Isolation of Herpes Simplex Virus Regulatory Protein ICP4 as a Homodimeric Complex

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The viral polypeptide ICP4 (or Vmw175) is synthesized during the immediate early phase of infection by herpes simplex virus and regulates the transcription of delayed early and late viral genes. We obtained a partially purified preparation of soluble ICP4 under nondenaturing conditions. Physical constants for native ICP4 were empirically determined by molecular sieve chromatography and sucrose density gradient ultracentrifugation. The Stokes radius of native ICP4 was 8.72×10^{-7} cm. The sedimentation coefficient of native ICP4 was 9.00S. From these values, the calculated molecular weight of native ICP4 was 342,000, a value which is twice that of monomeric ICP4, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The failure of any other polypeptides to specifically coprecipitate with native ICP4 in the presence of anti-ICP4 antibody indicates that the 342,000-dalton complex is a homodimer of ICP4. The frictional coefficient ratio of native ICP4, which is 1.9, indicates that the homodimer is a highly elongated molecule.

The genome of herpes simplex virus (HSV) is a linear, double-stranded DNA molecule containing approximately 150 kilobase pairs that encode at least 50 polypeptides. These polypeptides and their corresponding mRNAs have been classified on the basis of their temporal order of appearance within an infected cell as immediate early (α), delayed early (β), or late (γ). The immediate early genes encode five infected cell polypeptides (ICPs) designated as ICP4, ICP0, ICP22, ICP27, and ICP47 by Honess and Roizman (13) or as Vmw175, Vmw110, Vmw68, Vmw63, and Vmw12, respectively, by Watson et al. (32). Because of the lack of conditional mutations in the genes encoding ICP0, ICP27, and ICP47, there is no information currently available regarding the roles of these proteins in viral infection. Mutants with deletions in the gene encoding ICP22 grow normally in human and monkey cell lines (18). Several temperature-sensitive mutants belonging to HSV complementation group 1-2 (24) and having mutations in the gene encoding ICP4 (7) have been isolated. In cells infected by these mutants at the nonpermissive temperature, the viral reproductive cycle does not proceed beyond the immediate early phase; i.e., transcription of the delayed early and late genes does not occur (20, 31). These results indicate that functional ICP4 is required to initiate and maintain transcription of the delayed early viral genes.

The known properties of ICP4 are consistent with its role as a transcriptional regulatory protein. This viral polypeptide is maximally synthesized from 4 to 6 h postinfection and is rapidly translocated to the nucleus (7). Three electrophoretic variants (ICP4a, b, and c) with apparent molecular weights of 163,000, 165,000, and 170,000 can be isolated from HSV-infected cells (16). All three forms are phosphorylated (33). Phosphoserine and phosphothreonine residues have been isolated from ICP4 phosphorylated in vivo (S. Faber and K. W. Wilcox, submitted for publication). ICP4 is modified in vitro by poly(ADP-ribosyl)ation (21). Under physiological conditions, ICP4 is apparently associated with nonmembranous, particulate nuclear material. Detergents such as

Nonidet P-40 or CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate) are not sufficient to solubilize ICP4 from the particulate fraction produced when nuclei are disrupted in phosphate-buffered saline (PBS) (1). Extraction of ICP4 from this particulate fraction requires high ionic strength (19). The affinity of partially purified ICP4 for single-stranded DNA (9) suggests that ICP4 exists as a nucleoprotein complex in infected cells. This complex may be stabilized by cellular proteins. Freeman and Powell (9) have reported that more highly purified ICP4 does not bind to DNA; however, the addition of an extract from uninfected cells to this purified ICP4 restores its ability to bind to single-stranded DNA (9).

The properties of ICP4 synthesized in cells infected with either wild-type HSV or a temperature-sensitive strain with a mutation in the ICP4 gene have been investigated (Faber and Wilcox, submitted). Initial attempts to purify ICP4 from HSV-infected Vero cells were hindered by two problems, namely extensive proteolytic degradation of ICP4 that occurred when nuclei were isolated from infected cells and the insolubility of ICP4 in buffers with ionic strengths below 0.25 M. In the first part of this paper, we present our solutions to these two problems. In the second part of the paper, we present our results from studies on the size, molecular weight, and subunit composition of soluble, native ICP4.

MATERIALS AND METHODS

Cells and virus. Vero cells were grown in Dulbecco modified Eagle medium supplemented with 5% newborn bovine serum and 150 U of penicillin G and 150 μg of streptomycin sulfate per ml. The virus strain used was HSV-1(HFEM). Infected cells were incubated in medium 199V (199/Hanks balanced salt solution supplemented with 1% heat-inactivated newborn bovine serum and 150 U of penicillin G and 150 μg of streptomycin sulfate per ml).

Solutions. PBS contains 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4. Reticulocyte standard buffer (RSB) contains 10 mM Tris hydrochloride (pH 7.5), 10 mM NaCl, and 1.5 mM $MgCl_2$. REX-300 contains 10 mM Tris hydrochloride (pH 8.0), 1.0 mM EDTA, 10 mM β-

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mercaptoethanol, and 300 mM $(NH_4)_2SO_4$. Disruption buffer (DB) contains 50 mM Tris hydrochloride (pH 7.0), 2% sodium dodecyl sulfate (SDS), 0.7 M β-mercaptoethanol, and 10% glycerol.

Reagents, proteins, and enzyme assays. Aprotinin was obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind. L-1-tosylamide-2-phenylmethyl chloromethyl ketone (TPCK), CHAPS, and Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK) were obtained from Calbiochem-Behring, La Jolla, Calif. Phenylmethylsulfonyl fluoride, *Escherichia coli* β-galactosidase (no. G6512; 630 Sigma units/mg), bovine liver catalase (no. C100; 3,500 Sigma units/mg), and rabbit muscle phosphorylase *b* (no. P6635; 29 Sigma units/mg) were obtained from Sigma Chemical Co., St. Louis, Mo. Bovine serum albumin was obtained from Armour Pharmaceutical Co. The methods of Craven et al. (6), Beers and Sizer (3), and Illingworth and Cori (14) were used to assay the activities of β-galactosidase, catalase, and phosphorylase *b*, respectively. The polyclonal rabbit anti-ICP4 antibody was prepared against denatured type 1 ICP4 purified by electroelution from SDS-polyacrylamide gels (Faber and Wilcox, submitted). Immunoglobulin G (IgG) was partially purified from preimmune and immune sera by $(NH_4)_2SO_4$ fractionation.

