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Characterization of the DNA-Binding Properties of Herpes Simplex Virus Regulatory Protein ICP4

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ICP4 is a herpesvirus-encoded protein that is expressed during the immediate-early phase of productive infection and is required for efficient transcription of viral genes during the early and late phases of infection. Previous studies have shown that ICP4 is a component of specific protein-DNA complexes but have not revealed whether native ICP4 directly recognizes specific nucleotide sequences. Using DNA affinity chromatography, we have purified ICP4 to near homogeneity. The purified preparation consists primarily of dimeric and tetrameric forms of ICP4. As shown by DNase protection experiments, purified ICP4 binds directly to specific sequences in viral DNA. The stability of ICP4-DNA complexes is increased over 100-fold by shifting the temperature from 23 to 4°C. The equilibrium dissociation constant of ICP4-DNA complexes formed at 4°C in the presence of 100 mM NaCl was determined to be approximately 1.1 nM.

The genome of herpes simplex virus (HSV) contains approximately 152 kilobase pairs (kbp) and at least 70 genes (30). During the course of productive infection, transcription of viral genes is temporally and quantitatively regulated by *cis*-acting signals within the viral genome and by *trans*-acting factors encoded in both the host and viral genomes (4, 7, 8, 10, 11, 16, 18, 21, 22, 28, 35–37, 40, 45). The temporal pattern of expression for a given viral gene forms the basis for its classification as either an immediate-early (alpha), early (beta), or late (gamma) gene (6, 18, 19, 48).

One of the five immediate-early genes encodes a polypeptide designated ICP4 (α4 or IE175k), which acts as a positive regulator of early gene transcription and a negative regulator of ICP4 gene transcription (8, 9, 17, 37, 39. 47). Proposed mechanisms for ICP4-mediated induction or repression of viral gene transcription include (i) ICP4-dependent activation or inactivation of cell-encoded transcription factors, (ii) ICP4-dependent modification of RNA polymerase II, and (iii) formation of specific ICP4-DNA complexes (9). Direct tests of mechanism 1 have not been reported. With regard to mechanism 2, Beck and Millette (2) observed that RNA polymerase II from HSV-infected cells has altered chromatographic and enzymatic properties, but the molecular basis for these alterations has not been established. Relevant to mechanism 3 are reports by several investigators that incubation of radiolabeled DNA with HSV-infected cell extracts (24, 25, 34) or partially purified preparations of ICP4 (1, 12, 14, 32) can result in the formation of stable protein-DNA complexes that contain ICP4 and specific viral DNA sequences.

We have described a method for partial purification of ICP4 from HSV type 1 (HSV-1)-infected Vero cells (12). ICP4 was enriched approximately 100-fold and constituted approximately 5% of the total protein (on a mass basis) in this preparation, which we designated fraction VIII. DNA-binding immunoassays with fraction VIII and portions of the HSV genome revealed stable complexes that contained ICP4 and specific sequences in the promoters for viral genes encoding ICP0, ICP4, and glycoprotein D (1, 26–28). Because the data did not reveal whether formation of these ICP4-DNA complexes was mediated directly by ICP4 or indirectly by cellular and/or other viral proteins in fraction VIII, we designated these specific DNA sequences ICP4-protein-binding sites. To investigate the role of ICP4 in formation of protein-DNA complexes, we have purified ICP4 to near homogeneity by DNA affinity chromatography. In this article, we report that ICP4 itself is a sequence-specific DNA-binding protein that recognizes the same ICP4-protein-binding sites defined previously with fraction VIII.

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MATERIALS AND METHODS

Materials. Restriction endonucleases, *Escherichia coli* DNA polymerase I (Klenow), T4 DNA ligase, proteinase K, ribonuclease T_1, pancreatic RNase A, and *E. coli* polynucleotide kinase were obtained from commercial sources and used according to the recommendations of the supplier. Ammonium sulfate (Ultrapure) was from Schwarz/Mann. *N*-tosyl-L-phenylalanine chloromethyl ketone and 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS) were from Calbiochem-Behring. [α-^{32}P]dATP was purchased from Dupont, NEN Research Products. *Bgl*II linkers were from New England BioLabs, Inc. DNase I (fast-protein liquid chromatography-pure) and Sephacryl S400 were purchased from Pharmacia. DEAE-TrisAcryl M was obtained from LKB Instruments, Inc.

General methods. Protein concentrations were measured by a modification of the Lowry method (27) with bovine serum albumin (BSA) as a standard. Spot dot radioimmunoassays for ICP4 were performed as previously described (31). Plasmid DNA was prepared by the method of Birnboim and Doly (3). Calf thymus DNA (Sigma Chemical Co.) was extracted twice with phenol-chloroform (1:1), precipitated with ethanol, suspended in buffer TE (see below), and sonicated to an average chain length of 300 bp. Polypeptides were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (31). Before gel electrophoresis, protein samples from column fractions were precipitated by the addition of trichloroacetic acid to 10% (wt/vol); the precipitate was pelleted at 12,000 × *g* for 15 min, suspended by sonication in 0.25 M NaOH, and then diluted by addition of an equal volume of 2× buffer DB (see below).

Buffers. Buffer C contained 10 mM Tris hydrochloride (pH

8.0), 1 mM EDTA, 10 mM β-mercaptoethanol, 10% (vol/vol) glycerol, and 0.01% (wt/vol) CHAPS. Buffer DB contained 50 mM Tris hydrochloride (pH 7.0), 2% (wt/vol) SDS, 0.7 mM β-mercaptoethanol, and 10% (vol/vol) glycerol. Buffer DNAB contained 10 mM Tris hydrochloride (pH 7.5), 100 mM NaCl, 1 mM EDTA, 28 mM β-mercaptoethanol, 48 μg of nuclease-free BSA (Boehringer Mannheim) per ml, 0.05 mM phenylmethylsulfonyl fluoride, and 0.05% CHAPS. Buffer PV contained 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4 and 0.6 mM EDTA. Buffer RSB contained 10 mM Tris hydrochloride (pH 7.5), 10 mM NaCl, and 1.5 mM $MgCl_2$. Buffer TE contained 10 mM Tris hydrochloride (pH 8.0) and 1 mM EDTA.

Construction of pAT153/Bgl. Plasmid pAT153 was derived by Twigg and Sherratt (46) from pBR322 by deletion of the region from positions 1647 to 2352. Like pBR322, this vector contains a *Hin*dIII site at position 29, a *Bam*HI site at position 375, and two high-affinity ICP4-protein-binding sites that span positions 105 to 139 and 185 to 226 (12). The recessed 3′ termini of *Hin*dIII-digested pAT153 were filled in with deoxynucleoside triphosphates by DNA polymerase (Klenow fragment) and blunt-end ligated to phosphorylated *Bgl*II oligonucleotide linkers with T4 DNA ligase. The ligated plasmid was transformed into *E. coli* HB101. *Hin*dIII-resistant plasmid DNA was prepared from a selected colony, digested with *Bgl*II to remove multiple copies of the linker, and religated to yield plasmid pAT153/Bgl, which contains a *Bgl*II site in place of the *Hin*dIII site and has two ICP4-protein-binding sites within a 346-bp region that is flanked by *Bgl*II and *Bam*HI sites.

