or another posttranslational modification(s). To distinguish between these possibilities, we expressed these two proteins individually or together in 293 cells. EMSA of extracts prepared from transfected cells demonstrated that active ICP4 is produced, as judged by its ability to bind the high-affinity site from the α4 promoter (Fig. 9A). Surprisingly, ICP4-containing extracts from these cells bind the TKB site (Fig. 9A). Coexpression of ICP27 and ICP4 results in decreased overall amounts of ICP4-DNA complexes on both the high-affinity site and the TKB site, without affecting their relative abundance (Fig. 9A). Western blot analysis revealed that this reduction results from decreased ICP4 levels in extracts from cotransfected cells (data not shown) rather than inhibition of binding by ICP27. Furthermore, mixing experiments demonstrated that addition of ICP27 does not inhibit the DNA binding activity of ICP4 on either site (Fig. 9A). These results indicate that the presence of ICP27 is not required for ICP4 complex formation with TKB when ICP4 is overexpressed in 293 cells. It is not clear whether the ability of ICP4, when overproduced in 293 cells, to bind TKB results from the presence of a cellular factor that can functionally substitute for ICP27 or from the generation of a subpopulation of ICP4 molecules with altered DNA binding activity.

To determine if additional factors are required for ICP4 binding to TKB, we expressed the DNA binding domain of ICP4 (amino acids 245 to 523) in bacteria, as a fusion protein with GST. EMSA of the DNA binding activity of the purified fusion protein showed that it binds TKB as efficiently as it binds the high-affinity DNA binding site from the α4 promoter (Fig. 9B). The binding is specific, as a GST fusion with another basic domain of ICP4 (amino acids 573 to 686) fails to bind either site (Fig. 9B). These data clearly indicate that the DNA binding domain from ICP4 does not require ICP27, or other factors, to bind TKB. Therefore, the modulation of ICP4's TKB binding activity by ICP27, in the context of an infection, is likely to reflect changes in the modification state of ICP4.

DISCUSSION

In this report we demonstrate a direct physical interaction between ICP4 and ICP27, two essential regulatory proteins of HSV-1. Mapping of their interaction domains reveals that these proteins make multiple contacts. Furthermore, coimmunoprecipitation experiments indicate that ICP27 associates predominantly with particular isoforms of ICP4 in extracts from cells infected with HSV-1. We find both ICP27 and ICP4 in a DNA-protein complex formed on a region downstream of the TK promoter (TKB). ICP27 is absent, however, from the ICP4 complex that forms with the high-affinity site present in the α4 promoter. Additionally, the abundance of the ICP4-TKB complex is dramatically reduced when extracts from cells infected with an ICP27-deficient virus are used. Yet it is not the physical presence of ICP27 that is required for the formation of the ICP4-TKB complex, as evidenced by the fact that ICP4 overproduced in mammalian cells in the absence of