SDS-PAGE. For SDS-polyacrylamide gel electrophoresis (SDS-PAGE), protein samples were dissolved in DB, boiled for 1 min, and then subjected to electrophoresis through a diallyltartardiamide cross-linked 3.5% stacking acrylamide gel and 9% resolving acrylamide gel as described by Heine et al. (12).

Radioiodination of proteins. Proteins were radiolabeled with iodine-125 (Amersham Corp., Arlington Heights, Ill.) in tubes coated with IoDOgEN (Pierce Chemical Co., Rockford, Ill.), which serves as a mild, solid-phase oxidizing reagent (8, 23). A sample (125 μl) of *Staphylococcus aureus* protein A (Sigma or Calbiochem) at a concentration of 1 mg/ml in 0.1 M sodium borate buffer (pH 8.5) was mixed with 4 mCi of carrier-free iodine-125 in a tube that had been previously coated on the bottom with 10 μg of IODO-GEN. After 15 min at 20°C, the sample was transferred to a tube containing 0.3 ml of stop buffer (50 mM potassium phosphate buffer [pH 7.5], 50 mM β-mercaptoethanol). A solution of gelatin was added to yield a final concentration of 0.2% (wt/vol) and a final volume of 0.5 ml. This mixture was exhaustively dialyzed against 50 mM potassium phosphate buffer (pH 7.5). The initial specific activity of the radiolabeled protein was 3×10^7 to 5×10^7 dpm/μg.

To radioiodinate protein in fraction VII, we mixed a sample (0.5 ml) with 0.5 mCi of iodine-125 in an IODO-GEN-coated tube. After 15 min at 20°C, the iodinated sample was adjusted to contain 25 mM β-mercaptoethanol and 0.2% gelatin and exhaustively dialyzed.

ICP4 RIA. For radioimmunoassay (RIA), proteins in SDS-polyacrylamide gels were transferred to nitrocellulose (Schleicher & Schuell BA85) by electrophoresis at 100 mA for 20 h in 20 mM Tris base–150 mM glycine–20% methanol (5). Proteins in aqueous samples were spotted (5 to 20 μl) onto a sheet of nitrocellulose (Millipore HAHY; Millipore Corp., Bedford, Mass.) placed on top of two sheets of Whatman no. 3 filter paper (Whatman, Inc., Clifton, N.J.). The nitrocellulose was then sequentially immersed for the indicated periods at 20°C in the following solutions: 45 min in pre-antibody washing solution (0.15 M NaCl, 0.01 M Tris hydrochloride [pH 7.5], 5% bovine serum albumin, 0.02% sodium azide); 90 min in pre-antibody washing solution supplemented with 75 μg of rabbit IgG containing anti-ICP4

antibody per ml; 10 min in Tris-saline (TS) (0.15 M NaCl, 0.01 M Tris hydrochloride [pH 7.5]); two 10-min washes in TS40 (TS containing 0.05% Nonidet P-40); 10 min in TS; 60 min in pre-antibody washing solution supplemented with 150,000 cpm of ^{125}I-labeled protein A per ml; 10 min in TS; two 10-min washes in TS40; and 10 min in TS. The nitrocellulose was then dried and exposed to Kodak X-ray film with an intensifying screen at −80°C.

Partial purification of ICP4. Vero cells infected with HSV-1 (HFEM) at 10 PFU per cell were incubated at 37°C for 6.5 h. All subsequent operations were carried out at 4°C in buffers containing 0.1 mM TPCK. All volumes are based on extracts prepared from 2×10^8 cells. The growth medium was decanted, and the monolayer was rinsed with 20 ml of ice-cold PBS supplemented with 0.6 mM EDTA. Cells were scraped into 20 ml of PBS plus 0.6 mM EDTA, pelleted, suspended in 5 ml of RSB, swollen for 10 min, and then disrupted in a Dounce homogenizer. Sucrose was added to a final concentration of 8.3%, and the nuclei were collected by centrifugation at $1,000 \times g$ for 10 min. The nuclei were suspended in 3.75 ml of RSB containing 8.3% sucrose and 1% Triton X-100 and then pelleted at $1,000 \times g$ for 10 min. The detergent-washed nuclei were suspended in 2 ml of REX-300 and then disrupted by sonication. The nuclear extract was supplemented with 0.5% CHAPS and clarified by centrifugation at $100,000 \times g$ for 1 h. Powdered ammonium sulfate (Ultrapure; Schwartz/Mann, Orangeburg, N.Y.) was added to 20% saturation in the supernatant. Precipitated proteins were removed by centrifugation at $12,000 \times g$ for 10 min. Powdered ammonium sulfate was added to the resulting supernatant to a final concentration of 50% saturation. Precipitated proteins were recovered by centrifugation at $12,000 \times g$ for 10 min. The resulting pellet was suspended in 0.25 ml of REX-300. This solution was clarified by centrifugation at $12,000 \times g$ for 5 min and is referred to as fraction VI. It contains approximately 2% of the total cellular protein and 25 to 50% of the total ICP4 (unpublished data).

Measurement of the Stokes radius of ICP4. It has been shown by Ackers (2) that the volume (V_e) required to elute a protein from a gel filtration column is determined by the total volume (V_t) of the column, the void volume (V_o) of the column, the Stokes radius (a) of the protein, and the mean effective pore radius (r) of the column matrix. For any given protein that is retarded by a gel filtration column, one can obtain a value for ($V_e - V_o$)/($V_t - V_o$). This value is referred to as the distribution coefficient, or K_D, for that protein (10). Ackers (2) has published the following equation showing that for any protein, the value of K_D is a function of a/r: $K_D = (1 - a/r)^2[1 - 2.104(a/r) + 2.09(a/r)^3 - 0.95(a/r)^5]$. Thus, the value of r for a particular molecular sieve matrix can be calculated by experimentally determining the K_D values for proteins with known a's. Once the column is calibrated, i.e., once values for V_o, V_t, and r are known, then the experimental value of K_D obtained for an unknown protein can be used to calculate the a of that protein.