Construction of pXK13. We are indebted to Tom Kelly for introducing us to the logic of the following construction (42). All plasmids were propagated in *E. coli* HB101, which is a *recA* mutant. From a *Pst*I-*Bam*HI digest of pAT153/Bgl, we isolated a 1,127-bp fragment that included the two ICP4-protein-binding sites. From a *Pst*I-*Bgl*II digest of pAT153/Bgl, we isolated a 2,957-bp fragment that included the two ICP4-protein-binding sites. These two fragments were ligated together (the *Bam*HI and *Bgl*II termini are complementary) to yield pXK444-2, which has unique *Pst*I, *Bgl*II, and *Bam*HI sites and a head-to-tail repeat of the 346-bp region derived from pAT153/Bgl that spans two ICP4-protein-binding sites. From pXK444-2 we isolated the *Pst*I-*Bgl*II and *Pst*I-*Bam*HI fragments that span the head-to-tail repeat of the ICP4-protein-binding sites. These two fragments were ligated to yield pXK444-4, which contains four copies, as head-to-tail repeats, of the 346-bp region derived from pAT153/Bgl. This process was continued until we obtained pXK444-32, which contains 32 copies of the 346-bp *Bgl*II-*Bam*HI region from pAT153/Bgl and thus contains 64 ICP4-protein-binding sites. Plasmid pXK444-32 was digested with *Eco*RI and *Sal*I, and the 11.38-kbp fragment containing the ICP4-protein-binding sites was inserted between the *Eco*RI and *Sal*I sites in the high-copy-number vector pUC13 to yield pXK13. Transformants of HB101 were subcloned and screened to isolate a strain in which pXK13 was maintained as a 14-kbp plasmid.

Preparation of affinity DNA-cellulose. DNA was adsorbed to cellulose by the method of Litman (26) with the following modifications. Plasmid pXK13 was linearized by digestion with *Eco*RI, extracted with phenol, precipitated with ethanol, and suspended in 10 mM NaCl at 2 mg/ml. Acid-washed cellulose (0.375 g/ml of DNA solution) was added and mixed to yield a thick paste which was dried at 37°C for 24 h. The dried cellulose was ground to a fine powder and washed for 10 min at 20°C once with 100% ethanol, twice with 10 mM NaCl, and once with buffer C supplemented with 50 mM KCl. The washed DNA-cellulose was suspended in buffer C supplemented with 50 mM KCl to yield a matrix with approximately 2.7 mg of DNA bound per g of cellulose.

Purification of ICP4. All operations were carried out at 0 to 4°C. All buffers were treated with *N*-tosyl-L-phenylalanine chloromethyl ketone immediately before use. A high-salt extract from the nuclei of 8×10^9 HSV-1-infected Vero cells was prepared as described previously (31). Briefly, nuclei were isolated by disruption of infected cells in hypotonic buffer. Adhering membranes were removed with Triton X-100. The washed nuclei were disrupted by sonication in buffer C containing 300 mM $(NH_4)_2SO_4$. This extract was clarified by centrifugation at 100,000 × *g* for 1 h. Proteins in the supernatant were precipitated by addition of $(NH_4)_2SO_4$ to 50% saturation. The precipitated protein was suspended in buffer C supplemented with 300 mM $(NH_4)_2SO_4$ and lacking glycerol, clarified by brief centrifugation, and designated fraction V.

Fraction V was applied to a Sephacryl S-400 column (2.5 by 57 cm) equilibrated in buffer C supplemented with 300 mM $(NH_4)_2SO_4$ and lacking glycerol. Protein was eluted with buffer C supplemented with 300 mM $(NH_4)_2SO_4$ and lacking glycerol at a flow rate of 15.0 ml/h. Fractions of 3.0 ml were collected and assayed for ICP4 by spot dot radioimmunoassay and SDS-PAGE. Fractions enriched in ICP4 were pooled. Pooled proteins were precipitated by addition of $(NH_4)_2SO_4$ to 50% saturation and suspended in buffer C supplemented with 300 mM $(NH_4)_2SO_4$ to yield fraction VI.

Fraction VI was dialyzed against buffer C supplemented with 50 mM KCl. The resulting precipitate was removed by centrifugation at 16,000 × *g* for 10 min. The supernatant, designated fraction VII, was loaded onto a DEAE-TrisAcryl M column (1.3 by 4.7 cm) equilibrated in buffer C supplemented with 50 mM KCl. Protein was eluted at a flow rate of 5 ml/h with a linear gradient of 50 to 350 mM KCl in buffer C; this step was followed by a step wash with 500 mM KCl in buffer C. Fractions enriched in ICP4 were pooled. Pooled proteins were precipitated by addition of $(NH_4)_2SO_4$ to 50% saturation and suspended in buffer C supplemented with 50 mM KCl to yield fraction VIII.

Fraction VIII was dialyzed against buffer C supplemented with 50 mM KCl and loaded onto a pXK13 affinity DNA-cellulose column (1.3 by 4.7 cm) equilibrated in buffer C supplemented with 50 mM KCl. Protein was eluted with a linear gradient of 50 to 1,500 mM KCl in buffer C; this was followed by a step wash with 2.0 M KCl in buffer C. Fractions enriched in ICP4 were pooled. Pooled proteins were precipitated by addition of $(NH_4)_2SO_4$ to 50% saturation and suspended in 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA–10 mM 2-mercaptoethanol–50% glycerol. This preparation was designated fraction IX and stored at −80°C.

Preparation of radiolabeled target DNA. The termini of a fragment of DNA flanked by *Ava*I sites and containing the region from −128 to +57 relative to the mRNA start site in the HSV-1 ICP0 gene (28) were filled in with DNA polymerase (Klenow fragment) and ligated to the termini of *Sma*I-digested pUC13 to yield plasmid pXK23. Cleavage of pXK23 with *Eco*RI and *Hin*dIII yielded a 233-bp fragment containing the ICP0 gene insert and flanking vector sequences. This fragment was isolated by electroelution from a 2.5% agarose gel; extracted sequentially with butanol, phenol, phenol-chloroform (1:1), and chloroform; precipitated in 70% ethanol; and suspended in buffer TE. The 3′ termini were labeled by incorporation of [α-^{32}P]dATP with DNA polymerase (29).

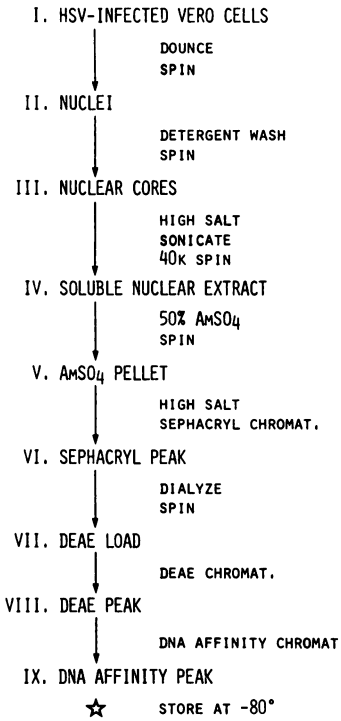

FIG. 1. Schematic representation of ICP4 purification procedure. See Materials and Methods for details. $AMSO_4$, Ammonium sulfate; CHROMAT., chromatography.

