An Immunoassay for the Study of DNA-Binding Activities of Herpes Simplex Virus Protein ICP8

CYNTHIA K. LEE† AND DAVID M. KNIPE*

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

Received 19 November 1984/Accepted 11 March 1985

An immunoassay was used to examine the interaction between a herpes simplex virus protein, ICP8, and various types of DNA. The advantage of this assay is that the protein is not subjected to harsh purification procedures. We characterized the binding of ICP8 to both single-stranded (ss) and double-stranded (ds) DNA. ICP8 bound ss DNA fivefold more efficiently than ds DNA, and both binding activities were most efficient in 150 mM NaCl. Two lines of evidence indicate that the binding activities were not identical: (i) ds DNA failed to compete with ss DNA binding even with a large excess of ds DNA; (ii) Scatchard plots of DNA binding with various amounts of DNA were fundamentally different for ss DNA and ds DNA. However, the two activities were related in that ss DNA efficiently competed with the binding of ds DNA. We conclude that the ds DNA-binding activity of ICP8 is probably distinct from the ss DNA-binding activity. No evidence for sequence-specific ds DNA binding was obtained for either the entire herpes simplex virus genome or cloned viral sequences.

The major DNA-binding protein encoded by herpes simplex virus type 1 (HSV-1) is an approximately 130,000-dalton protein commonly known as infected-cell polypeptide 8 (ICP8). This protein is expressed as a delayed early (beta) protein. Studies with temperature-sensitive mutants of both HSV-1 and HSV-2 have addressed several aspects of the functions of ICP8 in a lytic infection. The involvement of ICP8 in viral DNA replication is suggested by the fact that mutants with temperature-sensitive lesions in this gene do not replicate their DNA at the nonpermissive temperature (8, 9, 12, 23). This suggestion was further substantiated by the finding that purified wild-type ICP8 stimulates the viral DNA polymerase activity present in extracts from cells infected with a temperature-sensitive ICP8 mutant (19). Studies in our laboratory have examined the functions of ICP8 in the infected cell. A group of mutants were identified which produced ICP8 proteins that were thermolabile for binding to replicating viral DNA in the infected cell (17). These mutants also overproduced several species of viral mRNA and the proteins encoded by them at the nonpermissive temperature (10). This overproduction was independent of DNA replication, and all three temporal classes of viral genes were affected. Taken together, these data indicate that ICP8 is a protein with multiple functions, which include an involvement in viral DNA replication and the negative regulation of viral gene expression.

Biochemical studies from two laboratories provided evidence that ICP8 possesses some of the properties of helix-destabilizing proteins (HDPs). Two well-characterized HDPs are the bacteriophage T4 gene 32 protein and the *Escherichia coli* HDP (1, 6, 30). These proteins bind cooperatively to single-stranded (ss) DNA, and their affinity for double-stranded (ds) DNA is several magnitudes below that for ss DNA. Powell et al. (23) showed that purified ICP8 lowered the melting temperature of polydeoxyadenylate-polydeoxythymidylate [poly(dA)-poly(dT)] helices. This characteristic of DNA-binding proteins is commonly attributed to the

cooperative binding of protein molecules to ss regions that occur frequently in the unstable poly(dA)-poly(dT) duplex (1, 6). Ruyechan (26) showed by electron microscopy that ICP8 bound cooperatively to circular ss DNA. In the presence of purified ICP8, the circular ss DNA molecules were either fully extended or collapsed. The fully extended molecules were believed to be saturated with ICP8, whereas the collapsed molecules were not bound by ICP8. The apparent absence of partially extended molecules indicated that the binding was cooperative. The cooperative binding and poly(dA)-poly(dT) melting activities of ICP8 are attributed to interaction with ss DNA molecules. The ICP8 protein from cell extracts binds to both ss and ds DNA-cellulose columns (3, 14, 24). Although these investigators consistently observed that ICP8 bound ss DNA-cellulose more efficiently than ds DNA-cellulose, it is difficult to assess quantitatively the differences in binding in the presence of excess amounts of DNA on the columns. This investigation was initiated because we felt that the ds DNA-binding activity warranted further study.

We used an immunoassay to assess the interaction of ICP8 with DNA. The assay is similar to the procedure of Clark et al. (7), which was modified from that of McKay (22). These immunoassays have been used to show the specific and nonspecific DNA-binding activities of the T antigen of simian virus 40 (7, 22, 28). The advantage of this assay is that it allows us to test the affinity of ICP8 for various kinds of DNA without subjecting the protein to harsh conditions that might destroy specific forms or complexes.

MATERIALS AND METHODS

Cells and viruses. Vero cell monolayers were grown in Dulbecco modified Eagle medium containing 10% viable serum protein neonatal calf serum (Biocell Laboratories, Carson, Calif.). HSV-1 strain KOS 1.1 (13) was obtained from Myron Levine, University of Michigan, Ann Arbor. The virus was grown and titrated as described previously (15).