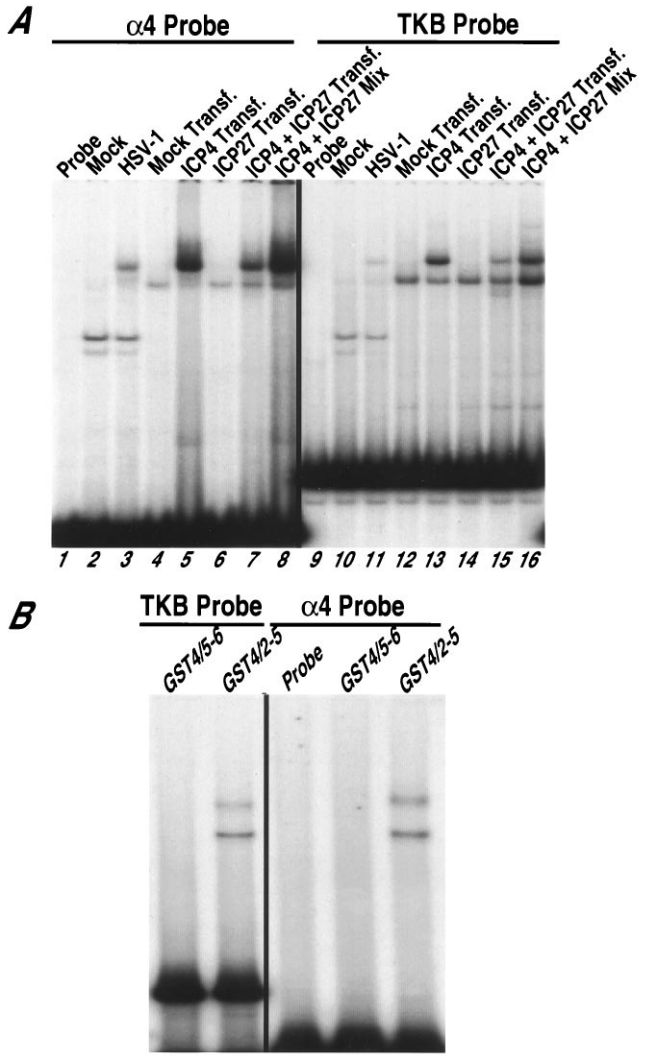

FIG. 9. ICP4 complex formation with extracts from transfected cells or purified bacterially expressed proteins. (A) Extracts from 293 cells transfected with plasmids expressing ICP4 (ICP4 Transf.) or ICP27 (ICP27 Transf.) under the control of the cytomegalovirus promoter were tested by EMSA (see Materials and Methods) for their ability to form ICP4-DNA complexes with DNA probes containing either the high-affinity binding site from the −17/+32 region of the α4 gene (α4 probe) or the noncanonical TKB site (TKB probe). Nuclear extracts from mock-infected or HSV-1-infected (6 h) HeLa cells were included as controls. Binding reactions in lanes 8 and 16 were performed by mixing extracts from cells individually transfected with either ICP4 or ICP27 expression constructs. (B) EMSA analysis of the binding activity of the purified DNA-binding domain of ICP4 (amino acids 245 to 523), expressed in bacteria as a GST fusion (GST4/2-5), to the canonical α4 gene site (α4 probe) and the noncanonical TKB site (TKB probe). A purified, bacterially expressed GST-fusion protein with amino acids 573 to 686 of ICP4 (GST4/5-6) was used as a negative control.

ICP27, or the ICP4 DNA binding domain expressed in bacteria, is able to form this complex. These processes could generate either partially modified or unmodified ICP4 polypeptides that, unlike more highly modified ICP4 molecules, are capable of binding to TKB DNA. Therefore, it is likely that ICP27 affects the functions of ICP4 by altering its posttranslational modification state.

ICP27 acts as both a positive and a negative regulator of gene expression in transfection experiments (18, 29, 69, 80, 89). With few exceptions (9, 68), ICP27 alone has little if any effect on the activities of target constructs. However, it exerts an effect when cotransfected with the transcriptional activators ICP4 and ICP0. The ICP27 effect appears to be independent of promoter regulatory elements in transfection experiments. Rather it depends on the presence of introns or 3′ untranslated sequences (45–47, 77, 79). These findings suggest that ICP27 functions in the regulation of gene expression at the posttranscriptional level. Furthermore, it was shown that ICP27 inhibits pre-mRNA splicing, both in vivo and in vitro (30, 63, 79), and that it mediates redistribution of the host cell pre-mRNA splicing factors and small nuclear ribonucleoprotein particles (snRNPs) during the course of an HSV-1 infection (42, 63, 78). This redistribution alone, however, is not sufficient for inhibition of splicing, as a temperature-sensitive α27 mutant that allows snRNP redistribution is unable to inhibit splicing (78). Inhibition of mRNA splicing could provide a selective advantage to the virus, whose mRNAs are predominantly unspliced (44, 93). Indeed, an ICP27-dependent decrease in the cellular mRNA levels is observed after HSV-1 infection (30). ICP27 was also shown to increase the 3′ processing efficiencies and poly(A) site usage of some late HSV-1 gene transcripts in vitro, while having no effect on the 3′ processing or polyadenylation of immediate-early and early transcripts (45). This may explain the requirement for ICP27 in the expression of true late genes (75), even though ICP27 does not influence poly(A) site usage of all late genes (45).

Genetic analysis of α27 mutant viruses suggested that ICP27 may have a role in transcription (48, 84). Recent work with viral mutants expressing defined domains of ICP4 but unable to express ICP27 also suggested that ICP27 is required for the expression of some early HSV-1 genes and that this effect is both transcriptional and posttranscriptional (76). ICP27 also stimulates RNA polymerase III transcription of the cellular *Alu* repeated sequences by increasing the activity of transcription factor TFIIIC (34).

ICP27 could regulate transcription of HSV-1 genes by one of several mechanisms. It might influence the compartmentalization of other HSV-1 regulatory proteins. Indeed, ICP27 was shown to affect the intracellular localization of ICP4 and ICP0, two HSV-1 transcriptional regulators (54, 103, 104). Specifically, the presence of ICP27 inhibits the nuclear localization of ICP4 and ICP0 both in transfection experiments and in the context of virus infection (54, 103, 104). It is not clear, however, whether the above effects result from (i) direct physical interactions between ICP27 and ICP4 and/or ICP0, (ii) ICP27 effects on the conformations or posttranslational modifications of these proteins, or (iii) another, as yet unknown, mechanism. The ICP27 region responsible for the inhibition of ICP4 nuclear localization was mapped to amino acids 262 to 512 (104), which is consistent with our finding that amino acids 179 to 406 of ICP27 bind directly to ICP4 (Fig. 3). Therefore, it appears that the inhibitory effect of ICP27 on ICP4's nuclear localization could be mediated by physical interactions between these two proteins.

