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Functional Relevance of Specific Interactions between Herpes Simplex Virus Type 1 ICP4 and Sequences from the Promoter-Regulatory Domain of the Viral Thymidine Kinase Gene

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The herpes simplex virus (HSV) type 1 immediate-early regulatory protein ICP4 is required for induced expression of HSV early and late genes, yet the mechanism by which this occurs is not known. We examined the promoter and flanking sequences of the HSV early gene that encodes thymidine kinase for the ability to interact specifically with ICP4 in gel retardation assays. Protein-DNA complexes containing ICP4 were observed with several distinct regions flanking the *tk* promoter. *cis*-Acting elements that interact with cellular transcription factors were apparently not required for these interactions to form. Purified ICP4 formed protein-DNA complexes with fragments from these regions, and Southwestern (DNA-protein blot) analysis indicated that the interaction between ICP4 and these sequences can be direct. None of the *tk* sequences that interact with ICP4 contains a consensus binding site for ICP4 (S. W. Faber and K. W. Wilcox, Nucleic Acids Res. 14:6067–6083, 1986), reflecting the ability of ICP4 to interact with more than one DNA sequence. A mutated ICP4 protein expressed from the viral genome that retains the ability to bind to a consensus binding site but does not bind specifically to the identified sites flanking the *tk* promoter results in induced transcription of the *tk* gene. These data support hypotheses for ICP4-mediated transactivation of the *tk* promoter in Vero cells that do not require the intrinsic ability of ICP4 to bind specifically in or near the promoter of the *tk* gene.

Expression of herpes simplex type 1 (HSV-1) genes is coordinately and sequentially controlled such that three major phases of protein synthesis occur (24, 25). These produce the immediate-early (IE), early, and late gene products. IE gene products are expressed in the absence of prior viral protein synthesis (2, 5, 46), and functional IE proteins are required to induce expression of both early and late genes (24, 25).

One IE protein, ICP4, has been shown to be required for induction of HSV-1 early and late genes and for repression of its own and possibly other IE genes (8, 13, 47, 54). Transient expression assays using cloned ICP4 and an appropriate reporter gene have shown that ICP4 transactivates early and late gene promoters (10, 18, 22, 40, 48), while genes under the control of the ICP4 promoter are repressed (10, 41, 42, 47). These studies are consistent with conclusions based on observations that temperature-sensitive (*ts*) and deletion mutants of ICP4 are impaired for early and late gene expression and overexpress the ICP4 gene and other IE genes (9, 13).

It previously has been shown that ICP4 has the ability to form protein-DNA complexes with DNA sequences including the hexanucleotide ATCGTC (19). One such sequence is present at the transcription initiation site of the gene for ICP4 (20, 28, 39). Several findings show that ICP4 binding to this site contributes to autoregulation by ICP4 (11, 12, 41, 42, 45, 49), including the observation that a mutation in this site introduced into the viral genome causes elevated levels of ICP4 (N. DeLuca, unpublished data).

The transactivating function of ICP4 is not as well understood. ICP4 is required for induced expression of early and late genes during viral infection; however, attempts to demonstrate *cis*-acting sequences responsible for induction have not been definitive. The promoter region for the HSV-1 thymidine kinase gene (*tk*) is perhaps the best characterized among ICP4-inducible gene products. Classified as an early gene, *tk* can be expressed at a basal level in heterologous systems, such as frog oocytes and mouse L cells (33, 34). Four *cis*-acting elements that correspond to binding sites for cellular transcription factors (26, 32, 35, 36) mediate this basal level of expression. However, in the context of the virus, the *tk* gene is efficiently expressed only in the presence of IE gene products. To address the possibility that expression under the control of HSV-1 *trans*-acting factors is regulated differently from the basal level of expression, several studies have attempted to define *tk* promoter domains required for induced expression. While earlier reports suggested that induction-specific sequences exist (15, 57), other studies (6, 14) have demonstrated that the promoter domains required for basal expression are also required during viral infection.

In contrast, studies of another HSV gene promoter, glycoprotein D (gD), show the existence of consensus and nonconsensus binding sites for ICP4 and suggest that these sites contribute to transactivation (3, 52, 53). Similarly, the IE protein of pseudorabies virus, a protein analogous to the HSV-1 ICP4 protein (31), binds to sites of limited homology on gene promoters which can be stimulated in vitro by the IE protein (7). Furthermore, multiple ICP4-binding sites, including consensus and nonconsensus sequences, have been identified in an HSV late gene (38), although no function has been attributed to them. In addition, mutational analysis of ICP4 has identified regions of the protein responsible for its regulatory activities based on the ability of mutant ICP4 polypeptides to retain transactivating and transrepressing functions in transient expression assays (11, 44, 45, 50) and in the background of the virus (12, 50). On the basis of these studies, it was determined that the region of the ICP4

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polypeptide which conferred DNA binding to consensus binding sites was required for regulatory activity. These observations raise the possibility that DNA binding plays a role in transactivation by ICP4.

In this report, we describe sequences near the HSV-1 *tk* promoter which interact with ICP4, confirming the ability of ICP4 to interact with multiple DNA sequences of, at best, limited homology to the consensus sequence. The behavior of a mutant ICP4 polypeptide with greatly reduced affinity for the binding sites in this region suggests that the specific sequence recognition of the −254 to +54 region of the *tk* gene by ICP4 is not required for transactivation.

MATERIALS AND METHODS

Virus and cells. Procedures for growth and maintenance of CV-1 and Vero cells have been described previously (8, 13). The wild-type strain of HSV-1 used was KOS. The ICP4 nonsense mutants *n*12 and *n*208 were previously described (12). The ICP4 insertion mutants *i*11 and *ni*11 are described below.

Whole-cell extracts. Approximately 10^6 Vero cells were mock infected or infected with wild-type KOS or an ICP4 mutant virus at a multiplicity of infection of 20 PFU per cell. At 6 h postinfection, cells were washed and harvested in TBS (140 mM NaCl, 5 mM KCl, 25 mM tricine [pH 7.4], 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$) and pelleted at 4°C. Pellets were stored at −80°C until use. Extracts were prepared by suspending the cells in 50 μl of cold lysis buffer (50 mM Tris hydrochloride [pH 8.0], 0.5 M NaCl, 2% Nonidet P-40 [NP-40], and 0.1 mM 1-chloro-3-tosylamido-7-amino-L-2-heptanone [TLCK] added immediately before use) and incubating them on ice for 30 min. The suspensions were then centrifuged for 15 min at 13,000 × *g*, and the resulting supernatant was carefully drawn off.

Radiolabeled probes. Probes were prepared from plasmid DNAs containing all or part of the *tk* promoter. Plasmids were cleaved at a relevant restriction site, treated with calf intestinal phosphatase (Boehringer Mannheim Biochemicals), end labeled with [γ-^{32}P]ATP (Dupont, NEN Research Products) by using T4 polynucleotide kinase (New England BioLabs), and cleaved again with a restriction endonuclease to release the desired end-labeled fragment. DNA fragments were separated on 8% or 10% polyacrylamide gels, stained with ethidium bromide, and cut out of the gel. DNA was recovered and purified by electroelution, followed by an Elutip column (Schleicher & Schuell, Inc.) and a Sephadex G50 spin column. Plasmids containing linker scanner (LS) mutations were a kind gift of Steven McKnight and Donald Coen and have been described previously (6, 35).