We chose to use Fractogel TSK HW-65F (MCB Manufacturing Chemists, Gibbstown, N.J.) as the molecular sieve matrix. This semirigid material fractionates globular proteins with molecular weights between 5×10^4 and 5×10^6 (11). The V_o and V_t of the column were measured with bacteriophage T4 and [^{3}H]acetate, respectively. The V_e for each protein species was calculated as the volume eluted from fraction 1 to the peak fraction, inclusive. The value of a/r for each value of K_D was determined from tables published by Ackers (2). The r of the column matrix was then calculated
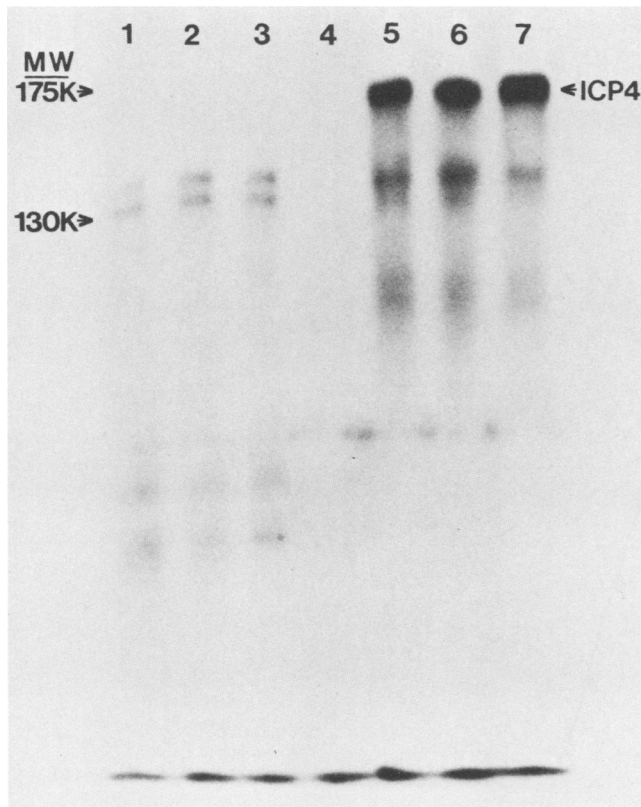
FIG. 1. Inhibition of proteolytic degradation of ICP4. Six flasks (2×10^6 Vero cells each) infected with HSV-1(HFEM) at 10 PFU per cell and one uninfected flask were incubated at 37°C for 6 h. The cells were then scraped into 1.0 ml of ice-cold PBS containing 0.6 mM EDTA and the indicated protease inhibitor (see below). The harvested cells were suspended in 1.0 ml of ice-cold RSB containing the indicated inhibitor and then disrupted on ice in a Dounce homogenizer. After sucrose was added to a final concentration of 8.3%, the nuclei were recovered by centrifugation at 4°C and then suspended in ice-cold RSB containing 8.3% sucrose, 1% Triton X-100, and the indicated inhibitor. The detergent-washed nuclei were pelleted, suspended in 0.4 ml of DB, sonicated on ice, and then boiled. A sample (25 µl) of each nuclear extract was subjected to SDS-PAGE, electroblotted to nitrocellulose, and subjected to the ICP4 RIA. Lane 4 contained a nuclear extract from uninfected cells; the other lanes contained nuclear extracts from infected cells. Lanes: 1, no inhibitor; 2, aprotinin at 60 Kallikrein units/ml; 3, 0.1 mM phenylmethylsulfonyl fluoride; 4, 0.1 mM TPCK; 5, 0.1 mM TPCK; 6, 0.1 mM TLCK; 7, 0.1 mM TPCK and 0.1 mM TLCK. MW, Molecular weight.

by using published values for the physical properties of the standard proteins, namely β-galactosidase (29) and catalase (28).

Calculations of the physical measurements of native ICP4. The free diffusion coefficient, D, of native ICP4 was calculated from the a of native ICP4 from the Stokes-Einstein equation: $D = kT/6\pi\eta a$, where k is the Boltzman constant, T is the absolute temperature, and η is the solvent viscosity. A temperature of 20°C and the viscosity of water at 20°C were employed in the calculation of $D_{20,w}$.

The molecular weight of native ICP4 was calculated from the free diffusion coefficient, $D_{20,w}$, and the sedimentation coefficient, $S_{20,w}$, of native ICP4 by use of the Svedberg equation: $M = S_{20,w}RT/D_{20,w}(1 - \bar{v}\rho)$, where R is the gas constant, $\bar{v}$ is the partial specific volume of ICP4, and ρ is the solvent density. For the $\bar{v}$ of ICP4, a value of 0.74 was

assumed, which corresponds to the mean value for proteins (27). A temperature of 20°C and the density of water at 20°C were employed in the calculation of the molecular weight.

The frictional ratio of native ICP4 was calculated from the expression: $f/f_o = a/[(3\nu M/4\pi N)^{0.33}]$, where N is the avogadro number.