ICP27 could also affect transcription of HSV-1 genes by altering the posttranslational modification state of ICP4. The presence of ICP27, either in HSV-1-infected or transfected cells, affects the electrophoretic mobility of ICP4 (48, 68, 89), which is highly modified posttranslationally by phosphorylation and nucleotidylation (4, 5, 62, 66, 97, 99). Protein phosphorylation alters the properties and activities of a number of transcriptional regulators and is one of the best understood biochemical regulatory processes (references 7 and 35 and references therein). Specifically, in vitro dephosphorylation of HSV-1-infected cell extracts differentially affects ICP4's ability to recognize gene regulatory regions (61). Here, we demonstrate that the presence of ICP27 during an HSV-1 infection

can modulate the ability of ICP4 to form a stable DNA-protein complex on the noncanonical TKB site but not on a high-affinity binding site (Fig. 8). The finding that ICP4 overexpression in 293 cells overcomes the ICP27 requirement for complex formation with TK DNA (Fig. 9A) indicates that there may be a 293 cell-specific factor that functionally substitutes for ICP27 or that ICP4 overproduction may result in the generation of a population of partially, or differentially, modified ICP4 molecules capable of binding TKB. The hypothesis that a cell factor is required, however, is eliminated by our observation that the bacterially expressed, and presumably unmodified, DNA binding domain of ICP4 (98) can bind TKB as efficiently as it binds a high-affinity site (Fig. 9B). Because this domain, when expressed in bacteria, has the same DNA binding specificity for a high-affinity site as the full-length ICP4 protein from HSV-1-infected cells (64), it is unlikely that our results are due to relaxed sequence specificity of the bacterially expressed ICP4. Therefore, it appears that ICP27 can alter the DNA binding specificity of ICP4 by modulating its posttranslational modification state. This conclusion is further supported by two observations. First, of the multiple electrophoretic forms of ICP4, the one with the slowest mobility, and presumably with the highest level of posttranslational modification, is unable to bind noncanonical sites but is able to bind high-affinity sites (53). It was this isoform that failed to coimmunoprecipitate with ICP27 in our experiments (Fig. 6). Second, the ability of nd8-10, a truncated ICP4 polypeptide missing amino acids 142 to 210 and 774 to 1298, to bind a high-affinity site in the OrfP (LS/T) promoter of HSV-1 (6) and repress its expression is diminished in the absence of ICP27 (76). Because this polypeptide contains an intact ICP4 DNA binding domain and is phosphorylated, it is thought that ICP27 may modulate its DNA binding activity at the level of posttranslational modification (76). Indeed, phosphopeptide analysis indicated that ICP4 phosphorylation is altered in cells infected with an ICP27 mutant virus (99). We note that the nd8-10 polypeptide retains one of the ICP27-interacting sites (amino acids 243 to 450) identified in this study (Fig. 4 and 5).

How ICP27 influences the modification state of ICP4 is not clear, as it has not yet been shown to possess any enzymatic activity. Perhaps, by its physical association with ICP4, ICP27 prevents some modification from occurring, e.g., by masking a potential phosphorylation site.

In addition to modulating ICP4's DNA binding activity, ICP27 is physically present in an ICP4-DNA complex with the TKB fragment (Fig. 7). Although ICP27 possesses potential RNA binding motifs (49) and binds RNA directly (9), no sequence-specific DNA binding activity has been described for this protein. Therefore, we postulate that ICP27 is brought to the complex with TK DNA by ICP4. The absence of ICP27 from ICP4 complexes formed on a high-affinity site (Fig. 8B) indicates that ICP27 interacts primarily with the isoelectric form of ICP4 that forms complexes with the noncanonical TKB site. The participation of ICP27 in nucleoprotein complexes formed in the proximity of gene regulatory regions may suggest a direct role for this protein in HSV-1 transcriptional regulation. While the direct participation of ICP27 in HSV-1 transcriptional regulation is merely speculation, the involvement of RNA binding proteins in transcription is not unprecedented and has been established in a number of systems (see references 11, 50, and 86 and references therein). We note that the amino-terminal domain of ICP27 is highly acidic and may act as a transcriptional activator (41, 88) when brought to a promoter by ICP4. Moreover, deletions of the acidic domain of ICP27 impair the lytic cycle of mutant viruses (71). These data support a role for the acidic amino terminus in ICP27's regulatory activities.

In conclusion our data suggest a role for ICP27 in HSV-1 transcriptional regulation, acting either directly or through its interaction with ICP4 and the modulation of its DNA binding activity. Therefore, in addition to studies addressing the post-transcriptional effects of ICP27, further effort is being directed towards understanding the potential role(s) of this protein in transcription.

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