The specific activity of the target DNA was approximately 1.0×10^7 dpm/μg.

Nitrocellulose DNA-binding assays. Radiolabeled target DNA and ICP4 were incubated at 4 or 23°C for 60 min in buffer DNAB supplemented with 10 mM $MgCl_2$. A sample (20 μl) from the reaction mixture was spotted directly onto a prewet 25-mm nitrocellulose disk (BA-85; Schleicher & Schuell, Inc.) which had been boiled twice for 10 min in distilled water and then chilled. The sample was filtered at a controlled rate of 0.5 ml/min at 4°C. The filter was then washed three times with 0.5 ml of ice-cold buffer DNAP lacking BSA. The washed filter was dried, and the amount of bound radiolabeled DNA was determined by scintillation counting.

RESULTS

Purification of ICP4. The ICP4 purification scheme is described in detail in Materials and Methods, and results are presented in Fig. 1. Nuclei prepared from HSV-1-infected Vero cells were washed with Triton X-100 to remove membranes and adhering cytoplasmic components. The detergent-washed nuclei (fraction III) contained more than 85% of the cell-associated ICP4 (estimated from radioimmunoassays) and less than 22% of the total cell protein (Table 1). The nuclei were disrupted by sonication and clarified by centrifugation. Most of the protein in the supernatant was precipitated by the addition of ammonium sulfate, whereas most of the nucleic acid remained soluble. The precipitated protein was suspended in a solution with an ionic strength greater than 0.5 M for optimal recovery of ICP4 in fraction V.

Fraction V was chromatographed through a molecular sieve matrix equilibrated in a high-ionic-strength buffer to resolve ICP4 from nuclear proteins and/or nucleic acids that cause aggregation of ICP4 at low ionic strength. The molecular sieve column yielded a partially purified preparation of ICP4 which was soluble at an ionic strength of 50 mM (fraction VII).

TABLE 1. Affinity purification of ICP4

Fraction	Fraction source	Protein	
		Concn (mg/ml)	Total (mg)
I	Cells[a]	1.8	1,424
II	Nuclei	4.9	613
III	TWN[b]	4.8	304
IV	40K supernatant	2.5	152
V	S400 load	6.3	62.5
VI	S400 peak	6.4	19.2
VII	DEAE load	4.2	15.2
VIII	DEAE peak	2.7	2.7
IX	DNA-cellulose peak	1.0	0.2

[a] 8×10^9 HSV-1-infected Vero cells.
[b] Triton-washed nuclei.

Fraction VII was loaded onto a DEAE-Tris acryl M column and eluted with a linear gradient of KCl. ICP4 was the major protein (on a mass basis) that eluted in the fractions containing 190 to 220 mM KCl (Fig. 2). These fractions were pooled and concentrated by ammonium sulfate precipitation. The suspended precipitate was designated fraction VIII. This preparation of fraction VIII was somewhat cleaner than our previously described fraction VIII preparation (12), which was prepared from frozen nuclei and chromatographed through a different molecular sieve matrix.

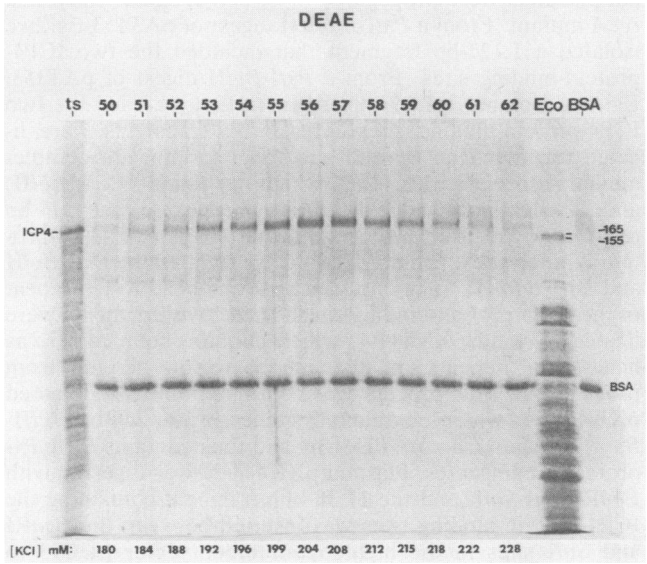

FIG. 2. Elution of ICP4 from DEAE-TrisAcryl column. Proteins eluted from the column with a gradient of KCl were collected in tubes containing 50 μg of BSA. Polypeptides from fractions 50 through 62, as indicated above the corresponding lanes, were subjected to SDS-PAGE (9% acrylamide) and stained with Coomassie blue. The concentration of KCl in each fraction was measured with a conductivity meter and is indicated below the lane. Lane ts contains polypeptides from an extract of cells infected with a temperature-sensitive mutant of HSV-1 that overproduces ICP4 at the nonpermissive temperature (32). Lane Eco contains polypeptides from an extract of *E. coli*; the β and β′ subunits of the bacterial RNA polymerase serve as molecular weight markers of 155,000 and 165,000.

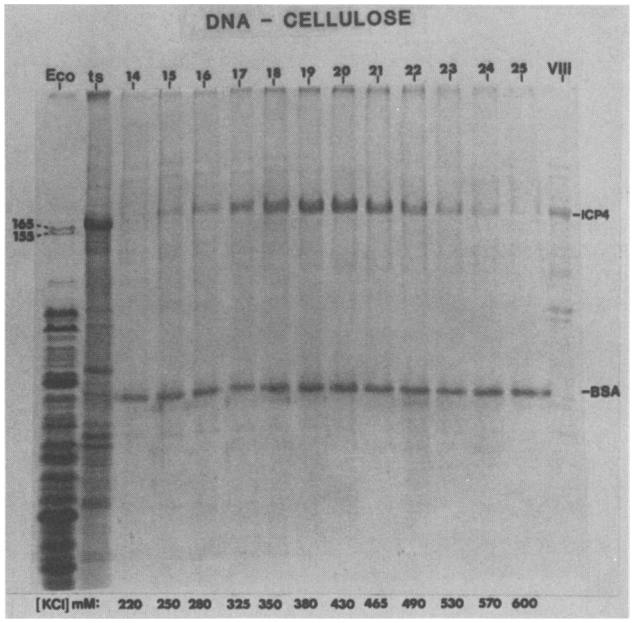

FIG. 3. Elution of ICP4 from affinity DNA cellulose. Fraction VIII was loaded onto a column containing 2.7 mg of *Eco*RI-cleaved pXK13 DNA per g of cellulose. Proteins were eluted from the affinity matrix with a linear gradient of 50 to 1,500 mM KCl and collected into tubes containing 25 μg of *N*-tosyl-L-phenylalanine chloromethyl ketone-treated BSA. A sample (approximately 2.5%) from fractions 14 to 25 was subjected to SDS-PAGE. The gel was stained with Coomassie blue and is shown here. The concentration of KCl in each tube was determined by conductivity measurements and is indicated below each lane. Samples for lanes ts and Eco were prepared as described in the legend to Fig. 2. A sample from fraction VIII was loaded in the lane labeled VIII.