RESULTS

Inhibition of proteolytic degradation of ICP4. Zezulak and Spear (34) reported that viral glycoproteins synthesized in HSV-infected Vero cells are susceptible to cleavage by one or more cell-associated proteases during cell extraction procedures. Their results indicate that the addition of either TLCK or p-hydroxymercuribenzoate to extraction buffers is sufficient to inhibit most proteolytic activity. We have observed that ICP4, which is a nonglycosylated immediate early HSV-encoded protein, is also highly susceptible to proteolytic cleavage during the initial stages of purification from HSV-infected Vero cells. In particular, we found that extensive proteolysis of ICP4 occurred during the isolation of nuclei from infected cells. We systematically tested a variety of extraction conditions and protease inhibitors to find a procedure that optimizes the recovery of intact ICP4 from nuclei. To conduct these tests, separate cell cultures infected with HSV-1(HFEM) were harvested by scraping into ice-cold PBS supplemented with 0.6 mM EDTA. Intact nuclei were obtained by homogenizing the cells in hypotonic buffer. The nuclei were washed with buffer containing 1% Triton X-100 and then subjected to SDS-PAGE. For each cell culture, a specific protease inhibitor was added to the extraction buffer immediately before use. RIA with polyclonal anti-ICP4 antibody after transfer of the separated polypeptides to nitrocellulose revealed that essentially no
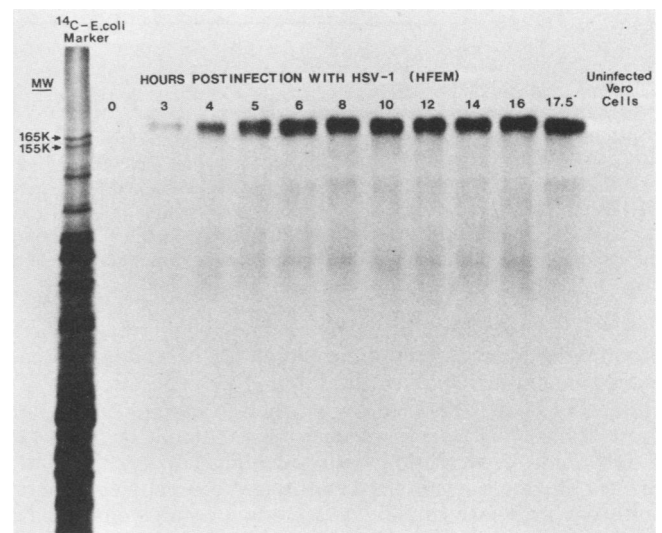

FIG. 2. Stability of ICP4 in vivo. Extracts were prepared from uninfected and HSV-1(HFEM)-infected Vero cells at the times indicated above each lane and analyzed as described in the legend to Fig. 1. TPCK (0.05 mM) was added to all extraction buffers immediately before use. All times postinfection were measured after a 1-h adsorption period at 20°C. E. coli cells grown in the presence of ^{14}C-labeled amino acids were disrupted by sonication in DB and coelectrophoresed in the same gel. The β and β' subunits of the E. coli RNA polymerase are visible as bands with molecular weights of 155,000 and 165,000, respectively.
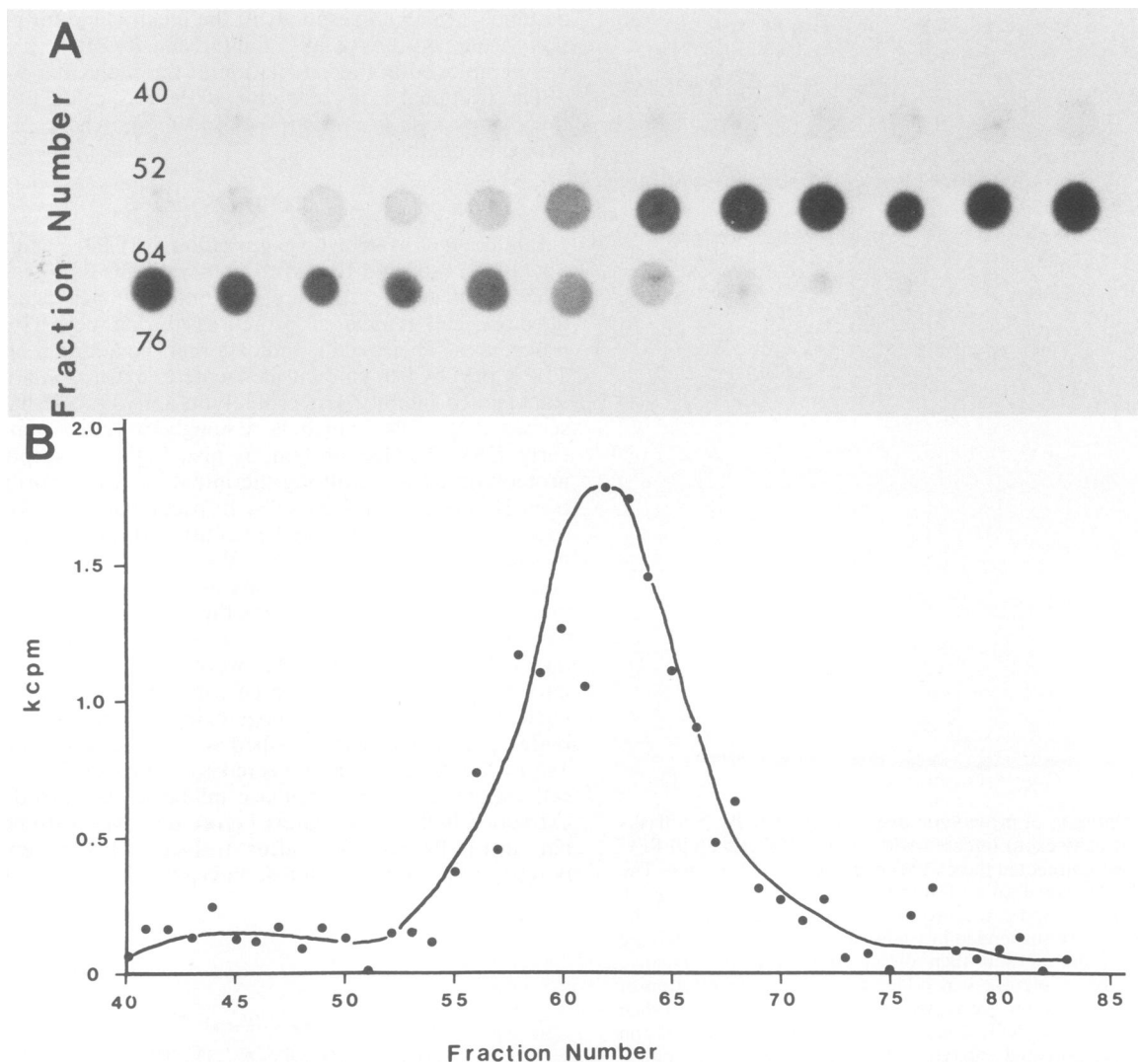
FIG. 3. Chromatography of ICP4 on a gel filtration column. Fraction VI (4 ml) supplemented with glycerol to a final concentration of 10% was loaded onto a column (3 by 45 cm) of Fractogel TSK HW-65F equilibrated in REX-300 at 4°C, and 100 fractions (0.3 ml) were collected. Fractions 40 and 81 corresponded to the peaks of V_o and V_t, respectively, as measured with radiolabeled high-molecular-weight DNA and [^{3}H]acetate, respectively (data not shown). (A) Samples (5 µl) from fractions 40 through 83 were spotted onto nitrocellulose and subjected to the ICP4 RIA. The fraction numbers appear above the corresponding spots. (B) Each radiolabeled spot shown in panel A was cut out from the nitrocellulose and counted in a scintillation counter. kcpm, Thousand counts per minute.

intact ICP4 remained in the extracted nuclei when either aprotinin or phenylmethylsulfonyl fluoride was added to the extraction buffer (Fig. 1, lanes 2 and 3). The addition of either TPCK or TLCK to the extraction buffers protected most of the ICP4 from significant degradation (Fig. 1, lanes 5 and 6). In combination with the report of Zezulak and Spear (34), these results indicate that Vero cells contain a sulfhydryl protease capable of degrading both glycosylated,

membrane-associated viral proteins and nonglycosylated, nucleus-associated proteins.