DNA-cellulose chromatography. Cell extracts were prepared and DNA-cellulose chromatography was performed by methods similar to those described before (14). Vero cells

* Corresponding author.

† Present address: Texaco, Inc., P. O. Box 509, Beacon, NY 12508.

were infected with HSV KOS 1.1 at a multiplicity of infection (MOI) of 20. The infected cells were incubated in low-methionine medium containing 1 μ Ci of [35 S]methionine and 250 μ g of sodium phosphonoacetate (a gift of Abbott Laboratories, North Chicago, Ill.) per ml. Cells were harvested 20 h after infection, suspended in lysis buffer (0.5 M NaCl, 20 mM Tris-hydrochloride [pH 7.6], 10 mM MgCl₂, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 500 μ g of bovine serum albumin per ml), sonicated for 15 s, and incubated with 100 U of bovine pancreatic DNase I (Millipore Corp., Freehold, N.J.) per ml for 1 h at 20°C. The extracts were clarified by centrifugation (27,000 $\times$ g for 15 min), dialyzed against D buffer (150 mM NaCl, 20 mM Tris-hydrochloride [pH 7.5], 1 mM EDTA, 1 mM 2-mercaptoethanol, 10% glycerol), and clarified again.

ss DNA-cellulose (1 mg of DNA per g of cellulose) was prepared by the method of Alberts and Herrick (2). Columns containing 1 ml of ss DNA-cellulose were prepared in 5-ml plastic pipettes. Clarified cell extracts (0.5 ml) were loaded onto the column, and the column was washed with 4 ml of D buffer. Proteins bound to the column were step-eluted with D buffer containing increasing concentrations of NaCl. The fractions were dialyzed against 150 mM NaCl, precipitated with acetone, and dissolved in protein gel sample buffer. Equal portions of each fraction were analyzed by electrophoresis in polyacrylamide gels, followed by autoradiography (15).

DNA used in binding assay. HSV DNA was prepared from Vero cells infected with KOS 1.1 for 24 h (MOI of 10). To obtain 3 H-labeled HSV DNA, cells were incubated with 10 μ Ci of [3 H]thymidine per ml from 5 to 24 h postinfection. After 24 h, the cells were washed and swelled in a buffer containing 10 mM Tris-hydrochloride (pH 7.6) and 5 mM MgCl₂. The nuclear and cytoplasmic fractions were isolated by Dounce homogenization. 3 H-labeled HSV DNA was isolated from the nuclear fraction only, whereas unlabeled HSV DNA was isolated from both the nuclear and cytoplasmic fractions. The extracts were treated with 0.4% sodium dodecyl sulfate and 1 mg of pronase per ml at 37°C for 16 h. Viral DNA was separated from cellular DNA by centrifugation through NaI equilibrium density gradients. The 3 H-labeled HSV DNA used in this study had a specific activity of 2.8×10^5 cpm/ μ g. Calf thymus DNA (Sigma Chemical Co., St. Louis, Mo.) was purified by sequential extractions with phenol, chloroform, and ethyl ether.

The HSV and calf thymus DNAs were digested with restriction enzymes to appropriate size fragments before binding. HSV DNA was digested with *Alu*I, and calf thymus DNA was digested with *Hae*III to produce blunt-ended fragments ranging from 70 to 2,600 base pairs. The digested DNAs were purified by phenol extraction, and the phenol was removed by two extractions with chloroform-isoamyl alcohol (24:1), followed by two extractions with water-saturated ethyl ether. The residual ether was evaporated by heating at 68°C for 10 min. Omission of either extraction resulted in interference with DNA binding, probably because of residual phenol in the DNA preparations. HSV DNA was denatured by boiling for 5 min.

Polydeoxynucleotides were purchased from P-L Biochemicals, Milwaukee, Wis. The chain length of poly(dT) was 1,000 nucleotides, and the chain length of poly(dA)-poly(dT) was 450 nucleotides before annealing.

Preparations of plasmid pSG18 were purified on CsCl density gradients (20). The plasmid was digested with *Bam*HI, *Bst*EII, and *Eco*RI, and the recessed 3' ends created by the restriction enzymes were labeled with [32 P]dCTP, unlabeled

dATP, dTTP, and dGTP, and the Klenow fragment of *Escherichia coli* DNA polymerase I (20). An excess of unlabeled nucleotides was then added to ensure that the ends were completely filled in. The labeled DNA was purified by chromatography through Sephadex G50-150 (Sigma).