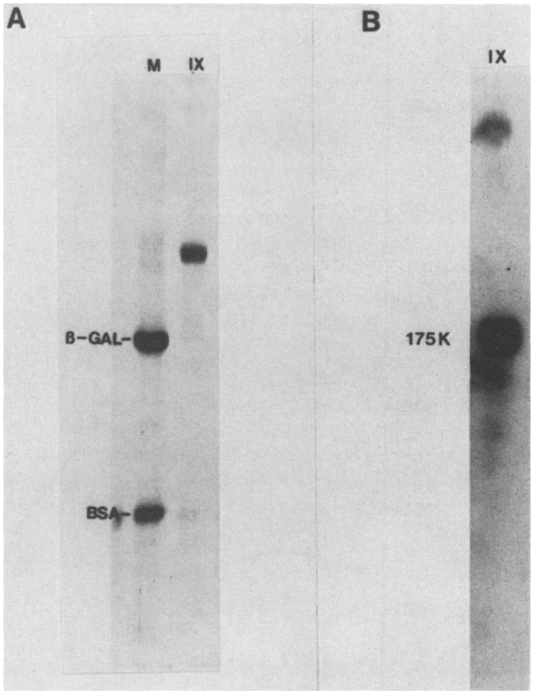

FIG. 4. Analysis of polypeptides in fraction IX. (A) Samples were subjected to electrophoresis through an SDS-polyacrylamide (9% acrylamide) gel and stained with Coomassie blue. Lane M contains approximately 1 μg each of the marker proteins β-galactosidase (β-GAL; 116,000 daltons) and BSA (66,000 daltons). Lane IX contains approximately 0.5 μl from fraction IX of the ICP4 purification scheme. (B) A 1-μl sample of fraction IX was subjected to electrophoresis through an SDS-polyacrylamide (9% acrylamide) gel. Polypeptides were then electroblotted to nitrocellulose, reacted with polyclonal anti-ICP4 antibody, and tagged with ^{125}I-labeled protein A as described previously (31). An autoradiogram of the nitrocellulose is shown here. The immunoreactive material near the top of the gel has an apparent molecular weight of 350,000.

Fraction VIII was loaded onto a DNA affinity matrix consisting of plasmid pXK13 bound to cellulose. This plasmid consists of 64 concatenated ICP4-protein-binding sites arranged in a head-to-tail orientation in the vector pUC13. The ICP4 in fraction VIII bound quantitatively to the column and was eluted with a gradient of KCl (Fig. 3). The ICP4 in fractions containing 325 to 490 mM KCl was pooled, concentrated by ammonium sulfate precipitation, and suspended in buffer to yield a solution designated fraction IX. It should be noted that ammonium sulfate precipitation of the pooled fractions from the DNA-cellulose column separates ICP4 from any DNA that may have leached from the column. Analysis of the polypeptides in fraction IX by SDS-PAGE revealed a nearly homogeneous 175,000-dalton polypeptide (Fig. 4A) that reacts with antibody specific for ICP4 (13) (Fig. 4B). A minor amount of ICP4 was also observed at a position corresponding to approximately 350,000 daltons (Fig. 4B).

Characterization of the ICP4 in fraction IX. We previously reported (31) that native ICP4 present in fraction VII is a noncovalent homodimeric molecule with a sedimentation coefficient of 9.0S. Analysis of the sedimentation coefficient of the ICP4 in fraction IX by sucrose gradient centrifugation revealed two immunoreactive peaks (Fig. 5). The major peak at 9.3S corresponds to the dimeric form of ICP4. Although the structure of the ICP4 in the more rapidly migrating peak at 14.2S has not been established, the sedimentation rate is consistent with a tetrameric form of ICP4. This result demonstrates that at least two different oligomeric forms of ICP4 are present in fraction IX. The apparent absence of a trimeric form suggests that ICP4 oligomers are formed by associations between dimers.

Sequence specificity of purified ICP4. The DNA-binding properties of the ICP4 in fraction IX were investigated by using a variety of target DNAs and binding conditions (P. Kattar-Cooley and K. W. Wilcox, manuscript submitted for publication). The results of these studies revealed that binding of ICP4 to specific target DNAs occurs at 4 to 37°C in a buffered (pH 7.5) solution containing 100 mM NaCl. The target DNA that was used for most of these experiments contained the region from −128 to +57 relative to the transcription initiation site for the HSV-1 gene that encodes ICP0 (α0 or IE110k) (Fig. 6). It has been reported (25) that addition of this target DNA to a crude extract from HSV-infected cells results in formation of a protein-DNA complex that includes ICP4 and that protects nucleotides −71 to −46 of the ICP0 promoter from digestion by exonuclease III.

To determine whether ICP4 alone is sufficient to protect nucleotides −71 to −46 of the ICP0 promoter from nucleolytic digestion, an end-labeled 233-bp fragment (Fig. 6) that contains the ICP0 gene promoter region was incubated with fraction IX and then briefly exposed to DNase I. We observed (Fig. 7) that the region from approximately −72 to −49 was protected by ICP4. This corresponds to essentially the same region protected by a crude preparation of ICP4 (25) and includes the hexanucleotide sequence 5′-ATCGTC-