To determine whether the rapid proteolysis of ICP4 in disrupted Vero cells is indicative of a high turnover rate of ICP4 in intact Vero cells, we harvested cells in the presence of TPCK at various times after infection with HSV-1 (HFEM). These extracts were analyzed by RIA of electrophoretically separated proteins. We observed no significant degradation of ICP4 during 17.5 h of infection (Fig. 2), a period sufficient for one cycle of viral replication. We also observed that the net amount of cellular ICP4 reached a plateau at about 8 h postinfection (Fig. 2) and remained constant thereafter. Since the synthesis of ICP4 is essentially shut off by 8 h postinfection, these results indicate that ICP4 is relatively stable in vivo. Apparently, neither ICP4 nor viral glycoproteins are accessible to the major Vero cell protease unless the cells are disrupted.

Isolation of a soluble form of native ICP4. High concentra-

TABLE 1. Calibration of analytical TSK-65 column

Protein	K_D	a/r	a(nm)	r(nm)
β-Galactosidase	0.654	0.0962	6.86	71.3
Catalase	0.722	0.0741	5.22	70.4
ICP4	0.573	0.1230	8.72	70.9[a]

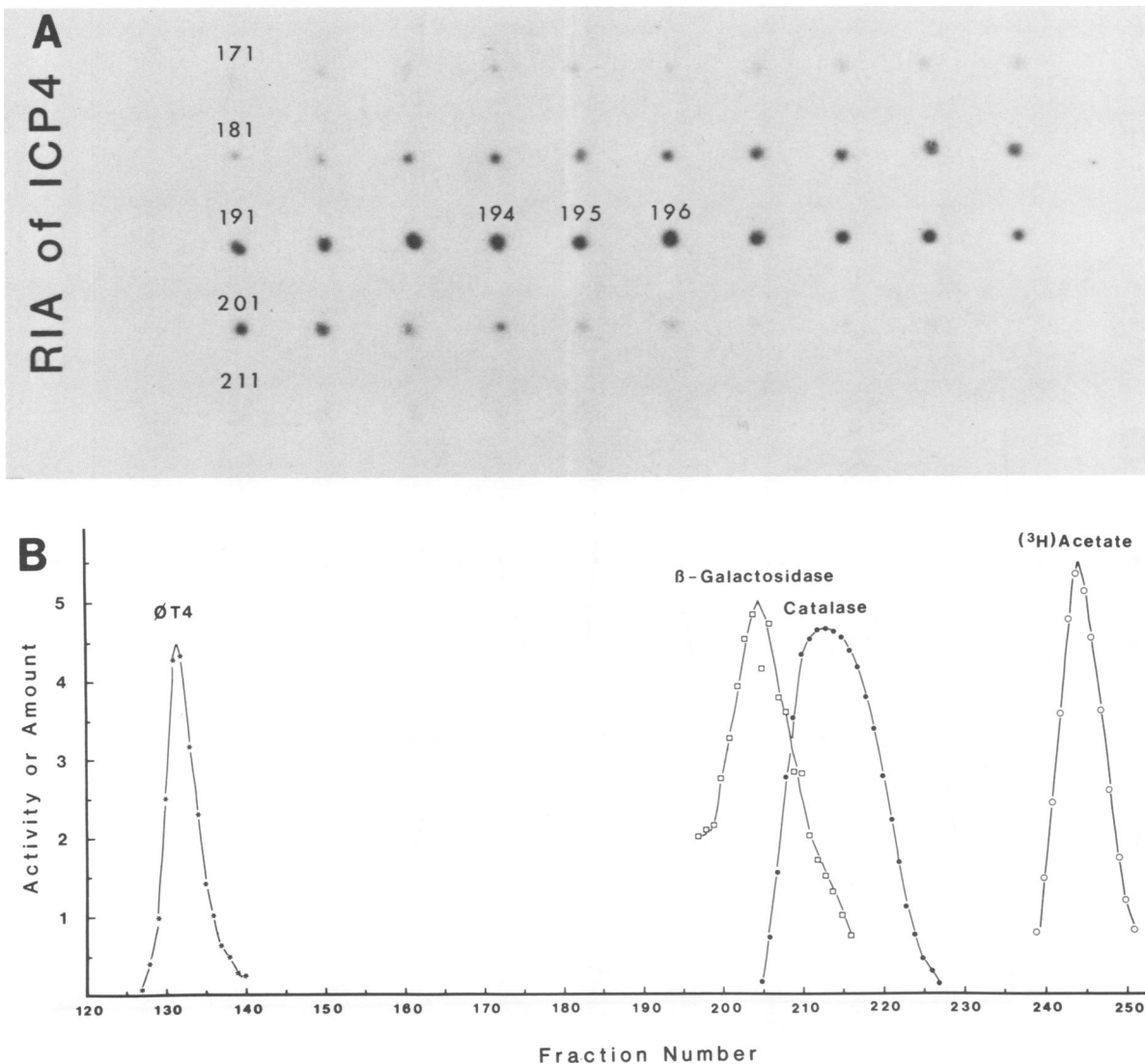[a] Average of the values for β-galactosidase and catalase.