Gel retardation assays. DNA-binding reactions were performed as follows. One nanogram of an end-labeled probe (3 × 10^4 to 6 × 10^4 cpm/ng) and 10 to 15 μg (~3 μl) of a whole-cell extract from uninfected or infected cells (determined by Bio-Rad protein assay) were combined in a buffer consisting of 10 mM Tris hydrochloride (pH 7.5), 1 mM EDTA, 0.1% NP-40, 5% glycerol, and 50 μg of bovine serum albumin per ml for 30 min at room temperature. A 2.0 to 5.0-μg sample of poly(dA-dT) · (dA-dT) or poly(dI-dC) (Pharmacia) was included as a nonspecific competitor. The total volume of the binding reaction was 30 μl. To identify protein-DNA complexes containing ICP4, 1 μl of a 1:10 dilution of 58S, a monoclonal antibody to ICP4 (51), was added to the binding mixture after the initial 30-min reaction and was allowed to incubate for an additional 15 min at room temperature. Binding reactions were electrophoretically separated on a 4% native polyacrylamide gel at 200 V as previously described (12). The gels were dried and exposed overnight to Kodak XAR-5 film with intensifying screens.

Purification of ICP4. Approximately 2 × 10^8 Vero cells were infected with KOS at a multiplicity of infection of 10 PFU per cell. At 12 h postinfection, the infected cells were scraped from culture bottles, pelleted at 4°C, suspended in cold TBS containing 0.1 mM TLCK, and pelleted. The cells were then suspended in RSB (10 mM NaCl, 10 mM Tris hydrochloride [pH 7.4], 3 mM $MgCl_2$, 0.1% NP-40) containing 0.1 mM TLCK, pelleted, suspended in 25 ml of RSB plus 0.1% NP-40, and subjected to six strokes in a Dounce homogenizer. Nuclei were isolated by centrifugation in a Beckman GPR centrifuge at 2,000 rpm for 5 min. For lysis, nuclei were suspended in 1.0 ml of RSB, followed by addition of an equal volume of cold 2× lysis buffer (100 mM Tris hydrochloride [pH 8.0], 1 M KCl, 4% NP-40, 0.2 mM TLCK). After 40 min on ice with stirring every 10 min, the sample was centrifuged for 1 h at 45,000 rpm in an SW50.1 rotor at 4°C. The supernatant was saved as the extract and subsequently fractionated on the basis of size in a manner similar to that reported previously (37). The extract was applied to a Sephacryl S-300 column (1.6 by 90 cm) equilibrated with CB0.5 {20 mM Tris hydrochloride [pH 8.0], 0.5 M KCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 0.01% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate [CHAPS], 0.1 mM TLCK} at a rate of 12 ml/h. Fractions were assayed by gel retardation and Western dot blot (immunoblot). Briefly, 1 μl of each fraction was spotted onto nitrocellulose and analyzed for the presence of ICP4 by incubation with a 1:100 dilution of antibody 58S (51) by using the Protoblot Western blot alkaline phosphatase system (Promega Biotec). Fractions containing ICP4 were pooled and mixed with 10% glycerol and 100 μg of bovine serum albumin per ml for storage.

ICP4 was further purified by specific DNA affinity. Two complementary single-stranded oligonucleotides specifying *Bam*HI sites at the ends and corresponding to the consensus binding site at the start site of ICP4 transcription were used to construct a 2-ml DNA affinity column by the method of Kadonaga and Tjian (27). The sequences of the oligonucleotides were GATCCGCCCCGATCGTCCACACGGAGCG CG and GATCCGCGCTCCGTGTGGACGATCGGGGCG. The column was equilibrated with CB0.05 buffer (same as CB0.5, except that CB0.05 contains 0.05 M KCl). The sample was dialyzed against CB0.05 and subsequently applied in 10 ml to the DNA affinity column at the rate of 12.5 ml/h. The column was washed with 10 to 20 ml of CB0.05 and then eluted with stepwise applications of 10 ml each of CB0.3, CB0.6, and CB0.9. These solutions contained 0.3, 0.6, and 0.9 M KCl, respectively. The flowthrough, wash, and eluted fractions were assayed by Western dot blot. ICP4-containing fractions corresponding to the 0.3 M elution were diluted to 0.05 M KCl and passed over the affinity column an additional time as already described. The final preparation of ICP4 was frozen at −80°C.

Methylation interference. End-labeled DNA probes were partially methylated as already described (30), ethanol precipitated, and treated with RNase to degrade the tRNA present in the stop buffer (30). The samples were phenol extracted and subsequently passed over a G50 spin column. The probe was then used in a gel retardation assay (described above), using affinity-purified ICP4. DNA was transferred to NA45 paper (Schleicher & Schuell) for 2 h at 225 mA in an electroblot apparatus, and the NA45 was exposed to film. Regions of the membrane corresponding to bound

and unbound DNAs were cut out and eluted in 50 mM arginine–1 M NaCl for 30 min at 70°C. Following ethanol precipitation, the samples were subject to piperidine treatment as already described (30). Bound and unbound DNAs were then electrophoresed on a 12% sequencing gel.

Southwestern and Western blots. Binding of the promoter sequences to ICP4 was analyzed by a Southwestern assay based on a modification of the procedure described by Michael et al. (38). Briefly, approximately 0.5 μg of purified ICP4 was electrophoresed on a sodium dodecyl sulfate (SDS)–9% polyacrylamide gel and subsequently electroblotted to nitrocellulose. The nitrocellulose was then cut into strips corresponding to individual gel lanes. The protein on the strips was renatured by washing three times for 1 h each time in buffer A (10 mM Tris hydrochloride [pH 7.2], 5% skim milk, 10% glycerol, 2.5% NP-40, 0.1% dithiothreitol, 150 mM NaCl) at room temperature. Following a brief wash in buffer B (10 mM Tris hydrochloride [pH 7.2], 50 mM NaCl, 0.25% skim milk), the strips were placed in hybridization bags containing 4 ml of buffer B plus 1 μg of salmon sperm DNA per ml and 5×10^5 cpm (approximately 5 to 10 ng) of the indicated ^{32}P-labeled probe. Inclusion of salmon sperm DNA was essential to eliminate nonspecific binding of the probe to ICP4. Hybridization proceeded with constant shaking overnight at 4°C. The strips were briefly rinsed in two changes of cold buffer B without skim milk, dried, and exposed to film. For Western analysis, one strip was removed from the nitrocellulose sheet immediately after the transfer procedure and subsequently analyzed for the presence of ICP4 by incubation with a 1:100 dilution of antibody 58S and the Protoblot Western blot alkaline phosphatase system (Promega).

Expression studies. Nuclear run-on transcription experiments were performed exactly as previously described (12), by using the modified procedure of Weinheimer et al. (55). Northern (RNA) blot analysis and analysis of [^{35}S]methionine-labeled SDS-peptides on 9% polyacrylamide–*N,N'*-diallytartardiamide-cross-linked gels, were also performed as previously described (12). To probe the Northern blot for *tk*, a gel purified *Sac*I-*Sma*I fragment internal to the *tk* mRNA sequence was used. To probe for UL42, a gel-purified *Pst*I fragment internal to the UL42 mRNA sequence was used. Chloramphenicol acetyltransferase (CAT) assays were performed as previously described (10), except that the transfection used lipofectin in accordance with manufacturer (Bethesda Research Laboratories, Inc.)-supplied instructions.