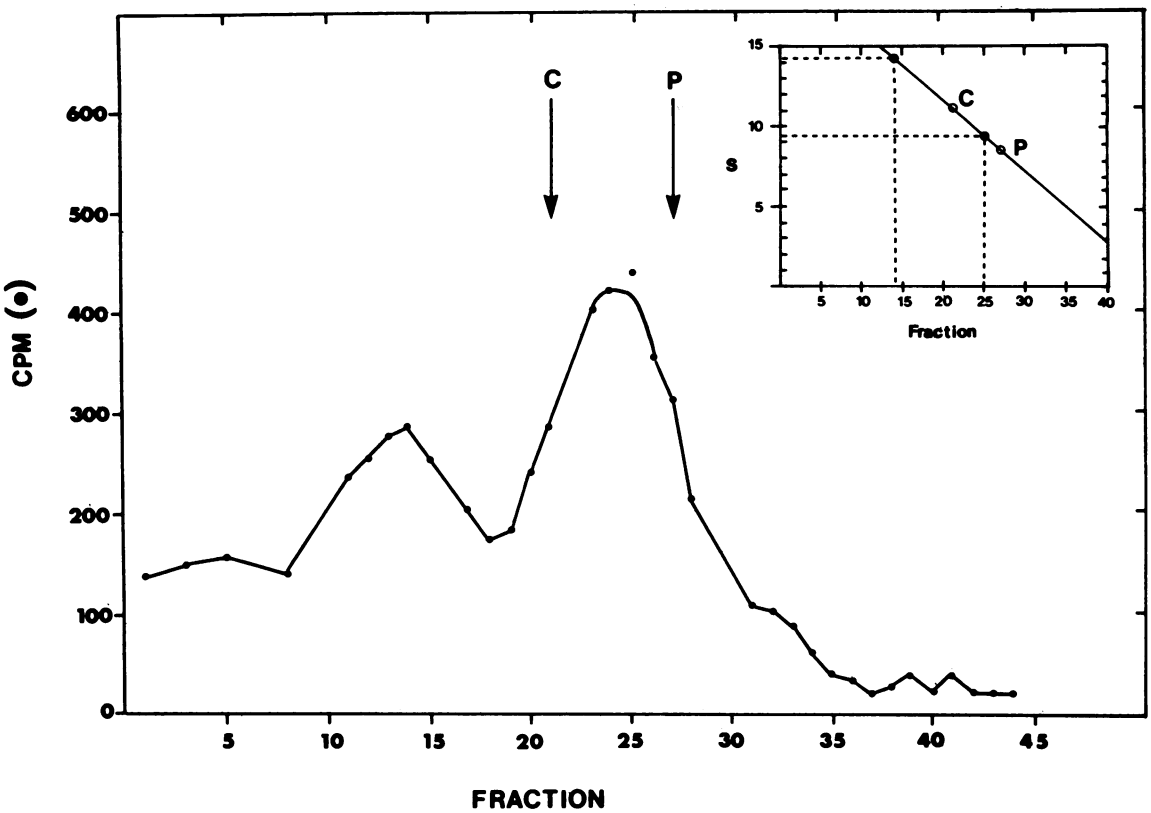

FIG. 5. Sedimentation velocity analysis of ICP4. A sample (1 μl) of fraction IX was mixed with catalase (400 U) and phosphorylase b (1.2 U) in a total volume of 100 μl and layered over a 5-ml gradient of 5 to 20% sucrose in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.3)–137 mM NaCl–5 mM KCl. The sample was centrifuged at 34,000 rpm in an SW50.1 rotor for 15 h at 4°C. A total of 44 fractions (100 μl each) were collected and numbered from the bottom of the tube. Quantitation of ICP4 by radioimmunoassay and measurements of catalase and phosphorylase b activities were performed and plotted as described previously (31). Arrows indicate the locations of the peak activity for catalase (C) and phosphorylase (P). CPM, Counts per minute. (Inset) Relationship between sedimentation coefficient and fractions obtained from this gradient, as established with the marker proteins catalase (C; 11.3S) and phosphorylase b (P; 8.4S). The sedimentation coefficients of the two major ICP4 peaks are indicated by dashed lines.

3′ that has been observed in all reported footprints produced by ICP4 (14). This result reveals that ICP4 binds in the absence of any other cellular or viral proteins to specific DNA sequences in HSV-1 DNA.

Stability of the ICP4-DNA complex. The half-life of complexes formed between ICP4 and the ICP0 gene promoter region was determined by measuring the dissociation of ICP4 from radiolabeled DNA at either 4 or 23°C. After the binding reaction reached equilibrium, a 10-fold excess of unlabeled specific competitor DNA (pXK13) was added as a "sink" for free ICP4; protein-bound radiolabeled DNA was quantitated at intervals thereafter by nitrocellulose filtration. We observed that both the rate and apparently the order of the dissociation reaction were different at the two temperatures. Of the bound DNA, 50% dissociated from the complexes within 5 min at 23°C (Fig. 8A), whereas at 4°C the half-life of the complexes was approximately 35 h (Fig. 8B). At 4°C the dissociation reaction was first order, whereas at 23°C the dissociation rate apparently varied with both time and concentration. Thus, it appears that temperature may have a profound effect on both the stability and the structure of ICP4-DNA complexes. To simplify interpretation of the data, we chose to measure additional kinetic parameters at 4°C.

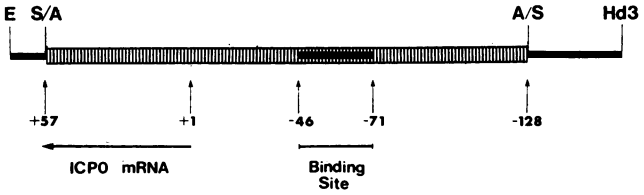

FIG. 6. Schematic diagram of target DNA for binding assays. A 177-bp *Aval* (A) fragment (▥) that spans the promoter for the HSV-1 gene encoding ICP0 was blunt-end ligated to the *Sam*I site (S) in pUC13 (▬) as described in Materials and Methods. Digestion of the resulting recombinant DNA with *Eco*RI (E) and *Hin*dIII (Hd3) yielded a 233-bp fragment with three unpaired thymidylates. Thus, the termini can be radiolabeled by incorporation of [α-^{32}P]dAMP. The solid bar from −71 to −46 corresponds to the ICP4-binding site (25).

Binding affinity of pure ICP4 for a specific DNA sequence. If it is assumed that formation of ICP4-DNA complexes proceeds by a bimolecular reaction in which freely diffusible molecules of ICP4 and DNA form specific complexes, then the ratio of protein-bound DNA to free DNA when the binding reaction is at equilibrium provides an indication of the relative affinity of ICP4 for that specific target DNA under a defined set of conditions (41). By obtaining measurements of PD/D (where PD is the amount of protein-bound

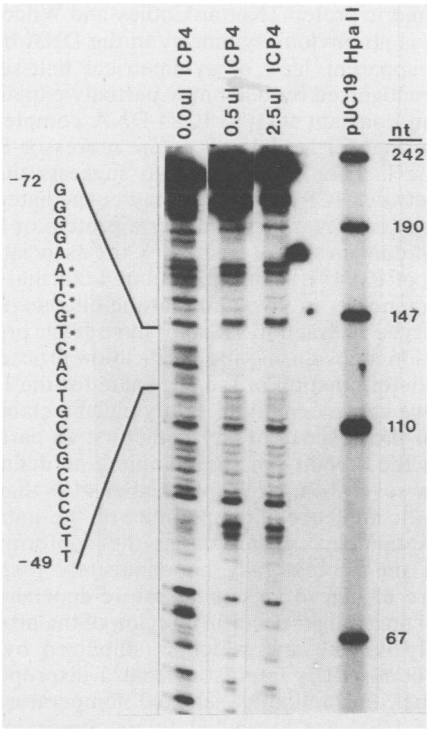

FIG. 7. DNase I footprint of the ICP4-binding site in the ICP4 gene promoter. A 223-bp fragment that included nucleotides −128 to +57 (relative to the transcription initiation site in the gene encoding ICP0) was labeled at the *Eco*RI-generated 3′ terminus (Fig. 6) with [α-^{32}P]dATP, incubated with fraction IX, and then briefly (90 s) digested with 0.25 U of DNase I at 20°C. Radiolabeled digestion products were denatured, separated by electrophoresis through an 8% polyacrylamide gel in the presence of 8.3 M urea, and detected by autoradiography. Lane pUC19 HpaII contains ^{32}P-labeled denatured size markers generated from an *Hpa*II digest of pUC19 DNA. The nucleotide sequence of the ICP0 gene promoter (28) from positions −72 to −49 is shown on the left. Asterisks indicate the position of the ATCGTC hexanucleotide. nt, Nucleotide.