Immunoassay for DNA binding. Vero cells were infected with HSV KOS 1.1 (MOI of 20) for 6 h, and the infected-cell pellets were frozen at -80°C. Immediately before use, the pellets were thawed in immunoprecipitation buffer (10 mM Tris-hydrochloride [pH 7.6], 150 mM NaCl, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride) at a concentration of 10^7 cells per ml, sonicated in 15 1-s pulses, and clarified by centrifugation at 12,800 $\times$ g at 4°C for 15 min. A 5- μ l volume of monoclonal antibody 39S ascites fluid was incubated with 50 μ l of clarified cell extract for 30 min on ice. A 25- μ l volume of a 10% suspension of formaldehyde-fixed *Staphylococcus aureus* cells (IgG-sorb; Enzyme Center Inc., Boston, Mass.) was added, and incubation was continued for 15 min on ice. The immune complexes were pelleted by centrifugation at 12,800 $\times$ g at 4°C for 0.5 min, washed twice with 100 μ l of wash buffer (50 mM Tris-hydrochloride [pH 7.6], 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride) and suspended in 50 μ l of binding buffer (10 mM Tris-hydrochloride [pH 7.6], 150 mM NaCl, 1 mM MgCl₂, 1.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). The appropriate DNA was added, and binding was allowed to proceed on ice for the times given. The *S. aureus* cells were washed twice with 100 μ l of binding buffer, and the associated DNA was released by incubation in dissociation buffer (20 mM Tris-hydrochloride [pH 7.6], 1% sodium dodecyl sulfate) for 30 min at 20°C. The amount of labeled DNA in each fraction was determined by liquid scintillation counting after the addition of 20 to 40 volumes of aqueous scintillation fluid (Ultrafluor; National Diagnostics, Somerville, N.J.).

Immune complexes prepared from 10^6 cells contained 0.7 μ g of ICP8, as estimated by staining of the eluted polypeptides on polyacrylamide gels (data not shown). The complexes from a similar number of cells contained ca. 6 ng of endogenous DNA, as estimated by ethidium bromide staining (data not shown). Because the binding of ss and ds DNAs continued to be linear for amounts many times above the endogenous level, it seems unlikely that the endogenous DNA affected the binding reactions except as described below.

RESULTS

DNA-binding assay for ICP8. As an initial step in developing our immunoassay for DNA binding, we determined whether the binding of monoclonal antibody 39S to ICP8 would interfere with the ability of ICP8 to bind to ss DNA-cellulose columns. One-tenth volume of ascites fluid containing 39S antibody was sufficient to react with more than 90% of the ICP8 in infected-cell extracts (Fig. 1, lanes 1 and 2). The ICP8 protein from cell extracts preincubated with 39S antibody bound to ss DNA-cellulose columns as efficiently as ICP8 from untreated-cell extracts (Fig. 1, lanes 8 through 12). We concluded from this experiment that 39S antibody does not block the DNA-binding site of ICP8.

To study the DNA-binding activities of ICP8, we used 39S antibody in a one-step purification procedure to adsorb ICP8 onto formaldehyde-fixed *S. aureus* cells. We will refer to the *S. aureus* cells with bound antibody and bound ICP8 as immune complexes. The immune complexes were washed

FIG. 1. Binding of ICP8 to DNA-cellulose in the presence of monoclonal antibody 39S. One-tenth volume of ascites fluid containing 39S antibody was added to ^{35}S -labeled cell extract and incubated for 60 min at 4°C . A portion of this material was incubated with protein A-Sepharose (Sigma) for 60 min at 4°C , and proteins from the supernatant (lane 1) and unwashed Sepharose beads (lane 2) were analyzed in a 9.25% polyacrylamide gel. Proteins from samples separated by column chromatography (lanes 3 through 12) were resolved in a 6.6% polyacrylamide gel. The other lanes contain chromatography fractions from a cell extract that was preincubated with 39S antibody (lanes 8 through 12) or not so treated (lanes 3 through 7). Lanes: 3 and 8, 0.15 M NaCl flowthrough; 4 and 9, 0.3 M NaCl eluate; 5 and 10, 0.5 M NaCl eluate; 6 and 11, 1.0 M NaCl eluate; 7 and 12, 5.0 M NaCl eluate. ○, ICP8. Molecular weight (indicated in thousands) markers were beta-galactosidase (117,000) and bovine serum albumin (68,000).

and suspended in appropriate buffers, and the association of radioisotope-labeled DNAs was measured.