RESULTS

This study was designed to determine whether ICP4, the HSV-1 IE protein essential for induction of expression of early and late genes, can form protein-DNA complexes with specific promoter sequences of the *tk* gene and, if so, to localize the site(s) responsible for the interaction(s). Previous reports have indicated that in the presence of nonspecific competitor DNAs, protein-DNA complexes containing ICP4 can be visualized in gel retardation assays using probes that include the start site of ICP4 transcription (20, 28, 39) or portions of the promoters from the genes for ICP0 (28, 29) and gD (52, 53). Similar complexes have been shown with promoter fragments from a late gene (38). To determine whether ICP4 could interact with sequences in the vicinity of the *tk* transcription initiation site, we performed gel retardation assays, using probes spanning the *tk* promoter from −480 to +54 (Fig. 1). ICP4-containing protein-DNA complexes were identified by addition of 58S, a monoclonal antibody to ICP4 (51), to the binding reaction. The complexes which were reduced in mobility by addition of the antibody were inferred to contain ICP4 (for example, KOS-infected lanes marked +Ab in Fig. 1A). The results (Fig. 1) indicate that ICP4 can form a protein-DNA complex with several regions of the *tk* promoter. The last two lanes of Fig. 1E showed two ICP4-containing complexes formed when a probe spanning −13 to +54 was used. The first portion of Fig. 1E and the second half of Fig. 1D show gel retardation assays using probes spanning −75 to −13 and −115 to +17, respectively. The −75 to −13 probe contains the TATA box and the proximal SP1-binding site, while the −115 to +17 probe contains all four of the recognized *cis*-acting sites for cellular transcription factors, as well as the start site of transcription. ICP4-containing protein-DNA complexes were not observed with either of these probes. The first portion of Fig. 1D shows a gel retardation assay with a probe spanning −221 to −115, which includes sequences up to the *Pst*I site, which is generally considered beyond the limit of the promoter. This fragment also contains an octamer motif, which has been implicated as a functional component of the *tk* promoter in some transient assays (43). No ICP4-containing protein-DNA complexes formed with this probe.

We then examined sequences further upstream for the ability to interact with ICP4. A fragment from −254 to −197 was able to form a complex with ICP4 (Fig. 1C). Since the fragment from −221 to −115 did not interact with ICP4, the binding site most probably is localized to the sequence between −254 and −221. The other two panels in Fig. 1 show two ICP4-containing complexes that formed with fragments spanning −385 to −254 (Fig. 1B) and −480 to −385 (Fig. 1A). A summary of the ability of *tk* promoter fragments to form protein-DNA complexes with ICP4 is shown schematically in Fig. 2.

To confirm that the formation of the observed ICP4-DNA complexes was not influenced by the presence of functional *cis*-acting elements that affect transcriptional efficiency, we performed gel retardation assays by using probes containing LS mutations in these sequences. Probes containing LS mutations in the proximal SP1-binding site (LS−59/−49 and LS−56/−46) were as capable as the wild-type probe of forming a complex with ICP4. Similar results were obtained with a probe containing LS−29/−18, which mutates the TATA box. In addition, ICP4 was able to form a complex with a probe containing LS+5/+15, a mutation downstream of the mRNA cap site that, when inserted into the background of the virus, has been shown to reduce *tk* transcription (6). ICP4 can also form a complex with a probe containing the LS+16+36/+10 mutation. LS+16+36/+10 is a mutation that deletes +16 to +36 and inserts a 10-base-pair *Bam*HI linker (35). The ability of LS+16+36/+10 to interact with ICP4, combined with the facts that a fragment from −115 to +17 does not interact with ICP4 and a fragment from +17 to +54 does (see Fig. 2), effectively localizes the region necessary for interaction with ICP4 to sequences between +36 and +54. The data for the LS mutants are summarized in Fig. 2.

To further test the specificity of these interactions, competition experiments were performed. ICP4-containing protein-DNA complexes that formed with each of the four described regions of the *tk* promoter were specifically competed away when increasing amounts of the same unlabeled fragment were added to the binding reaction. Figure 3 shows a series of competition experiments in which *tk* −254 to −197 was the labeled probe. Increasing amounts of the

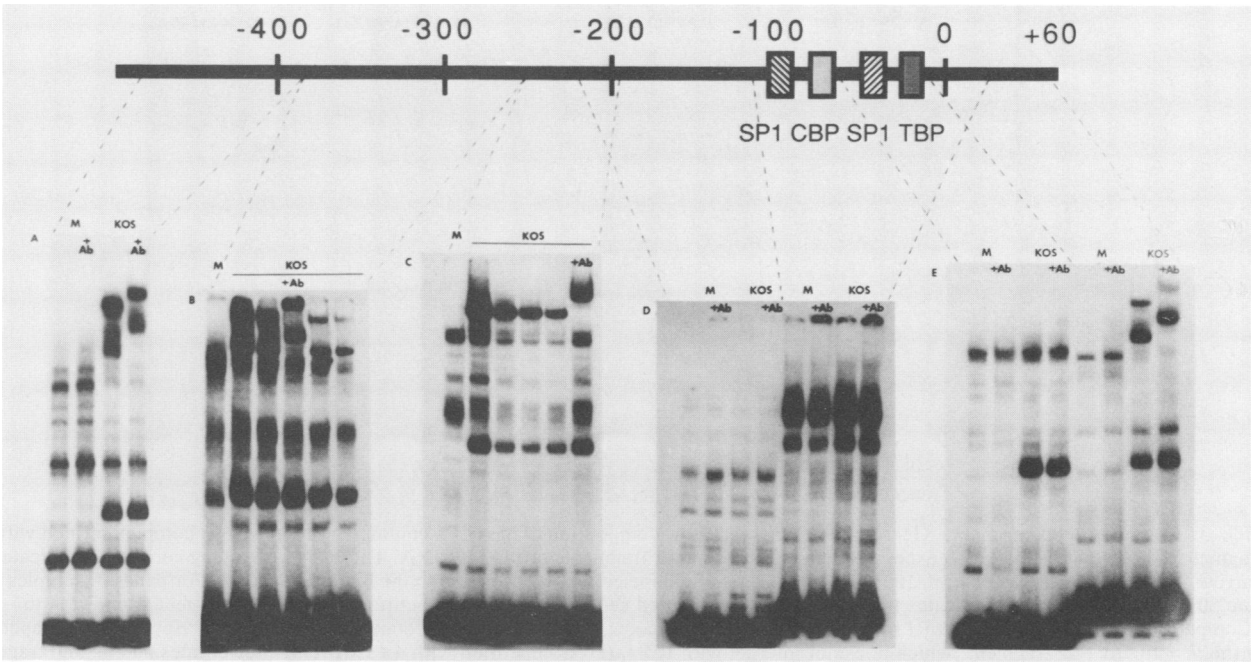

FIG. 1. Protein-DNA interactions of ICP4 with *tk* promoter fragments. A schematic diagram of the *tk* promoter is shown, including *cis*-acting sequences which interact with cellular transcription factor SP1, proteins that bind to CCAAT boxes (CBP), and TATA box-binding proteins (TBP). Nucleotides are indicated with reference to the transcription start site. (A) Gel retardation assay using the *tk* fragment from −480 to −385. Lanes 1 and 2 contained extract from mock-infected cells (M). Lanes 3 and 4 contained extract from KOS-infected cells. A monoclonal antibody to ICP4 was added to lanes 2 and 4 (+Ab). (B and C) Gel retardation assays using *tk* fragments from −385 to −254 and from −254 to −197, respectively. Lane 1 in each panel contained mock-infected cell extract. Lanes 2 to 6 contained extract from KOS-infected cells and increasing amounts of nonspecific competitor DNA. Lanes B4 and C6 contained a monoclonal antibody to ICP4. (D) Gel retardation assay using *tk* fragments from −221 to −115 (lanes 1 to 4) and from −115 to +17 (lanes 5 to 8). Addition of mock- and KOS-infected extracts and the monoclonal antibody was as for panel A. (E) Gel retardation assays using *tk* fragments from −75 to −13 (lanes 1 to 4) and from −13 to +54 (lanes 5 to 8). Addition of mock- and KOS-infected cell extracts and the monoclonal antibody was as for panel A.

unlabeled fragment (−254 to −197) were able to compete for most of the ICP4-containing complex (Fig. 3A) while not at all affecting the binding of a nonspecific DNA-binding protein (see the band located between the ICP4-containing complex and the free probe in each panel of Fig. 3). Competition was also observed when excess DNA containing the start site of ICP4 transcription (a consensus binding site) was used as an unlabeled competitor (Fig. 3B). Very little competition was observed when excess amounts of the *tk* −197 to −75 fragment or a piece of pUC19 DNA was used (Fig. 3C and D). ICP4 did not form a complex with either of these fragments in mobility shift experiments (Fig. 1D; data not shown).