FIG. 4. Elution of proteins from an analytical gel filtration column. A column (1.5 by 90 cm) containing Fractogel TSK-65 was equilibrated in PBS. A sample (0.21 ml) of fraction VII was mixed with phage T4, β-galactosidase (8 Sigma units), catalase (40 Sigma units), and [³H]acetate and loaded as a final volume of 0.28 ml onto the column. The column was pumped at a constant flow rate of 10 ml/h at 4°C. A total of 265 fractions (0.49 ml each) were collected. (A) Samples (5 μl each) from fractions 171 to 220 were spotted onto nitrocellulose and subjected to the ICP4 RIA. (B) Fractions 125 to 145 were assayed for protein to detect the peak of phage T4; one unit on the vertical axis corresponds to an optical density at 650 nm (OD_{650}) of 0.05. Fractions 195 to 220 were assayed for β-galactosidase; one unit on the vertical axis corresponds to an OD_{405} of 0.10. Fractions 205 to 230 were assayed for catalase; one unit on the vertical axis corresponds to a decrease in OD_{240} of 0.10. Fractions 235 to 255 were assayed for radioactivity; one unit on the vertical axis corresponds to 200 cpm.

tions of salt are required to extract ICP4 from the nuclei of HSV-infected cells (19, 33). Our initial experiments showed that the ICP4 present in clarified nuclear extracts is insoluble at ionic strengths below 0.25 M. This finding necessitated the development of an ICP4 purification scheme that could be carried out at high ionic strength. During the course of this investigation, we discovered that chromatography of a clarified nuclear extract through a molecular sieve matrix yielded a single peak of immunoreactive ICP4 that remained soluble at ionic strengths as low as 50 mM.

Details of the purification scheme are presented above. Briefly, nuclei were prepared 7 h postinfection from cells infected with HSV-1 (HFEM). The nuclei were washed in RSB containing Triton X-100 and then disrupted in REX-300. The nuclear extract was clarified by centrifugation at 100,000 × g. Proteins in the supernatant were precipitated by the addition of ammonium sulfate to a final concentration of 50%. The precipitate was suspended in REX-300 and chromatographed through a molecular sieve matrix equilibrated in the same buffer. A sample of each fraction was spotted onto nitrocellulose and subjected to RIA with anti-ICP4 antibody. A single peak of ICP4 eluted from the column (Fig. 3). Fractions 56 to 68 were pooled, concentrated by ammonium sulfate precipitation, and suspended in PBS. This solution of partially purified ICP4 is referred to as fraction VII. Centrifugation of this sample indicated that the partially purified ICP4 was soluble in PBS (data not shown). This material was therefore analyzed more extensively to determine the size, molecular weight, and composition of soluble, native ICP4.
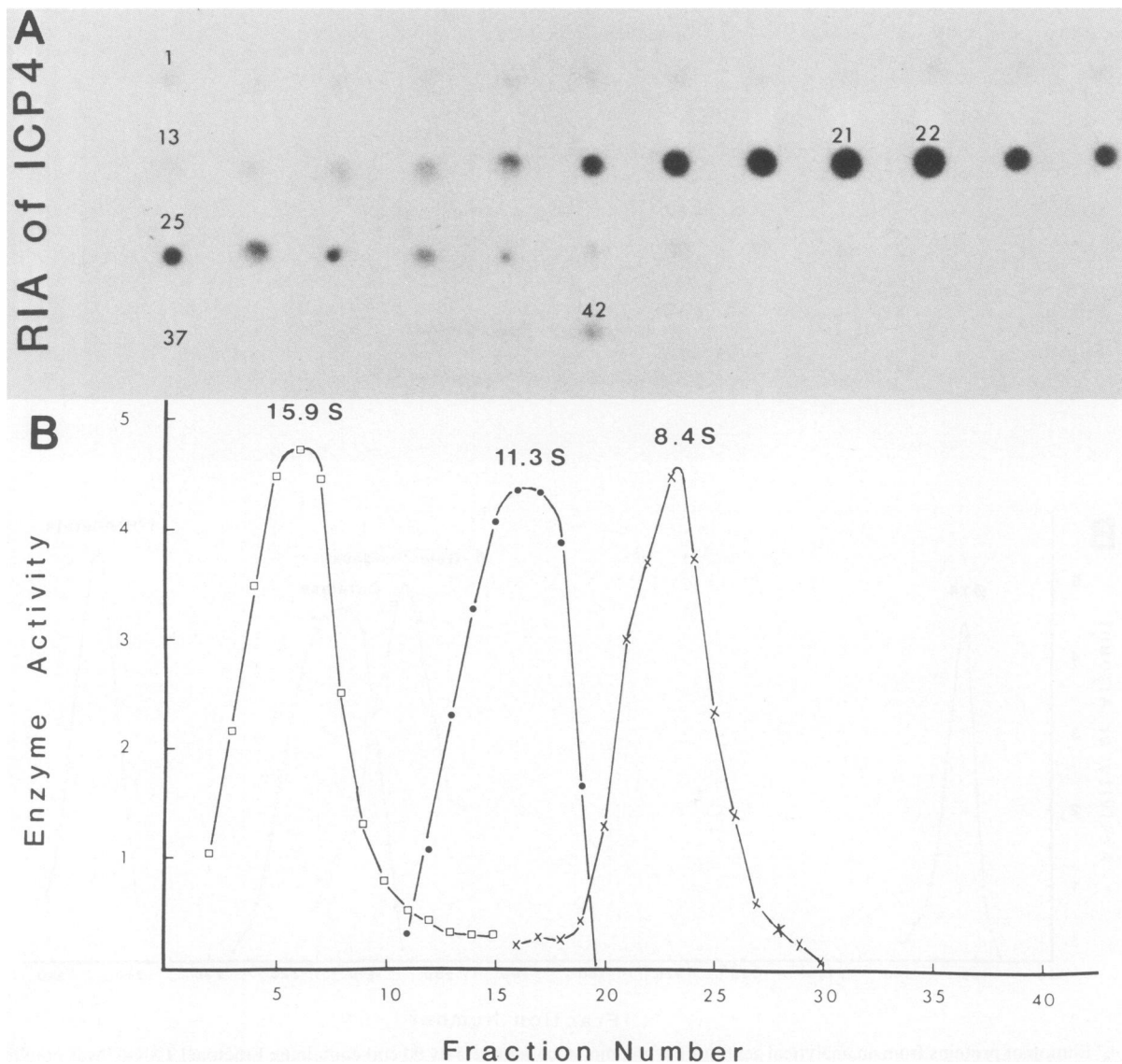
FIG. 5. Sedimentation velocity analysis. A sample (65 μl) of fraction VII was mixed with catalase (400 U), β-galactosidase (8 U), and phosphorylase b (1.5 U) in a total volume of 100 μl and layered over a gradient of 5 to 20% sucrose in PBS. The sample was centrifuged at 34,000 rpm in an SW50.1 rotor for 15 h at 4°C, and 45 fractions (100 μl each) were collected from the bottom of the tube. (A) Samples (5 μl each) from fractions 1 to 44 were spotted onto nitrocellulose and subjected to the ICP4 RIA. (B) Enzymatic activities were measured on samples from each fraction as described in the text. For β-galactosidase (□), catalase (●), and phosphorylase b (×), one unit on the vertical axis corresponds to an optical density at 405 nm (OD_{405}) of 0.4, a decrease in OD_{240} of 0.04, and an OD_{660} of 0.2, respectively, as obtained in the enzymatic assays.