Requirements for DNA binding. The binding of ss and ds DNA to the immune complexes was directly proportional to the amount of ICP8 present (Fig. 2). ss DNA was bound ca. fivefold more efficiently than ds DNA. At the highest concentration of cell extract used (Fig. 2), 67% of the ss DNA and 14% of the ds DNA were associated with the immune complexes. When either infected-cell extract or 39S antibody was omitted from the reaction mixture, only 0.3 to 1.9% of either DNA was associated with the immune complexes. Therefore, the association of DNA with the immune complexes was dependent on the presence of both infected-cell extracts and 39S antibody. Because ICP8 was the major protein precipitated from infected-cell extracts with 39S (Fig. 3), it was likely that the associated DNA represented DNA bound by ICP8. To determine whether other minor viral or cellular proteins recognized by 39S antibody may have been responsible for the DNA-binding activities, the following experiment was performed. Extracts prepared from cells infected with the HSV ICP8 mutant KOS 1.1 *ts*18 (12, 17) at the nonpermissive temperature were used in the immunoassay. Less than 10% of the mutant protein was

immunoprecipitated with 39S antibody, whereas 5 and 2.5% of the ss and ds DNAs, respectively, became associated with the immune complexes. Therefore, DNA binding to the complexes required ICP8, and we conclude that the DNA associated with the immune complexes was bound to ICP8.

Binding of ds and ss DNA was nearly maximal within approximately 20 min (Fig. 4). Optimal binding was achieved in 150 mM NaCl, and binding was abolished by 300 mM NaCl (Fig. 5), although the association of ICP8 with the immune complexes remained stable at NaCl concentrations up to and including 1 M (data not shown). The salt sensitivity of DNA binding in this immunoassay was similar to that observed for the binding of ICP8 to DNA-cellulose columns (Fig. 1). Based on the above parameters, our routine binding assay was carried out in a solution containing 150 mM NaCl for 60 min.

Saturation of DNA-binding sites. The binding of various amounts of DNA by a constant amount of protein was measured, and the absolute amount of DNA bound was plotted (Fig. 6). When ss DNA binding was analyzed, the amount of bound DNA initially increased proportionally to the amount of DNA available for binding. At DNA concen-

FIG. 2. Degree of binding of ss (Δ) and ds (○) DNA to immune complexes is dependent on the amount of cell extract. Various amounts of cell extract were adsorbed onto *S. aureus* cells. Binding of 10 ng of ^3H -labeled HSV DNA after a 60-min incubation was measured.

trations of greater than 100 ng per reaction, the amount of bound DNA increased more slowly until saturation was achieved at ca. 300 ng of DNA per reaction. At saturation, the ICP8-to-DNA weight ratio was approximately 2:1.

The binding of ds DNA by ICP8 also increased proportionally to the amount of DNA present at DNA concentrations of less than 100 ng per reaction. At higher DNA concentrations, the percentage of bound DNA decreased when the reaction approached saturation at 900 ng of DNA per reaction (ICP8-DNA weight ratio of ca. 1:1.4).

The data shown in Fig. 6 were also analyzed by standard Scatchard plots, assuming DNA to be the ligand and the immune complexes to be the receptor. The binding curves for ss and ds DNA were very different. The binding curve for ss DNA (Fig. 7A) was a concave downwards curve, which is characteristic of positive cooperation (5, 16), whereas the binding curve for ds DNA (Fig. 7B) was a concave upwards curve, which reflects either negative cooperation or multiple binding sites of differing affinity (5). Because the curves were nonlinear and because both the DNA molecules (21) and the immune complexes potentially have multiple sites for interaction, we feel that meaningful values for the dissociation constants or the number of nucleotides bound cannot be

FIG. 3. HSV proteins associated with immune complexes used for DNA binding. Vero cells were infected with KOS 1.1 (MOI of 20) for 6 h in medium containing 6 μ Ci of [35 S]methionine per ml. Cell extracts were prepared, and proteins were adsorbed onto *S. aureus* cells with 39S antibody as described in the text. Equivalent portions of the supernatant fraction from the immunoadsorption (lane 1), the combined washes of the immune complexes (lane 2), and the dissociated fraction from the immune complexes (lane 3) were loaded onto a 9.25% polyacrylamide gel, and the autoradiogram of the resulting gel is shown.

FIG. 4. Kinetics of DNA binding. Binding of 10 ng of either ss (O) or ds (Δ) 3 H-labeled HSV DNA was measured after various incubation times. The reaction was stopped by pelleting the immune complexes by centrifugation.

determined from these data. The curves show clearly the difference in the nature of the binding of the two kinds of DNA to the immune complexes.

Competition of DNA binding. To examine further the relationship between the ss and ds DNA-binding activities of ICP8, we performed competition experiments. In these

FIG. 5. Effect of NaCl on DNA binding. Binding of 10 ng of ss (O) or ds (Δ) 3 H-labeled HSV DNA was measured after 60 min on ice. The binding buffers contained various concentrations of NaCl.

experiments, the ratio of protein to ^3H -labeled DNA was approximately equal to that at the lowest points of the saturation curves shown in Fig. 6 (10 ng), and cold competing DNA was added in increasing amounts until they were well beyond those in the plateau regions of the curves. The binding of ^3H -labeled ss HSV DNA was efficiently competed by cold ss DNA that was either homologous (HSV) or heterologous [poly(dT)] (Fig. 8). Cold ds HSV DNA and poly(dA)-poly(dT) duplexes did not compete with ss DNA binding even when present in 100-fold excess, suggesting that the ds DNA-binding activity does not reflect binding to ss regions within ds DNA.