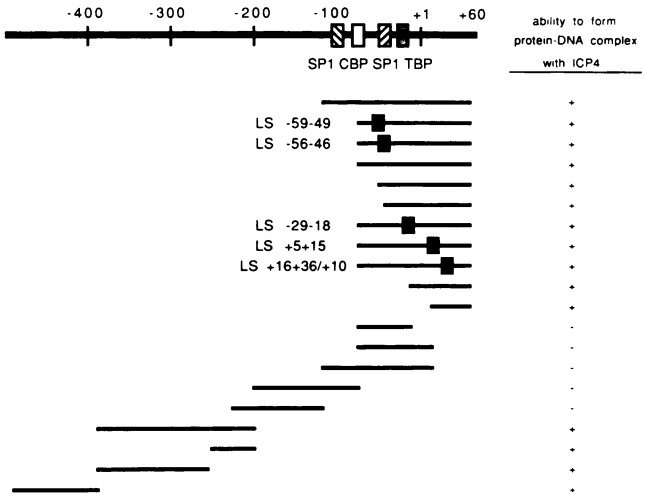

FIG. 2. Schematic diagram of the *tk* promoter and all *tk* promoter fragments tested for the ability to form ICP4-containing protein-DNA complexes. Fragments containing LS mutations (35) and the positions of the mutations are indicated. For abbreviations, see the legend to Fig. 1.

Binding of purified ICP4 to the *tk*-binding site. To confirm these results and better address the nature of the ICP4-DNA interaction, we purified ICP4 from infected cell nuclei. Nuclear extracts were applied to a Sephacryl S-300 gel filtration column, and fractions were assayed for immunological reactivity to 58S antibody by Western dot blot and for DNA-binding activity by gel retardation assay. Figure 4A shows results of a gel retardation assay of the relevant fractions from the Sephacryl S-300 column. The peak of DNA-binding activity also corresponded to the peak of 58S antibody reactivity (data not shown). The appropriate fractions were pooled and applied to a DNA affinity column as described in Materials and Methods. The affinity column was eluted stepwise with 10-ml washes of column buffer containing 0.3, 0.6, and 0.9 M KCl. The fractions were analyzed by Western dot blot. The elution profile is shown in Fig. 4B. A single, sharp peak was obtained with application of the column buffer containing 0.3 M KCl. The material from this peak was used for subsequent analyses. Figure 4C is a Coomassie brilliant blue-stained SDS-polyacrylamide gel of

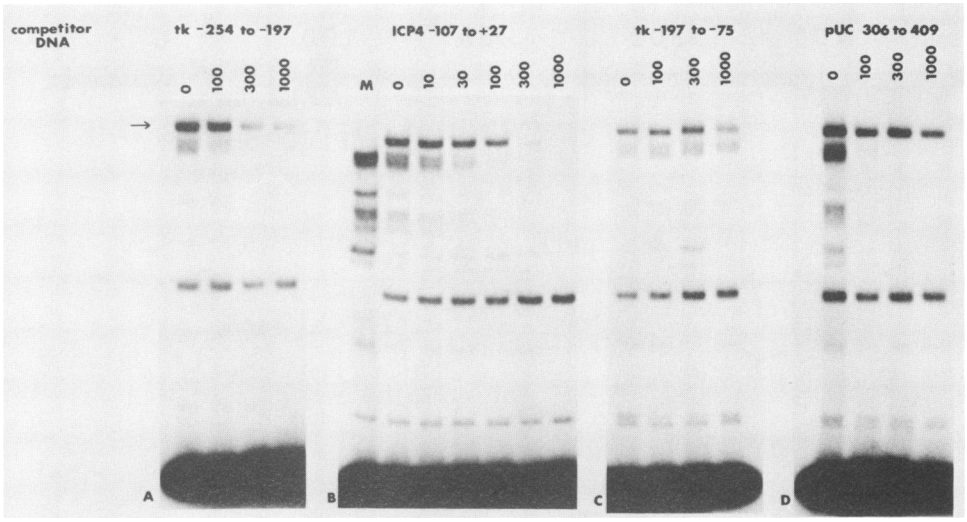

FIG. 3. Competition experiments. Gel retardation assays show competition of the ICP4-containing protein-DNA complex formed with the *tk* fragment from −254 to −197 with excess, unlabeled *tk* promoter fragments or pUC19 DNA. The ICP4-containing complex is indicated by the arrow. For each panel except B, 0, 100, 300, or 1,000 ng of unlabeled DNA was added to the binding reaction. For panel B, samples with 10 and 30 ng were also run. M indicates the lane with mock-infected cell extract. (A) Competition with the *tk* fragment from −254 to −197. (B) Competition with the *Bam*HI-*Eco*RI fragment of the ICP4 promoter, which contains the start site of ICP4 transcription. (C) Competition with the *tk* −197 to −75 fragment, which does not interact with ICP4. (D) Competition with a *Pvu*II-*Rsa*I (nucleotides 306 to 409) fragment of pUC19.

this peak showing a single band of 170 kilodaltons (lane 1). This band comigrated with immunological reactivity to the monoclonal antibody to ICP4 (lane 2).

Purified ICP4 was subsequently used in gel retardation assays with the consensus binding site in the ICP4 promoter (Fig. 5A), as well as with two of the *tk* promoter sequences which interact with ICP4 (Fig. 5B and C). The protein-DNA complexes seen here were further shifted by addition of 58S monoclonal antibody (lanes marked +Ab) and comigrated with complexes seen when whole-cell extracts were used. To further demonstrate that ICP4 can specifically and directly bind DNA, purified ICP4 was used in Southwestern analysis with probes containing a consensus binding site, a mutant consensus site, and the *tk* site from −254 to −197 (Fig. 6). Approximately 0.5 μg of purified ICP4 was electrophoresed, transferred to nitrocellulose, renatured, and probed with end-labeled DNA fragments. Bands that comigrated with immunological reactivity to 58S (lane 4) were seen when probes containing the consensus site in the ICP4 promoter (lane 1) and the *tk* sequence from −254 to −197 were used (lane 2), reflecting the ability of these DNAs to interact directly with ICP4. A probe containing a mutation in the consensus site (ΔAT probe) did not interact with ICP4, reflecting the specificity of the interaction (lane 3). Therefore, the results of the Southwestern analysis agree with those of the band shift experiments.

Figure 7 shows methylation interference data obtained with the *tk* −254 to −197 fragment. The end-labeled probe was partially methylated and used in a binding reaction with purified ICP4. The products of the binding reaction were separated by electrophoresis on a nondenaturing gel. The entire gel was electroblotted to NA45 paper and subsequently exposed to film to identify bound and unbound DNAs. The bound and unbound DNAs were eluted from the paper, cleaved with piperidine, and electrophoretically separated on a denaturing gel. The results of this assay indicate that methylation of a number of G residues on both strands between residues −240 and −231 interferes with ICP4 binding. The relevant sequence and affected nucleotides are indicated in Fig. 7C.