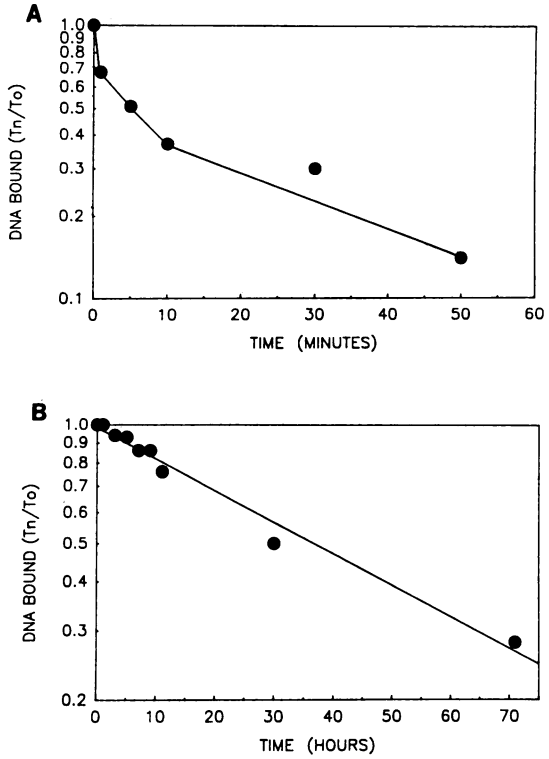

FIG. 8. Stability of ICP4-DNA complexes. Complexes were formed at 23°C (A) or 4°C (B) by preincubating 200 ng (5.8 pmol) of ICP4 and 4.5 pmol of radiolabeled DNA (233-bp ICP0 gene promoter fragment) for 60 min in 400 μl of buffer DNAB supplemented with 10 mM $MgCl_2$. At the end of this incubation period, 8.5 μg of unlabeled *Eco*RI-digested pXK13 DNA (which contains 61 pmol of ICP4-binding sites) was added. Samples (20 μl) were removed at the indicated times, and protein-bound DNA was measured by nitrocellulose filtration. The ratio of DNA bound at each time point (Tn) versus DNA bound at time zero (T0) is plotted as a function of time after the addition of pXK13 DNA.

DNA and D is the amount of free DNA) over a range of initial DNA concentrations, the equilibrium constant (K_D) for the binding reaction between ICP4 and a specific target DNA may be determined (43). This procedure also provides information on the amount of active ICP4 present in a binding reaction. The results of such an analysis are shown in Fig. 9. In Fig. 9A, each point represents a sample from a separate binding reaction in which a fixed amount of ICP4 (approximately 290 fmol, assuming a pure population of homodimers) was incubated with a different amount of radiolabeled target DNA at 4°C in a binding solution at pH 7.5 that contained 100 mM NaCl. Approximately 200 fmol of DNA was sufficient to saturate the binding capacity of an estimated 290 fmol of dimeric ICP4. Furthermore, the maximum amount of DNA bound did not exceed approximately 115 fmol. Taken together, these results indicate either that approximately 30 to 50% of the ICP4 in the reaction was capable of binding DNA or that the amount of dimeric ICP4 in the reaction was overestimated by a factor of 2 to 3 (see Discussion).

The data shown in Fig. 9A can be represented in an alternative format, as described by Scatchard (43), by plotting PD/D versus PD. In this format, the slope of the line is equal to the negative inverse of the equilibrium dissociation constant (K_D) and the x intercept is equal to the molar concentration of active ICP4 in the reaction. From this representation of the data (Fig. 9B), we calculated that the value of K_D is approximately 1×10^{-9} M and that the concentration of active ICP4 is 6.5 nM, which corresponds to 130 fmol of active ICP4 per 20-μl reaction. If our estimate of the total amount (290 fmol) of dimeric ICP4 per reaction is correct, then approximately 45% of the ICP4 in fraction IX is capable of binding to DNA under the conditions of this assay.

DISCUSSION

Our primary conclusion from the experimental results presented here is that ICP4 binds directly to specific sequences in double-stranded DNA in the absence of any other cellular or virus-encoded proteins. Additional evidence for this conclusion was recently provided by Michael et al. (32), who reported that specific DNA fragments were bound by ICP4 that had been resolved from other proteins by SDS-PAGE, transferred to nitrocellulose, and renatured.

The experimentally determined value of 1.1×10^{-9} M for the equilibrium dissociation constant (K_D) of the ICP4-DNA complex formed at 4°C in 100 mM NaCl is several orders of magnitude higher than the K_D values for specific complexes formed between DNA and either the *E. coli lac* repressor (1

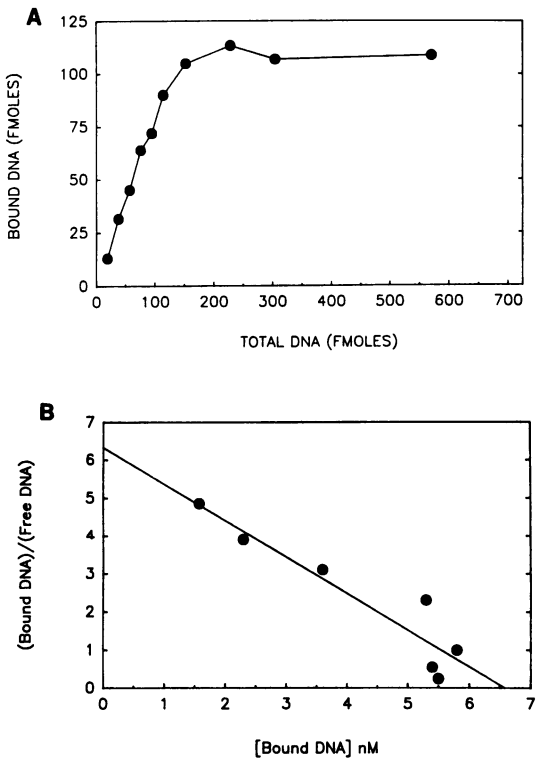

FIG. 9. Scatchard analysis of the interaction between ICP4 and the ICP0 gene promoter. (A) Binding reactions (20 μl) containing approximately 100 ng of ICP4 (290 fmol for a dimer with a mass of 300 kilodaltons) and the indicated amount of the 233-bp radiolabeled target DNA shown in Fig. 6 were incubated at 4°C for 60 min. The amount of protein-bound DNA was determined by nitrocellulose filtration. (B) The data from the plot shown in panel A were replotted as a Scatchard plot. Free DNA was calculated by subtracting the amount of bound DNA from the total amount of DNA. The experimentally determined slope ($-1/K_D$) of the linear regression line resulted in a calculated equilibrium dissociation constant of 1.1×10^{-9} M. The concentration of total binding protein was calculated from the intercept with the abscissa.

$\times 10^{-13}$ M) (41) or phage lambda *c*I protein (3×10^{-13} M) (20) but is similar to the K_D value for complexes formed between DNA and either simian virus 40 T antigen (0.4×10^{-9} to 4×10^{-9}) (33) or the *E. coli* tryptophan (*trp*) repressor (2.6×10^{-9} M) (23, 44). Direct comparisons among these dissociation constants are inappropriate because the values were determined under different binding conditions. However, from preliminary results (Kattar-Cooley and Wilcox, unpublished results), it is clear that assay conditions alone are not sufficient to explain the relatively high K_D values for ICP4-DNA complexes compared with either lambda *c*I-DNA or *lac* repressor-DNA complexes.