Determination of physical parameters for native ICP4. Chromatography of ICP4 through a calibrated gel filtration column allows one to calculate the a of native ICP4 (for details, see above). A sample of fraction VII was co-chromatographed through an analytical TSK-65 column along with bacteriophage T4, β-galactosidase, catalase, and sodium [^{3}H]acetate. The ICP4 eluted as a symmetrical peak centered at fraction 195 (Fig. 4A). The peaks for the calibration markers phage T4, β-galactosidase, catalase, and [^{3}H]acetate corresponded to fractions 132, 205, 213, and 244, respectively (Fig. 4B). As described above, the calculated r of the column matrix was 71.3 nm with β-galactosidase as the calibrating standard and 70.4 nm with catalase as the calibrating standard. From the average of these two values, the calculated a of native hydrated ICP4 was 8.72 nm. The

relevant data used to make these calculations are presented in Table 1.

The sedimentation coefficient for native ICP4 was obtained by sedimentation velocity analysis. Proteins with known sedimentation rates were sedimented along with a sample of fraction VII through an isokinetic sucrose gradient. Under the conditions of this centrifugation, the peak of ICP4 was located in fractions 21 and 22, which corresponded to the middle of the gradient (Fig. 5A). The peak activities of β-galactosidase (15.9S), catalase (11.3S), and phosphorylase (8.4S) were located in fractions 6, 16, and 23, respectively (Fig. 5B). From the linear calibration curve for this gradient (Fig. 6), the value of $S_{20,w}$ for ICP4 was 9.00S.

By the equations described above, the calculated $D_{20,w}$ of native ICP4 was 2.46×10^{-7}. By the calculated values for
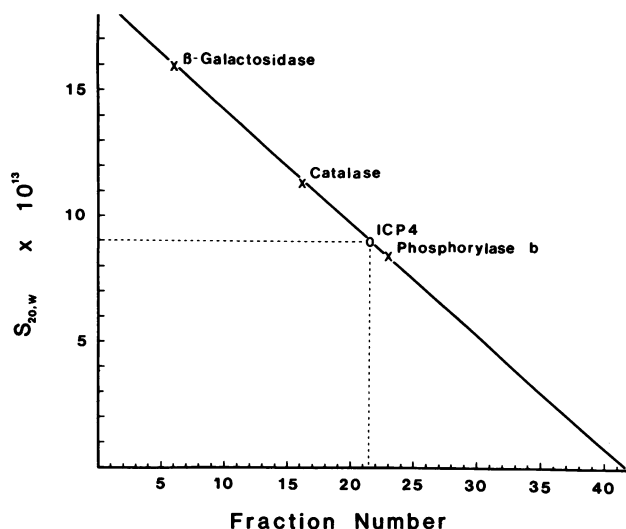
FIG. 6. Calibration of sucrose gradient. The sedimentation co-efficients of the marker proteins are plotted as a function of the corresponding peak fraction from the gradient described in the legend to Fig. 5. The line through the points was obtained by linear regression analysis. The published values for the sedimentation coefficients of the marker proteins are: β-galactosidase, 15.9S (29); catalase, 11.3S (28); and phosphorylase b, 8.4S (26). $S_{20,w}$, Calculated sedimentation coefficient at 20°C and the density of water.

$D_{20,w}$ and $S_{20,w}$, the molecular weight of native, hydrated ICP4 was 342,000. The f/f_o for native ICP4 was 1.88.

Subunit composition of native ICP4. Monomeric, denatured ICP4 has an apparent molecular weight between 165,000 (16) and 175,000 (22) based on its relative migration rate in an SDS-polyacrylamide gel. Our calculated value for the molecular weight of native, hydrated ICP4 is approximately twice the value of monomeric ICP4. The most obvious possibility is that native ICP4 exists as a homodimer under the conditions of our purification procedure. However, it is also possible that one or more host or viral proteins are associated with ICP4, forming a complex with a molecular weight of 342,000. To determine the polypeptide composition of the 342,000-molecular-weight complex, the proteins in fraction VII were radiolabeled with ^{125}I and then immunoprecipitated with either immune IgG (containing anti-ICP4 antibody) or with preimmune (control) IgG. Radiolabeled proteins in the precipitates were separated by SDS-PAGE and detected by autoradiography (Fig. 7). Only one band, corresponding to ICP4, was specifically precipitated (Fig. 7). Two bands with apparent molecular weights between 40,000 and 45,000 were observed in equal amounts in both control and immune precipitates (Fig. 7). Analysis of these same samples on a 15% polyacrylamide gel revealed only one or two minor bands with molecular weights below 40,000; these minor bands were found equally in both the control and the immune precipitates (data not shown). We conclude that the soluble, native ICP4 which we have partially purified is a homodimer.