Association of a mutant ICP4 protein with the *tk* promoter. In a previous study, mutant ICP4 proteins with 2-amino-acid insertions in the amino-terminal 774 amino acids were tested in transient assays for the ability to bind to a consensus binding site, transactivate tkCAT, and repress IE3CAT (50). From one of the mutant plasmids, a virus (*vni*13) was constructed that expressed a mutant ICP4 protein that interacted with an apparently low affinity for a consensus binding site and failed to interact with the nonconsensus binding site between −254 and −197 relative to the *tk* transcriptional start site. Another virus constructed in this study, *vni*16, expressed an ICP4 protein that failed to interact with both the consensus and nonconsensus binding sites. On the basis of these results, a hypothesis was presented that the structural requirements for interaction with the nonconsensus sites were greater than those for interaction with consensus binding sites. Described below is the phenotypic characterization of two mutant viruses containing an insertion of two alanine residues at amino acid 274 in the gene for ICP4. The properties of the mutant proteins expressed from viruses containing the insertion at amino acid 274 support the above-described hypothesis and address the requirement for specific interactions with the nonconsensus sites described in the first part of this study with regard to transactivation of *tk* transcription by ICP4.

Plasmid pi11 (50) contains both a 2-amino-acid insertion at residue 274 and a nonsense codon at residue 774 (11). For reasons of clarity which will become evident, this plasmid was redesignated pni11. Plasmid pni11 was used to construct two viruses, *vi*11 and *vni*11, both of which contain the 2-amino-acid insertion, with the latter also containing the nonsense mutation. These viruses were constructed by standard techniques (12, 50), and the details of their construction will be described elsewhere (manuscript in preparation).

To determine the DNA-binding properties of the *vi*11 and

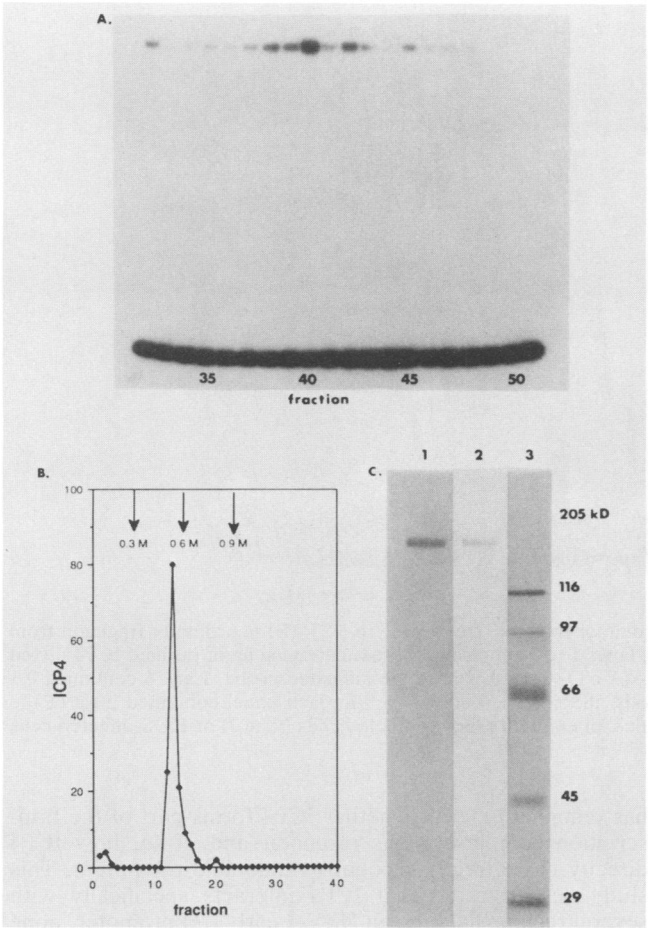

FIG. 4. Purification of ICP4. (A) Gel retardation assay of relevant column fractions from a Sephacryl S-300 column. Approximately 0.1 ng of the *Bam*HI-*Eco*RI (−107 to +27) fragment from the ICP4 promoter containing a consensus binding site was used in a binding reaction with 0.03 μg of poly(dI-dC) and 2 μl of each column fraction. (B) Elution profile from the DNA affinity column. Arrows indicate the points at which the respective elution buffers were applied. The *y* axis represents arbitrary units of ICP4 based on densitometric scans of the Western dot blot (see Materials and Methods). (C) SDS-gel of 1 μg of affinity-purified ICP4 (lane 1). A duplicate lane was assayed for immunological reactivity to α ICP4-specific antibody 58S (lane 2). Molecular mass markers and their corresponding sizes are shown in lane 3. kD, Kilodaltons.

*vin*11 proteins relative to those of the full-length KOS protein and the *n*208 (parent of *vni*11) protein, whole-cell extracts were prepared and used in gel retardation assays with probes containing a consensus binding site and the *tk* sites described in Fig. 1. Both the *vi*11 and *vni*11 proteins formed protein-DNA complexes with the consensus binding site but not with the sites in the *tk* promoter region (Fig. 8). To more closely examine the DNA-binding properties of an ICP4 molecule containing the *i*11 insertion, the *vi*11 protein was purified from *vi*11-infected Vero cells by using the procedure described above for the wild-type protein. Binding of purified KOS ICP4 to the indicated probes was compared with that of *vi*11 in the presence of different concentrations of a nonspecific competitor. Protein-DNA complexes were evident with the KOS protein at the highest concentration (1 μg) of the nonspecific competitor used, while *vi*11-DNA complexes were not detected at the lowest concentration (0.03 μg) of the competitor (Fig. 8B). Therefore, the specific affinity of the *vi*11 protein for the *tk* sites was drastically reduced or eliminated. In a similar experiment, it was determined that the purified *vi*11 protein retained the ability to bind to a consensus binding site (data not shown). This is consistent with the results of Fig. 8A and also with the fact that DNA affinity was successfully used as a method for purification. In the absence of competitor DNA, a relatively intense heterogeneous shift was seen for both the KOS and *vi*11 proteins. This most probably reflects nonspecific binding of the *vi*11 and KOS proteins, because an antibody further shifted this material (data not shown). However, despite the relatively homogeneous nature of the ICP4 preparations used in the above-described experiments (Fig. 4C), we cannot rule out the possibility that the heterogeneous material was in part due to a small degree of contamination with other nonspecific DNA-binding proteins or degradation products of ICP4.

Activities of *i*11 insertion mutants. The observation that both the *vni*11 and *vi*11 proteins had dramatically reduced abilities to associate specifically with the wild-type binding sites within the −254 to +54 region of the *tk* gene provides a test for the involvement of these interactions in ICP4-mediated transinduction of the *tk* gene and ICP4 function in general. Initial analysis of the phenotypes of *vi*11 and *vni*11 was performed by comparing the viral peptides synthesized in *n*12-, *n*208-, KOS-, *vi*11-, and *vni*11-infected cells at 4 and 9 h postinfection. *n*12 contains a nonsense mutation at amino acid 251 and does not specify ICP4 activities; therefore, it efficiently synthesizes only IE (α) proteins and ICP6 (12). *n*208, like *vni*11, contains a nonsense mutation at amino acid 774 and is semipermissive for early and "leaky" late (γ_1) gene expression (12). As can be seen from the SDS-gel profile of Fig. 9, the *i*11 insertion had no detectable effect in the KOS or *n*208 background; therefore, gene expression as measured by protein levels was not greatly affected by the *i*11 insertions. Consistent with the polypeptide profile shown in Fig. 9 is the observation that the virus *vi*11 grew as well as KOS on cells that do not provide ICP4 in *trans* (data not shown). *n*12, *n*208, and *vni*11 require the wild-type ICP4 provided by E5 cells for growth because of the presence of the nonsense mutations in the genes for ICP4.

A direct examination of the effect of the *i*11 mutation on the transcription of the *tk* gene was performed by nuclear run-on transcription. Nuclei were prepared at 6 h postinfection from uninfected cells and from cells infected with *n*12, *vi*11, or KOS. Phosphonoacetic acid (300 μg/ml) was included to inhibit DNA synthesis and therefore restrict the measurement of transcription to that from input templates. Nascent RNA molecules were elongated by in vitro incubation of nuclei with nucleoside triphosphates, including [α^{32}P]GTP. RNA was isolated and used to probe filters containing serial dilutions of single-stranded DNA homologous and complementary to the indicated mRNA. The difference between uninduced transcription and ICP4-induced transcription of the genes for *tk* and ICP8 is demonstrated in Fig. 10A by comparison of the *n*12 and KOS signals, respectively. The *i*11 mutation did not affect induced expression, since the rates of transcription of the genes for *tk* and ICP8 in the *vi*11 background were similar to those in the KOS background. Also shown in Fig. 10A is the ability to repress representative IE gene (ICP4 and ICP27) transcription as a consequence of either the *vi*11 or KOS ICP4 protein.