The K_D value for a given protein-DNA complex is inversely proportional to the strength and number of specific interactions between the protein and DNA. Both the lambda and *lac* repressors bind as oligomeric proteins that make multiple contacts with symmetrical sites in DNA. The lambda *c*I protein binds as a dimer to DNA (5); each of the two protomers makes specific contact with a separate half-site of approximately 8 bp. The *lac* repressor binds as a tetramer to DNA (15); at least two of the four protomers make specific contacts with separate half-sites of approximately 12 bp each. Although ICP4 also binds to DNA in vitro as an oligomeric protein (Kattar-Cooley and Wilcox, submitted), there is no obvious symmetry in the DNA binding site (14). The apparent lack of symmetrical half-sites in the sequence recognized by ICP4 may partially explain why the dissociation constant for the ICP4-DNA complex is much higher than that for lambda *c*I- or *lac* repressor-DNA complexes. The lack of symmetry also suggests that specific contacts between ICP4 and DNA may be mediated either by only one protomer within the dimeric protein or by a DNA recognition domain that is created by the association of two monomers of ICP4. It should be pointed out that ICP4 may exist as a monomer in vivo and that the oligomeric forms of ICP4 observed in fraction IX may have been produced by concentration steps during the purification procedure.

For the determination of the K_D value for the ICP4-DNA complex, we chose conditions that yielded a stable, specific complex in the absence of any cofactors. In particular, we found that the stability of the complex, as defined by the half-life, is several hundred-fold higher at 4 than at 23°C. This dramatic influence of temperature on the stability of the ICP4-DNA complex suggests that the conformation of a portion of the protein may be exquisitely responsive to temperature changes. This temperature-dependent conformational change might occur in a region of the protein which is relatively flexible and which is stabilized by extended hydrogen bonds. It is interesting that a disproportionately high number of randomly selected temperature-sensitive mutants of HSV are attributed to mutations in the ICP4 gene. This is consistent with the hypothesis that the active site of ICP4 is in a region of the protein which is highly responsive to amino acid changes resulting from mutations or circumstances such as environmental conditions or post-translational modifications (phosphorylation) that alter the tertiary structure of ICP4.

Previous reports have established that there may be at least three structural variants of ICP4 (designated ICP4a, ICP4b, and ICP4c in order of decreasing mobility during SDS-PAGE) in an HSV-infected cell (38). It has been proposed that these structural variants have the same primary amino acid sequence but differ in the number and location of phosphate and/or other moieties added post-translationally (49). It is tempting to speculate that these structural variants represent functional variants of ICP4. Analysis of ICP4-DNA complexes by the Southwestern (DNA-protein) blot procedure (32) suggests that both the b and the c forms of ICP4 can bind DNA (the a form was not resolved in this experiment), although specificity of binding may differ somewhat between the two forms. It should be noted that the migration profile of ICP4 in an SDS gel is strain dependent; in particular, ICP4 present in extracts from cells infected with the HFEM strain of HSV-1 migrates as a single broad band on SDS gels (31). The ICP4 in fraction IX was obtained from HFEM-infected cells and migrates as a single broad band during SDS-PAGE (Fig. 4). Furthermore, we presume that the ICP4 in fraction IX is heterogeneous with respect to phosphorylated forms. Thus, our results do not address the issue of binding affinities of different forms of ICP4.

Our value for the molar concentration of ICP4 in binding assays is based on a measurement of the total protein concentration in fraction IX, an estimate (from a Coomassie-stained gel) that greater than 90% of the protein mass in fraction IX is ICP4, and the assumption that all of the ICP4 exists as a dimer under the conditions of the binding assay. We presume that this value is within a factor of 2 of the actual value. Data from the Scatchard plot (Fig. 9) imply that approximately 45% of the ICP4 in fraction IX is capable of

binding to DNA. Given the potential error in our estimate of the concentration of ICP4 in fraction IX and the fact that a significant proportion of the ICP4 in fraction IX apparently exists as a tetramer, it is more accurate to state that 22 to 90% of the ICP4 in fraction IX is capable of binding to DNA. That a significant proportion of the ICP4 in fraction IX is able to bind to DNA is not unexpected, since the last step of the purification procedure is selective for this population. More important is the observation that the ICP4 in fraction VIII bound quantitatively to the DNA-cellulose column (data not shown). If it is assumed that the first eight steps in the purification of ICP4 are not selective with respect to the DNA-binding ability of ICP4, then this would suggest that at least 22% of the ICP4 present in HSV-infected cells 6 h postinfection is capable of binding to DNA.