DISCUSSION

We report here the isolation and characterization of a soluble form of native ICP4 from the nuclei of Vero cells infected with HSV-1(HFEM). The isolation conditions required the use of a protease inhibitor and a buffer with a high ionic strength during the initial purification steps. Chromatography of a clarified nuclear extract through a molecular

sieve column equilibrated in 300 mM ammonium sulfate yielded a peak of ICP4 that was soluble at low ionic strength. Apparently, this chromatographic step separated ICP4 from the one or more factors that cause aggregation under low-salt conditions. It is not clear what these factors are, nor is it clear whether the precipitation of ICP4 in nuclear extracts prepared at low ionic strength is due to the formation of a specific high-molecular-weight complex or results from non-specific coprecipitation with numerous other insoluble nuclear proteins. We are currently investigating this phenomenon to determine what factors in nuclear extracts promote the precipitation of ICP4 at low ionic strength.

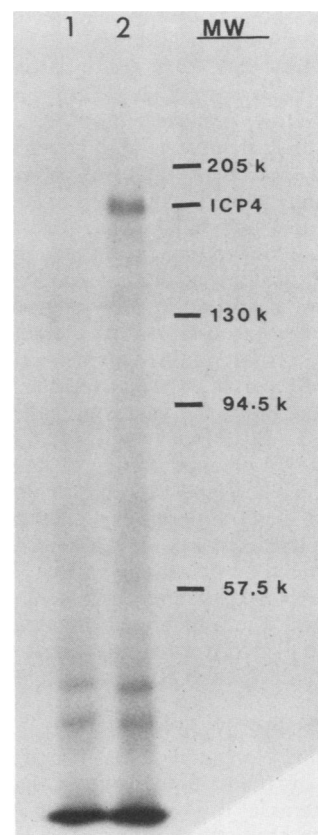Based on our measurements, the soluble, native form of

FIG. 7. Immunoprecipitation of native ICP4. A sample of iodinated fraction VII, prepared as described in the text, was mixed with 7 volumes of immunoprecipitation buffer (15 mM Tris hydrochloride [pH 7.5], 225 mM NaCl, 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 1.5 mg of bovine serum albumin per ml, 0.05 mM TPCK) and divided into two tubes. Immune IgG (containing anti-ICP4 antibody) was added to one tube and preimmune IgG was added to the other tube. After 4 h at 4°C, a suspension of protein A-bearing, formaldehyde-fixed $S. aureus$ was added to each tube. After an additional hour, the mixtures were centrifuged at 12,000 × g for 30 s. The pellets were washed three times in immunoprecipitation wash buffer (10 mM Tris hydrochloride [pH 7.2], 150 mM NaCl, 0.3% Nonidet P-40, 0.3% sodium deoxycholate). The washed pellets were suspended in DB, boiled for 30 s, and centrifuged at 12,000 × g for 30 s to pellet the bacteria. A sample of each supernatant was subjected to SDS-PAGE. Iodinated proteins were detected by exposure of the fixed, dried gel to X-ray film. Marker proteins, loaded in a separate lane, were detected by Coomassie blue staining (not shown). Lanes: 1, preimmune IgG; 2, immune IgG. MW, Molecular weight, k, thousand.

ICP4 isolated under these conditions is a 342,000-dalton complex composed of two monomers of ICP4. In this purification scheme, the use of potentially denaturing agents such as urea, chaotropic salts, and strong detergents has been purposely avoided to minimize alterations in the native structure of ICP4. The use of 300 mM ammonium sulfate in the extraction buffers was based on the use of similar conditions for the extraction of active RNA polymerase II from mouse myeloma cells (25). In the case of RNA polymerase, the use of a high salt concentration is necessary to dissociate the enzyme from a transcription complex composed of DNA, RNA, and protein (15). Given the role of ICP4 in the regulation of viral DNA transcription, it is reasonable to speculate that ICP4 is associated with a similar complex within the nucleus.

Although the extraction buffer REX-300 facilitates the dissociation of ICP4 from other nuclear components, it does not dissociate the dimeric ICP4 complex. Hence, the dimer is quite stable. However, there are apparently no covalent bonds between the two monomer components, since the addition of SDS alone is sufficient to disrupt the complex (unpublished results; also, see Fig. 5 in reference 4).

The calculated molecular weight of native ICP4 is based on empirically determined values of a and $S_{20,w}$ and an assumed value of $\bar{v}$ for ICP4. The accuracy of the two measured values is dependent on the accuracy of the published values for the corresponding standard proteins and our designation of the peak fraction containing ICP4. The error introduced by these two factors is no greater than 5%. A somewhat greater potential for error is introduced by the assumed value of $\bar{v}$ for ICP4. We used the mean value for proteins of 0.74, although the values range from 0.69 to 0.78 (27). Even if the $\bar{v}$ of ICP4 was as low as 0.69 or as high as 0.78, the calculated molecular weight of native ICP4 would fall between 287,000 and 403,000. These values also indicate that ICP4 is most likely dimeric, especially if one considers that polypeptide molecular weights determined by SDS-PAGE may be in error by as much as 15%. Thus, the close agreement between the calculated molecular weight of the native protein and the predicted molecular weight of a protein composed of two ICP4 monomers is strong evidence that the polypeptide composition of the native protein is (ICP4)$_2$.

The value of 1.88 for f/f_o of native ICP4 indicates that the protein has a highly elongated shape. If one were to assume that the shape of ICP4 approximates that of a prolate ellipsoid, then the axial ratio of the protein would be about 18 (30). Although the actual shape of native ICP4 is unknown, this high value for the axial ratio illustrates the extended configuration of native ICP4.

The composition and structure of native ICP4 should be considered in formulating a model for its biological function. For example, if the protein stimulates the transcription of delayed early genes by binding to specific sequences within the viral genome, then one might predict that the binding site would consist of a pair of sequences as a direct or indirect repeat (in keeping with the dimeric structure of the protein) separated by one or more turns of the helix (in keeping with the extended conformation of the protein). Although such a model is purely conjectural at this time, there are some data pertinent to the DNA-binding properties of ICP4. Freeman and Powell (9) reported that ICP4 binds to single-stranded DNA via interaction with an unidentified component from uninfected cells. Preliminary studies indicate that our partially purified ICP4 binds both single-stranded and double-stranded DNA. We have no evidence as to whether this DNA-binding activity of dimeric ICP4 requires one or more of the cell-encoded proteins which are also present in the partially purified preparation, nor do we have any evidence that native ICP4 binds to specific regions of the HSV genome. Studies are in progress to address these questions and to extend our investigations of the mechanism whereby ICP4 promotes the transcription of specific viral genes.

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