Figure 10B shows a Northern blot reflecting stable transcript levels from cells infected with KOS, *vi*11, or *n*12. Both

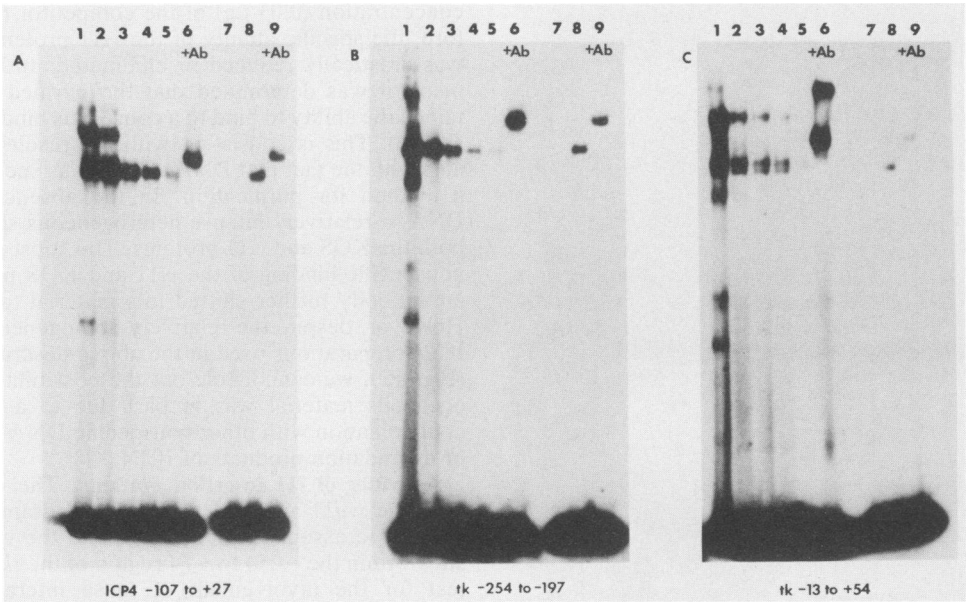

FIG. 5. Gel retardation assays with affinity-purified ICP4. (A) ICP4 promoter fragment from −107 to +27. (B) *tk* promoter fragment from −254 to −197. (C) *tk* promoter fragment from −13 to +54. In each panel, lanes 1 to 5 represent approximately 60 ng of purified ICP4 mixed with 0, 0.03, 0.1, 0.3, or 1.0 μg of poly(dI-dC) (A and B) or poly(dA-dT–dA-dT) (C) and 1 ng of the indicated probe. Lane 6 contained 0.1 μg of nonspecific competitor DNA plus 1 μl of a 1:10 dilution of antibody 58S (+Ab). Lanes 7 to 9 in each panel contained 2 μg of the nonspecific competitor DNA indicated and approximately 10 μg of a whole-cell extract of mock-infected cells (lane 7) or KOS-infected cells (lanes 8 and 9). Lane 9 also contained 58S antibody.

tk mRNA levels and UL42 (also an early gene) mRNA levels in *i*11-infected cells approached wild-type levels, also indicating the transcriptional competence of the mutant virus.

As an additional test of the activity of the *i*11 ICP4 molecule, we examined the ability of the *i*11 protein to transactivate tkCAT in transient assays in the absence of other viral proteins. Plasmid pi11 encodes a full-length ICP4 protein containing insertion *i*11. pi11 was constructed from pni11 (50) in the following manner. pni11 was digested with *Sac*I, which cleaves the plasmid twice, once on either side of the *Hpa*I linker (11) that specifies the nonsense mutation. Therefore, the nonsense mutation is excised by removal of the 0.5-kilobase *Sac*I fragment. The corresponding wild-type *Sac*I fragment from pK1-2 (11) was then inserted into the plasmid, replacing the *Sac*I fragment containing the nonsense mutation. When pi11 was compared with pK1-2 for the ability to transactivate ptkCAT, similar levels of induction were observed (Fig. 11). Therefore, in the absence of other viral proteins, the mutant ICP4 protein encoded by pi11 is proficient for transactivation of *tk*, despite the DNA-binding deficiency documented above.

DISCUSSION

Biochemical and genetic studies of HSV-1 IE protein ICP4 have indicated that it contributes to repression of IE gene expression (10, 41, 42, 47) and is required for induced expression of viral early and late genes (10, 18, 22, 47, 54). A known biochemical property of the ICP4 molecule is that it possesses sequence-specific DNA-binding activity (19, 20, 28, 29, 39). In addition, several reports have indicated that ICP4 can interact with a number of different DNA sequences that deviate from the consensus binding site (28, 29, 38, 52). These results raise possibilities for the mechanisms that underlie the regulatory activities of ICP4.

With regard to the *trans*-activating properties of ICP4, it has remained unclear whether ICP4 forms part of the transcription complex on the promoter and, if so, how ICP4 directly or indirectly recognizes inducible promoters. This study demonstrated that ICP4 interacts specifically with several regions flanking an HSV-1 early (*tk*) promoter, none of which contains a consensus binding site. The functional relevance of these interactions was addressed by characterizing a mutant ICP4 peptide that is altered in its ability to bind specifically to these sites. These results show that a mutant ICP4 peptide that does not detectably possess the

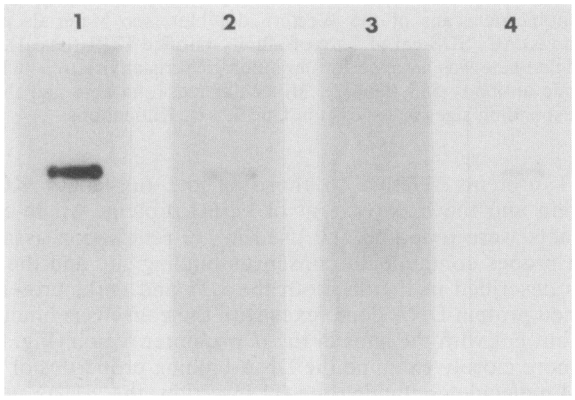

FIG. 6. Southwestern analysis using affinity-purified ICP4. Each lane contained approximately 0.5 μg of purified ICP4. Lane 1 was probed with a fragment spanning −107 to +27 of the ICP4 promoter which contains a consensus binding site. Lane 2 was probed with the *tk* fragment from −254 to −197. Lane 3 was probed with a fragment identical to that in lane 1, except that the AT pair within the consensus binding site was deleted. This fragment has previously been shown not to bind ICP4 (12). A duplicate lane was tested for immunological reactivity to 58S (lane 4).