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LITERATURE CITED

1. **Beard, P., S. Faber, K. W. Wilcox, and L. I. Pizer.** 1986. Herpes simplex virus immediate early infected-cell polypeptide 4 binds to DNA and promotes transcription. Proc. Natl. Acad. Sci. USA **83**:4016–4020.
2. **Beck, T. W., and R. L. Millette.** 1982. In vitro transcription of herpes simplex virus genes. Partial purification and properties of RNA polymerase II from uninfected and infected HEp-2 cells. J. Biol. Chem. **257**:12780–12788.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
4. **Campbell, M. E. M., J. W. Palfreyman, and C. M. Preston.** 1984. Identification of herpes simplex virus DNA sequences which encode a trans-acting polypeptide responsible for stimulation of immediate early transcription. J. Mol. Biol. **180**:1–19.
5. **Chadwick, P., V. Pirotta, R. Steinber, N. Hopkins, and M. Ptashne.** 1971. The lambda and 434 phage repressors. Cold Spring Harbor Symp. Quant. Biol. **35**:283–294.
6. **Clements, B. J., R. J. Watson, and N. M. Wilkie.** 1977. Temporal regulation of herpes simplex virus type 1 transcription: location of transcripts on the viral genome. Cell **12**:275–285.
7. **Coen, D. M., S. P. Weinheimer, and S. L. McKnight.** 1986. A genetic approach to promoter recognition during trans induction of viral gene expression. Science **234**:53–59.
8. **DeLuca, N., and P. A. Schaffer.** 1985. Activation of immediate-early, early, and late promoters by temperature-sensitive and wild-type forms of herpes simplex virus type 1 protein ICP4. Mol. Cell. Biol. **5**:1997–2008.
9. **Dixon, R. A. F., and P. A. Schaffer.** 1980. Fine-structure mapping and functional analysis of temperature-sensitive mutants in the gene encoding the herpes simplex virus type 1 immediate early protein VP175. J. Virol. **36**:189–203.
10. **Everett, R. D.** 1984. A detailed analysis of an HSV-1 early promoter: sequences involved in trans-activation by viral immediate-early gene products are not early-gene specific. Nucleic Acids Res. **12**:3037–3056.
11. **Everett, R. D.** 1984. Transactivation of transcription by herpes virus products: requirement for two HSV-1 immediate-early polypeptides for maximum activity. EMBO J. **3**:3135–3141.
12. **Faber, S. W., and K. W. Wilcox.** 1986. Association of the herpes simplex virus regulatory protein ICP4 with specific nucleotide sequences in DNA. Nucleic Acids Res. **14**:6067–6083.
13. **Faber, S. W., and K. W. Wilcox.** 1986. Characterization of a herpes simplex virus regulatory protein: aggregation and phosphorylation of a temperature-sensitive variant of ICP4. Arch. Virol. **91**:297–312.
14. **Faber, S. W., and K. W. Wilcox.** 1988. Association of herpes simplex virus regulatory protein ICP4 with sequences spanning the ICP4 gene transcription initiation site. Nucleic Acids Res. **16**:555–570.
15. **Geisler, N., and K. Weber.** 1976. Isolation of a set of hybrid lac repressors made in vitro between normal lac repressor and its homogeneous tryptic core. Proc. Natl. Acad. Sci. USA **73**: 3103–3106.
16. **Gelman, I. H., and S. Silverstein.** 1985. Identification of immediate early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. Proc. Natl. Acad. Sci. USA **82**:5265–5269.
17. **Gelman, I. H., and S. Silverstein.** 1987. Dissection of immediate-early gene promoters from herpes simplex virus: sequences that respond to the virus transcriptional activators. J. Virol. **61**: 3167–3172.
18. **Godowski, P. J., and D. M. Knipe.** 1986. Transcriptional control of herpesvirus gene expression: gene functions required for positive and negative control. Proc. Natl. Acad. Sci. USA **83**:256–260.
19. **Honess, R. W., and B. Roizman.** 1973. Proteins specified by herpes simplex virus. XI. Identification and relative molar rates of synthesis of structural and nonstructural herpesvirus polypeptides in the infected cell. J. Virol. **12**:1347–1365.
20. **Johnson, A. D., C. O. Pabo, and R. T. Sauer.** 1980. Bacteriophage lambda repressor and cro protein: interactions with operator DNA. Methods Enzymol. **65**:839–856.
21. **Jones, K. A., and R. Tjian.** 1985. Sp1 binds to promoter sequences and activates herpes simplex virus 'immediate-early' gene transcription in vitro. Nature (London) **317**:179–182.
22. **Jones, K. A., K. R. Yamamoto, and R. Tjian.** 1985. Two distinct transcription factors bind to the HSV thymidine kinase promoter in vitro. Cell **42**:559–572.
23. **Klig, L. S., I. P. Crawford, and C. Yanofsky.** 1987. Analysis of trp repressor-operator interaction by filter binding. Nucleic Acids Res. **15**:5339–5351.
24. **Kristie, T. M., and B. Roizman.** 1986. α4, the major regulatory protein of herpes simplex virus type 1, is stably and specifically associated with promoter-regulatory domains of α genes and of selected other viral genes. Proc. Natl. Acad. Sci. USA **83**: 3218–3222.
25. **Kristie, T. M., and B. Roizman.** 1986. DNA-binding site of major regulatory protein α4 specifically associated with promoter-regulatory domains of α genes of herpes simplex virus type 1. Proc. Natl. Acad. Sci. USA **83**:4700–4704.
26. **Litman, R. M.** 1968. A deoxyribonucleic acid polymerase from *Micrococcus luteus* isolated on deoxyribonucleic acid-cellulose. J. Biol. Chem. **243**:6222–6233.
27. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
28. **Mackem, S., and B. Roizman.** 1982. Structural features of the herpes simplex virus α gene 4, 0, and 27 promoter-regulatory sequences which confer α regulation on chimeric thymidine kinase genes. J. Virol. **44**:939–949.
29. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor.** 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69**:1531–1574.
31. **Metzler, D. W., and K. W. Wilcox.** 1985. Isolation of herpes simplex virus regulatory protein ICP4 as a homodimeric complex. J. Virol. **55**:329–337.
32. **Michael, N., D. Spector, P. Mavromara-Nazos, T. M. Kristie, and B. Roizman.** 1988. The DNA-binding properties of the major regulatory protein α4 of herpes simplex virus. Science **239**: 1531–1534.

33. **Müller, D., I. Ugi, K. Ballas, P. Reiser, R. Henning, and M. Montenarh.** 1987. The AT-rich sequence of the SV40 control region influences the binding of SV40 T antigen to binding sites II and III. Virology **161**:81–90.
34. **Muller, M. T.** 1987. Binding of the herpes simplex virus immediate-early gene product ICP4 to its own transcription initiation start site. J. Virol. **61**:858–865.
35. **O'Hare, P., and G. S. Hayward.** 1985. Evidence for a direct role for both the 175,000- and 110,000-molecular-weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J. Virol. **53**:751–760.
36. **O'Hare, P., and G. S. Hayward.** 1985. Three *trans*-acting regulatory proteins of herpes simplex virus modulate immediate-early gene expression in a pathway involving positive and negative feedback regulation. J. Virol. **56**:723–733.
37. **O'Hare, P., and G. S. Hayward.** 1987. Comparison of upstream sequence requirements for positive and negative regulation of a herpes simplex virus immediate-early gene by three virus-encoded *trans*-acting factors. J. Virol. **61**:190–199.
38. **Pereira, L., M. H. Wolff, M. Fenwick, and B. Roizman.** 1977. Regulation of herpesvirus macromolecular synthesis. V. Properties of α polypeptides made in HSV-1 and HSV-2 infected cells. Virology **77**:733–749.
39. **Preston, C. M.** 1979. Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature-sensitive mutant of *tsK*. J. Virol. **29**:275–284.
40. **Quinlan, M., and D. M. Knipe.** 1985. Stimulation of expression of a herpes simplex virus DNA-binding protein by two viral functions. Mol. Cell. Biol. **5**:957–963.
41. **Riggs, A. D., S. Bourgeois, and M. Cohn.** 1970. The lac repressor-operator interaction. III. Kinetic studies. J. Mol. Biol. **53**:401–417.
42. **Rosenfeld, P. J., and T. J. Kelly.** 1986. Purification of nuclear factor I by DNA recognition site affinity chromatography. J. Biol. Chem. **261**:1398–1408.
43. **Scatchard, G.** 1949. The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. **51**:660–672.
44. **Schevitz, R. W., Z. Otwinowski, A. Joachimiak, C. L. Lawson, and P. B. Sigler.** 1985. The three-dimensional structure of trp repressor. Nature (London) **317**:782–786.
45. **Shapira, M., F. L. Homa, J. C. Glorioso, and M. Levine.** 1987. Regulation of the herpes simplex virus type 1 late (gamma 2) glycoprotein C gene: sequences between base pairs −34 to +29 control transient expression and responsiveness to transactivation by the products of the immediate early (alpha) 4 and 0 genes. Nucleic Acids Res. **15**:3097–3111.
46. **Twigg, A. J., and D. Sherratt.** 1980. Trans-complementable copy-number mutants of plasmid ColE1. Nature (London) **283**: 216–218.
47. **Watson, R., and J. B. Clements.** 1980. A herpes simplex virus type 1 function continuously required for early and late virus RNA synthesis. Nature (London) **285**:329–330.
48. **Watson, R. J., C. M. Preston, and J. B. Clements.** 1979. Separation and characterization of herpes simplex virus type 1 immediate-early mRNAs. J. Virol. **31**:42–52.
49. **Wilcox, K. W., A. Kohn, E. Sklyanskaya, and B. Roizman.** 1980. Herpes simplex virus phosphoproteins. I. Phosphate cycles on and off some viral polypeptides and can alter their affinity for DNA. J. Virol. **33**:167–182.