The binding of ^3H -labeled ds HSV DNA was easily competed by the ss DNA poly(dT) (Fig. 9). Other ss DNAs, such as those from HSV and calf thymus, competed to a similar extent (data not shown). The homologous ds DNA (nuclear HSV DNA) competed as well as ss DNA up to a 10-fold excess of cold DNA. At higher concentrations of cold DNA, ds DNA competed less efficiently than ss DNA. ds calf thymus DNA competed as well as nuclear HSV DNA, but HSV DNA isolated from the cytoplasm competed less efficiently. The ability of ss DNA to compete efficiently with the binding of ds DNA suggested that the binding sites for the two types of DNAs either overlap or demonstrate an allosteric interaction.

Binding of ICP8 to HSV and bacterial DNA sequences. The biphasic Scatchard plot obtained for the binding of ds DNA

FIG. 6. Saturation of DNA-binding sites. The binding of various amounts of ss (○) or ds (△) DNA to immune complexes formed with a fixed amount of infected-cell extract was measured after 60 min. The cell extract in each reaction was derived from 9×10^5 infected cells.

FIG. 7. Scatchard plot analysis of ss (A) and ds (B) DNA binding. The data shown in Fig. 6 were analyzed in Scatchard plots. The amount of labeled DNA bound was plotted on the abscissa, and the ratio of bound and free counts per minute was plotted on the ordinate.

(Fig. 7B) suggested that ICP8 molecules have two independent classes of binding sites for ds DNA. We wanted to determine whether the higher-affinity binding reaction which occurred at low DNA concentrations represented binding to specific DNA sequences. Therefore, binding reactions were carried out at a ratio of protein to DNA similar to that at the lowest point of the saturation curve shown in Fig. 6 (10 ng). Initially, we examined the binding of ICP8 to *Bgl*II-digested HSV DNA. Before binding, the ends of the 16 restriction

FIG. 8. Competition of ss DNA binding. Various amounts of cold DNA and 10 ng of ss ^3H -labeled HSV DNA were added at the same time to immune complexes prepared from 9×10^5 infected cells. Binding was measured after 60 min. Cold competing DNAs were poly(dT) (○), ss HSV DNA (□), ds HSV DNA (△), and poly(dA)-poly(dT) (▽). The poly(dA)-poly(dT) homopolymers were reannealed for 5 min at 40°C and cooled on ice before addition.

fragments (5 to 36 kilobases) were filled with a combination of ^{32}P -labeled and cold nucleotides. When the bound and unbound DNAs were analyzed by electrophoresis through agarose gels, we found that all 16 fragments were bound equally by ICP8 (data not shown).

A possibility remained that the putative specific sequence may be common to all the *Bgl*II fragments. Therefore, we tested the binding of ICP8 to smaller DNA fragments. As substrate we used plasmid pSG18 (11), which consists of the bacterial plasmid pBR325 with an insert derived from the 16-kilobase *Eco*RI F fragment of HSV DNA (0.314 to 0.420 map units). This plasmid contains sequences required for the expression of ICP8, as determined by transient expression (25). Because ICP8 has been shown to affect its own expression (10), we wanted to determine whether it might bind specifically to sequences around the ICP8 gene. A triple restriction endonuclease digest (*Eco*RI, *Bam*HI, and *Bst*EII) of the plasmid produced fragments ranging from 400 to 4,000 base pairs. ICP8 bound slightly better to the two pBR325 fragments than to the HSV DNA fragments (Fig. 10). With the exception of the two lower-molecular-weight fragments (400 and 460 base pairs), all the viral fragments were bound equally. The increased affinity for the two bacterial DNA fragments may be due to a preference for adenine-plus-thymine-rich sequences.

DISCUSSION

We describe here a simple assay for the binding of ICP8 to various DNAs. ICP8 molecules from infected-cell extracts were adsorbed onto a solid matrix by using a monoclonal antibody which did not inhibit DNA binding, and the subsequent association of several different types of DNA to this immune complex was measured. This procedure was similar

to an assay described by Clark et al. to show the specific binding of simian virus 40 T antigen to the viral origin of replication (7). In addition to its simplicity, this immunoassay allows us to preserve the integrity of the protein and the DNA. The DNAs used as substrate have not been subjected to harsh procedures, such as those used for coupling DNA onto cellulose matrixes, and therefore they possess more predictable secondary structures. The ICP8 used for the reaction was directly immunoprecipitated from sonicated cell extracts. High-salt-concentration treatments used in protein purification procedures were avoided in this study because we were interested in preserving any multimeric forms, complexes, or labile conformations that may be required for particular interactions of ICP8 with DNA.

ss DNA-binding activity. Theoretically, cooperative binding of protein molecules onto DNA is required if the DNA molecule is to be saturated with protein (29). One of the functions of ss DNA-binding proteins, such as the gene 32 protein of T4 and the HDP of *E. coli*, is to protect ss DNA from nuclease digestion. Therefore, it is important for these proteins to bind ss DNA without leaving gaps. This is accomplished by the ability to bind cooperatively to ss DNA.