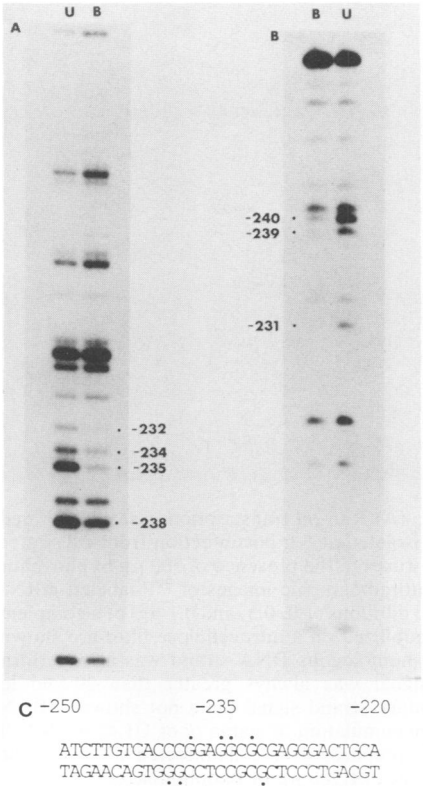

FIG. 7. Methylation interference. End-labeled fragments containing the *tk* −254 to −197 fragment were treated as indicated in Materials and Methods. (A) G cleavage pattern of the coding strand (U, unbound DNA; B, bound DNA). Underrepresented G nucleotides are marked by dots and position numbers relative to the mRNA start site. (B) G cleavage pattern of the noncoding strand. (C) Relevant nucleotide sequence. Underrepresented nucleotides are marked with dots.

intrinsic ability to interact specifically with these nonconsensus binding sites flanking the *tk* promoter induces transcription of the *tk* gene to the same level and rate as wild-type ICP4. Our interpretation of these results is that such interactions are not absolutely required for activation of transcription by ICP4.

ICP4 specifically interacts with DNA sequences adjacent to the *tk* promoter. One is in the 5′-untranslated region downstream of the promoter; the other three are located in upstream regions beyond the conventional limits of the *tk* promoter. None of these sequences contains a consensus (ATCGTCnnnnYCGRC) binding site for ICP4. Similarly, protein-DNA complexes have been observed in regions of other HSV-1 gene promoters that also do not contain consensus binding sites (28, 29, 38, 52). This confirms that ICP4 can interact with a variety of DNA sequences. The mechanism by which this occurs is not understood. A possible explanation involves the existence of a single domain on the protein capable of recognizing divergent DNA sequences. This implies that the nonconsensus ICP4-binding sites may be degenerate homologies of the consensus and that protein-DNA contacts in flanking regions may contribute to a stable interaction. In some of the nonconsensus sites identified for ICP4, loose homologies of the consensus ATCGTCnnnnY CGRC can be identified. For example, the site located between −254 and −197 contains the sequence ATCt TgnnnnCCGGa (capital letters match the consensus) from −250 to −236. The nucleotides which interfere with binding when methylated span residues −240 to −231 (Fig. 7). The experimentally determined contacts at positions −238, −239, and −240 are contained in the sequence given above, while four additional contacts are adjacent to it. Alternatively, certain regions of the protein may act to modify the specificity of binding, as certain insertion mutations differentially affect binding to different sites (this report; 50). Diversity of sequence recognition may also be generated by protein-protein contacts between ICP4 and other viral or cellular proteins. While this remains a possibility, our results indicate that diverse DNA-binding properties are inherent in

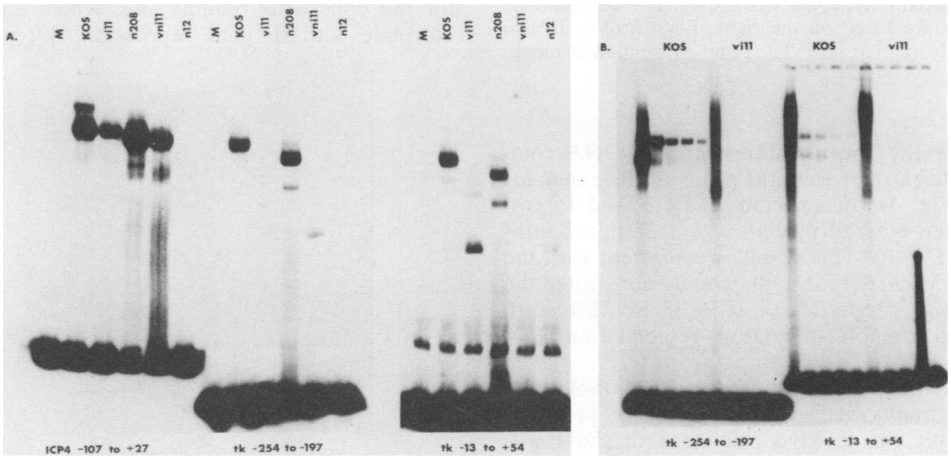

FIG. 8. Gel retardation analysis of *i*11 and *ni*11 ICP4 molecules. (A) The indicated viruses were used to prepare infected cell extracts for use in gel retardation assays with probes containing the consensus binding site in the ICP4 promoter, spanning *tk* −254 to −197 or spanning *tk* −13 to +54. (B) Gel retardation assay with purified wild-type (KOS) or *i*11 ICP4 with *tk* promoter fragments −254 to −197 (left half) and *tk* −13 to +54 (right half). The first five lanes in each half represent 1.0 ng of the *tk* promoter fragment from −254 to −197 with approximately 60 ng of purified KOS ICP4 in the presence of 0, 0.03, 0.1, 0.3, and 1.0 μg of poly(dI-dC). The second five lanes in each half were identical, except that 60 ng of purified *i*11 ICP4 was used. The right half of the figure was identical, except that the probe used was the *tk* −13 to +54 fragment.

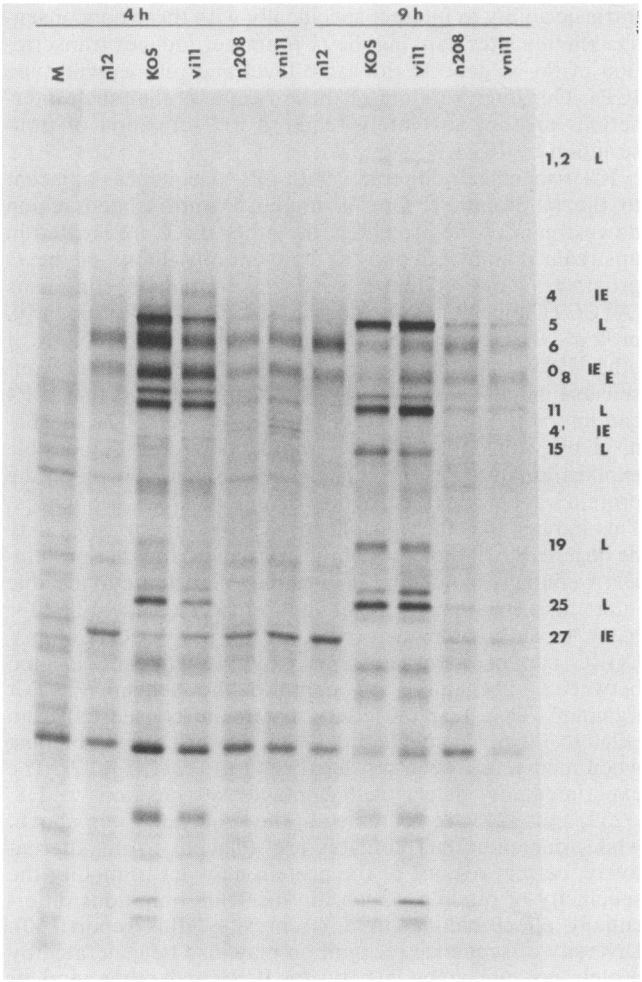

FIG. 9. Polypeptide profiles of the *vi*11 and *vni*11 viruses. Cells were infected at a multiplicity of infection of 20 in Ham F12 medium and labeled for 30 min with [^{35}S]methionine at 4 or 9 h postinfection. Samples were electrophoresed on a 9% polyacrylamide gel. An autoradiogram of labeled polypeptides is shown. ICP designations for selected proteins are listed on the right. Each protein is also classified as IE, early (E), or late (L). Lane M contained mock-infected cell extract.