In this study, we found that a Scatchard plot analysis of the binding of various amounts of ss DNA by a constant amount of protein produced a concave downwards curve (Fig. 7A) which resembled that predicted for positive cooperative binding (5, 16). Because we were measuring the binding of various amounts of DNA, the positive cooperativity observed here represents the cooperative binding of DNA molecules by ICP8. This is in contrast to the positive cooperation described by Ruyechan (26), which represents

FIG. 9. Competition of ds DNA binding. Various amounts of cold DNA and 10 ng of ds ^3H -labeled HSV nuclear DNA were added at the same time to immune complexes prepared from 9×10^5 cells. Binding was measured after 60 min. The cold competing DNAs were ds nuclear HSV DNA (○), ds cytoplasmic HSV DNA (△), ds calf thymus DNA (□), and poly(dT) (◇).

FIG. 10. Binding of pSG18 DNA sequences by ICP8. The binding of 20 ng of 32 P-labeled DNA (pSG18 digested with *Eco*RI, *Bam*HI, and *Bst*EII) to cell extract from 2.5×10^6 cells was measured after 60 min. The bound and unbound fractions were extracted with phenol, and the DNA was analyzed by electrophoresis in a 1.5% neutral agarose gel. The gel was fixed in 95% ethanol for 30 min and dried, and an autoradiogram of the gel was obtained on Kodak XRP-1 film with two Cronex Lightning-Plus intensifying screens (E. I. duPont de Nemours & Co., Inc., Wilmington, Del.). Lanes: 1, 2 ng of DNA; 2 through 4, DNA from immunoassay without cell extract; 5 through 7, DNA from immunoassay containing cell extract; 2 and 5, unbound DNA; 3 and 6, DNA in washes; 4 and 7, bound DNA. Lane 2 was loaded with an amount representing 25% of the total sample; lanes 3 through 7 were loaded with amounts representing 40% of the total sample. The lengths (in base pairs) of ϕ X174 DNA digested with *Hae*III are shown. ○, pBR325 fragments.

the cooperative binding of several ICP8 molecules onto a single DNA molecule, presumably through protein-protein interactions. If protein-protein interactions are invoked to explain the cooperative binding of DNA molecules to ICP8 in our immunoassay, we have to postulate that the ICP8 molecules immobilized on the *S. aureus* cells are still free to interact with each other, and that this interaction would allow a second molecule of DNA to become bound. Two alternative explanations which do not invoke protein-protein interactions could be proposed.

The first explanation is that the binding of ICP8 to a DNA molecule enhanced the ability of that DNA molecule to form base pairs with its complementary strand. This would result in both strands' becoming associated with the immune complex. At higher DNA concentrations, a DNA network may be formed. There is evidence that both the *E. coli* HDP protein and the T4 gene 32 protein catalyze the renaturation of ss cDNAs (1, 6). These proteins bind the sugar-phosphate backbone of ss DNA and unstack the bases (29). This extends the ss molecules and promotes base pair formation between complementary strands. The ability of ICP8 to promote renaturation of ss DNA has not been tested.

The second explanation is that the observed cooperativity of ss DNA binding could be due to multiple DNA-binding

sites on the ICP8 molecule. In this case, the binding of DNA to one site would cause an allosteric effect and activate the other binding sites. Our data are consistent with positive cooperativity. Although a typical curve for multiple binding sites was not obtained, this possibility cannot be ruled out.

ds DNA-binding activity. We have two lines of evidence which indicate that the binding of ICP8 to ds DNA is a separate activity from ss DNA binding. The first line of evidence is the lack of competition of ss DNA binding by ds DNA. If the binding to ds DNA represents binding to ss regions contained within that DNA, we would expect an excess of ds DNA to compete with the binding of ss DNA. We failed to find competition for ss DNA binding with a 100-fold excess of ds DNA (Fig. 8). This amount of ds DNA was sufficient to saturate the binding sites of the ICP8 molecules in a reaction in which no ss DNA was present (Fig. 6). Therefore, we conclude that the ds DNA-binding activity of ICP8 does not consist of binding to ss regions within ds DNA in a manner identical to that for ss DNA. The lack of competition by ds DNA probably reflects a lower affinity for ds DNA than for ss DNA, resulting in the binding of the relatively small amount of ss DNA in the reaction. Similar competition binding results with purified ICP8 have been published by Ruyechan and Weir (27).