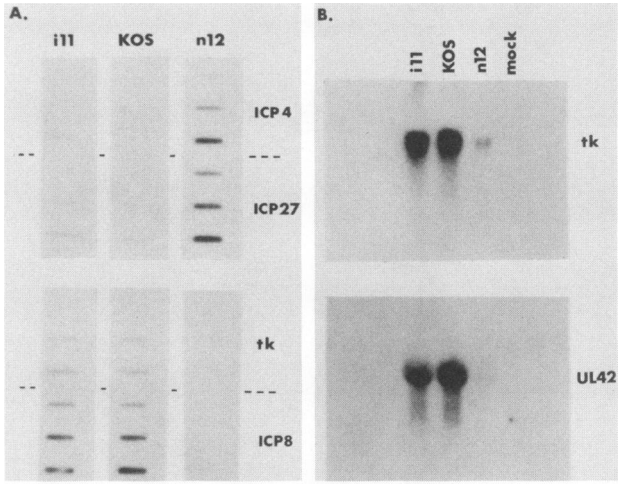

FIG. 10. (A) Run-on transcription assay performed on infected cell nuclei isolated at 6 h postinfection from cells infected with the indicated viruses in the presence of 300 μg of phosphonoacetic acid per ml. Autoradiographic images of ^{32}P-labeled mRNA hybridized to threefold dilutions (1.0, 0.3, and 0.1 μg) of a complementary DNA strand immobilized on a nitrocellulose filter are shown. Hybridization to the homologous DNA strand was also performed, and the resulting signal was always greater than 10-fold less than the complementary strand signal (data not shown). (B) Northern blot indicating accumulation of stable *tk* or UL42 mRNA. Total cellular RNAs were prepared from cells infected with the indicated viruses in the presence of 300 μg of phophonoacetic acid per ml and then electrophoretically separated and transferred to nitrocellulose. Filters were probed with a gel-isolated, ^{32}P-labeled *Sac*I-*Sma*I fragment of the *tk* gene or a *Pst*I fragment of the UL42 gene.

the ICP4 protein itself. The mobilities of ICP4-DNA complexes seen in crude extracts were the same as those seen for purified protein (Fig. 5). In addition, ICP4 bound to two divergent sites when purified protein was used in a Southwestern protocol (Fig. 6). This result is consistent with the previous results of Michael et al. (38), who demonstrated the diverse DNA-binding properties of ICP4 in Southwestern experiments with a promoter-regulatory region of an HSV late gene.

Examination of the properties of the ICP4 molecules produced by the mutant viruses *vi*11 and *vni*11 has provided a means to examine the in vivo relevance of the ICP4-binding sites near the *tk* promoter. Neither the ICP4 in whole-cell extracts from cells infected with *vi*11 or *vni*11 nor purified ICP4 from *vi*11-infected cells specifically bound to *tk* sequences (Fig. 8). Therefore, analyses of their regulatory activities were performed to assess function in the apparent absence of binding by ICP4. The expression assays examining *vi*11 and *vni*11 are shown in Fig. 9 to 11. At the level of protein expression, *vi*11 and *vni*11 are almost indistinguishable in the production of IE, early, and late proteins relative to KOS and *n*208, respectively. In addition, the transcription of the early genes, *tk*, ICP8, and UL42 in the KOS and *vi*11 backgrounds was similar (Fig. 10). The level of IE gene (ICP4 and ICP27) transcription appeared to be slightly elevated in the *vi*11 sample. This may reflect a lower affinity for the consensus binding site, which has been shown to correlate with IE gene repression (49; unpublished data).

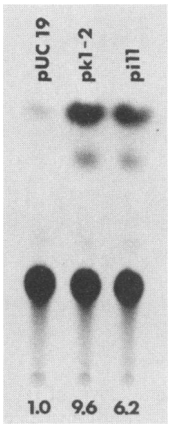

FIG. 11. Transient expression assay comparing expression of ptkCAT (10) following cotransfection with pUC19, pi11, or pK1-2 (which encodes wild-type ICP4). The numbers indicate the fold increase in percent acetylation of chloramphenicol as a function of pi11 and pK1-2 relative to that in the cotransfection with pUC19.

The ability of the *i*11 protein to transactivate the *tk* promoter in the absence of other viral gene products was demonstrated by the ability of *pi*11 to transactivate tkCAT in transient assays nearly as efficiently as the wild-type protein coded for by pK1-2 (Fig. 11). On the basis of these data, we propose that the interactions between ICP4 and the DNA sequences adjacent to the *tk* promoter, at best, marginally contribute to transactivation of *tk* gene expression and are not essential for the transinducing properties of ICP4.

It remains possible that the in vitro mobility shift experiments are not sensitive enough to measure functional ICP4-DNA interactions within a cell. If so, it is possible that the *i*11 protein retains some sequence-specific binding activity in vivo, which may confer activity. Arguing against this possibility are the results of previous studies which showed that deletion of the sequence that encodes the untranslated region (23) or the region upstream of −119 relative to the *tk* mRNA start site (14) had little or no effect on transactivation. Alternatively, functional ICP4-DNA interactions may differ from those detectable by in vitro band shift experiments.

Studies of the *tk* promoter have shown that the *cis* sequences which interact with cellular transcription factors are required for both *tk* basal-level expression in transient assays and *tk* expression in the context of the virus, leading to the proposal that viral *trans* induction is mediated through the interaction of viral *trans*-activators with cellular transcription factors (6). Similar mechanisms have been proposed for the simian virus 40 large-T antigen (21), the E1A protein of adenovirus (56; reviewed in reference 4), and the IE protein of pseudorabies virus (1). We favor a hypothesis involving the interaction of ICP4 with one or probably several cellular transcription factors which interact with the *tk* promoter. The argument for multiple interactions follows from the observation that mutations in any one of the sites which specify an interaction with a cellular factor(s) do not lower *tk* expression below the uninduced level (6). Nonspecific contacts by ICP4 with the DNA may help to stabilize these interactions. Note that purified *i*11 protein retained the ability to bind to the nonconsensus sites in the absence of a nonspecific competitor (Fig. 8B) and that most mutations in the DNA-binding domain of ICP4 that affect specific DNA binding also reduce or eliminate transactivation (45, 50). The effects of these mutations on the nonspecific DNA-binding properties of ICP4 have not been extensively studied. In view of the experimentally determined size of the ICP4 molecule relative to that of the promoter, contact among ICP4, promoter sequences, and one or more cellular *trans*-activating factors is a reasonable possibility. Tests of these hypotheses are under way in our laboratory.

While our data tend to minimize the importance of specific DNA binding for transactivation of the *tk* promoter, it remains possible that this activity is important for transactivation of other HSV genes. Other investigators who have examined the expression of the HSV-1 gene for gD have proposed, on the basis of transient assays and in vitro transcription reactions, that the ICP4-binding sites present near the promoter of the gene for gD contribute to transactivation (52, 53). In view of the size and complexity of ICP4, it is possible that it transactivates genes by a variety of mechanisms. Some genes may require the sequence-specific binding of ICP4 to give the level of expression seen in productive infection, while other genes, like *tk*, may not. This may hold in particular for genes such as that for gD, which contains a consensus binding site located at −105 with respect to the gD mRNA start site (19, 52, 53). The presence of this site could contribute to transactivation by a different mechanism used by ICP4, or, more specifically, *i*11 ICP4, to transactivate the *tk* gene and numerous other genes which are transactivated but do not contain consensus binding sites. However, it was previously shown that this sequence is not necessary for induced expression of the gene for gD in transient assays (16, 17).

The possible contribution of the binding sites described herein for transactivation may become more significant in cell types other than those used to propagate productive infections in the laboratory. The relative abundance and activities of cellular transcription factors which mediate *tk* transcription may vary in different cell types, such as epithelial cells and neurons. Therefore, direct sequence-specific binding of ICP4 could possibly be used as an alternative mechanism to transactivate a gene in one cellular environment and not be required in another.

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