The second line of evidence is the fundamentally different Scatchard plots obtained for the two binding reactions. A biphasic or concave upwards curve obtained for ds DNA binding (Fig. 7B) is predicted for either negative cooperative binding or multiple independent binding classes. In the absence of other evidence for negative cooperation, it is more likely that the curve represents multiple binding classes (5). We can rule out the possibility that the multiple classes represent sites for ss versus ds DNA. The possibility remains that the multiple classes represent different affinities for specific DNA sequences. Specific sequence recognition has not been observed, but it is conceivable that the small amount of endogenous DNA blocks a sequence-specific binding activity. We observed that ds HSV DNA isolated from the infected-cell cytoplasm did not compete as well as other DNAs for binding of nuclear HSV DNA by ICP8 (Fig. 9). The significance of this observation is yet to be determined.

Correlation of in vivo and in vitro DNA-binding properties. Previous in vitro studies on the interaction between ICP8 and DNA have focused on the ss DNA-binding activity (19, 23, 26). In addition to this ss DNA-binding activity, we have characterized the binding of ICP8 to ds DNA in vitro by an immunoassay. Two previous studies have suggested that ICP8 is bound to ds DNA in the infected cell. We found that at early times after the initiation of viral DNA replication (6 h postinfection at an MOI of 20), immunoprecipitation of ICP8 from infected-cell extracts also brought down virus-specific DNA (17). This DNA was susceptible to restriction endonuclease digestion and subsequently migrated as ds molecules in agarose gels. Leinbach and Casto (18) isolated ICP8-containing deoxyribonucleoprotein complexes by sucrose gradient sedimentation from nuclear extracts of cells infected with HSV-1. Analyses of the DNA in cesium chloride gradients showed that both the cellular and viral DNAs associated with these complexes were predominantly ds DNA, and there was no evidence for the presence of ss DNA. It is possible that, in both studies, ss DNA bound by ICP8 reannealed during extraction. Therefore, although these data suggest that ICP8 is bound to ds DNA in the infected cell, it has not been proven.

The function of the ds and ss DNA-binding activities of

ICP8 in the life cycle of the virus are not known. It is possible that the ss DNA-binding activity is required for protecting and imparting rigidity to ss DNA present at replication forks, whereas the ds DNA-binding activity is required for binding progeny DNA and protecting it from nuclease attack until encapsidation occurs, as proposed for the pseudorabies virus DNA-binding protein (4). The relationships of these DNA-binding activities to viral DNA replication and gene expression remain important questions in the study of this multifunctional protein.

We conclude that ICP8 has at least two DNA-binding activities, one for ds DNA and a second for ss DNA. The binding sites for these two types of DNA either overlap or demonstrate an allosteric interaction. The immunoassay used in this study may be used to test other monoclonal antibodies that might inhibit all or some of the DNA-binding activities of ICP8. Conditional lethal mutants may also be tested to identify mutant proteins that possess only a subset of the activities.

ACKNOWLEDGMENTS

We are most grateful to Martin Zweig for providing us with monoclonal antibody 39S. We thank M. L. Levin and Janet L. Smith for technical assistance.

This work was supported by Public Health Service grant CA26345 from the National Cancer Institute. C.K.L. was supported by the Damon Runyon-Walter Winchell Cancer Fund and Public Health Service training grant T32CA09031. D.M.K. was supported by a Faculty Research Award from the American Cancer Society.

LITERATURE CITED

1. Alberts, B., and L. Frey. 1970. T4 bacteriophage gene 32: a structural protein in the replication and recombination of DNA. *Nature* (London) **227**:1313-1318.
2. Alberts, B., and G. Herrick. 1971. DNA-cellulose chromatography. *Methods Enzymol.* **21D**:198-217.
3. Bayliss, G. J., H. S. Marsden, and J. Hay. 1975. Herpes simplex virus proteins: DNA-binding proteins in infected cells and in the virus structure. *Virology* **68**:124-134.
4. Ben-Porat, T., R. Veatch, and H. Hampl. 1983. Functions of the major nonstructural DNA-binding protein of a herpesvirus (pseudorabies). *Virology* **124**:411-424.
5. Cantor, C. R., and P. R. Schimmel. 1980. The behavior of biological macromolecules. *Biophys. Chem.* **3**:852-873.
6. Christiansen, C., and R. L. Baldwin. 1977. Catalysis of DNA reassociation by the *Escherichia coli* DNA-binding protein. A polyamine-dependent reaction. *J. Mol. Biol.* **115**:441-454.
7. Clark, R., K. Peden, J. M. Pipas, D. Nathans, and R. Tjian. 1983. Biochemical activities of T-antigen proteins encoded by simian virus 40 A gene deletion mutants. *Mol. Cell. Biol.* **3**:220-228.
8. Conley, A. J., D. M. Knipe, P. C. Jones, and B. Roizman. 1981. Molecular genetics of herpes simplex virus. VII. Characterization of a temperature-sensitive mutant produced by in vitro mutagenesis and defective in DNA synthesis and accumulation of γ polypeptides. *J. Virol.* **37**:191-206.
9. Dixon, R. A. F., D. J. Sabourin, and P. A. Schaffer. 1983. Genetic analysis of temperature-sensitive mutants which define the genes for the major herpes simplex virus 2 DNA-binding protein and a new late function. *J. Virol.* **45**:343-353.
10. Godowski, P. J., and D. M. Knipe. 1983. Mutations in the major DNA-binding protein gene of herpes simplex virus type 1 result in increased levels of viral gene expression. *J. Virol.* **47**:478-486.
11. Goldin, A. L., R. M. Sandri-Goldin, M. Levine, and J. C. Glorioso. 1981. Cloning of herpes simplex virus type 1 sequences representing the whole genome. *J. Virol.* **38**:50-58.
12. Holland, L. E., R. M. Sandri-Goldin, A. L. Goldin, J. C. Glorioso, and M. Levine. 1984. Transcriptional and genetic analyses of the herpes simplex virus type 1 genome: coordinates 0.29 to 0.45. *J. Virol.* **49**:947-959.
13. Hughes, R. G., Jr., and W. H. Munyon. 1975. Temperature-sensitive mutants of herpes simplex virus type 1 defective in lysis but not in transformation. *J. Virol.* **16**:275-283.
14. Knipe, D. M., M. P. Quinlan, and A. E. Spang. 1982. Characterization of two conformational forms of the major DNA-binding protein encoded by herpes simplex virus 1. *J. Virol.* **44**:736-741.
15. Knipe, D. M., and A. E. Spang. 1982. Definition of a series of stages in the association of two herpesviral proteins with the cell nucleus. *J. Virol.* **43**:314-324.
16. Koshland, D. E., Jr. 1970. The molecular basis for enzyme regulation. In P. D. Boyer (ed.), *The enzymes*. Academic Press, Inc., New York.
17. Lee, C. K., and D. M. Knipe. 1983. Thermolabile in vivo DNA-binding activity associated with a protein encoded by mutants of herpes simplex virus type 1. *J. Virol.* **46**:909-919.
18. Leinbach, S. S., and J. F. Casto. 1983. Identification and characterization of deoxyribonucleoprotein complexes containing the major DNA-binding protein of herpes simplex virus 1. *Virology* **131**:274-286.
19. Littler, E., D. Purifoy, A. Minson, and K. L. Powell. 1983. Herpes simplex virus non-structural proteins. III. Functions of the major DNA-binding protein. *J. Gen. Virol.* **64**:983-995.
20. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. McGhee, J. D., and P. H. von Hippel. 1974. Theoretical aspects of DNA-protein interactions: co-operative and non-co-operative binding of large ligands to a one-dimensional homogeneous lattice. *J. Mol. Biol.* **86**:469-489.
22. McKay, R. D. G. 1981. Binding of a simian virus 40 T-antigen related protein to DNA. *J. Mol. Biol.* **145**:471-488.
23. Powell, K. L., E. Littler, and D. J. M. Purifoy. 1981. Nonstructural proteins of herpes simplex virus. II. Major virus-specific DNA-binding protein. *J. Virol.* **39**:894-902.
24. Purifoy, D. J. M., and K. L. Powell. 1976. DNA-binding proteins induced by herpes simplex virus type 2 in HEP-2 cells. *J. Virol.* **19**:717-731.
25. Quinlan, M. P., L. B. Chen, and D. M. Knipe. 1984. The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. *Cell* **36**:857-868.
26. Ruyechan, W. T. 1983. The major herpes simplex virus DNA-binding protein holds single-stranded DNA in an extended configuration. *J. Virol.* **46**:661-666.
27. Ruyechan, W. T., and A. C. Weir. 1984. Interaction with nucleic acids and stimulation of the viral DNA polymerase by the herpes simplex virus type 1 major DNA-binding protein. *J. Virol.* **52**:727-733.
28. Scheller, A., L. Covey, B. Barnett, and C. Prives. 1982. A small subclass of SV40 T antigen binds to the viral origin of replication. *Cell* **29**:375-383.
29. Von Hippel, P. H., D. E. Jensen, R. C. Kelley, and J. D. McGhee. 1977. Molecular approaches to the interaction of nucleic acids with "melting" proteins, p. 65-90. In H. J. Vogel (ed.), *Nucleic acid-protein recognition*. Academic Press, Inc., New York.
30. Weiner, J. H., L. L. Bertsch, and A. Kornberg. 1975. The deoxyribonucleic acid unwinding protein of *Escherichia coli*. Properties and functions in replication. *J. Biol. Chem.* **250**:1972-1980.