JOURNAL OF VIROLOGY, Mar. 1988, p. 810–817
0022-538X/88/030810-08$02.00/0

Vol. 62, No. 3

N-Ethylmaleimide Inhibition of the DNA-Binding Activity of the Herpes Simplex Virus Type 1 Major DNA-Binding Protein

WILLIAM T. RUYECHAN

Department of Biochemistry, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814-4799

Received 20 July 1987/Accepted 24 November 1987

The major herpes simplex virus DNA-binding protein, designated ICP8, binds tightly to single-stranded DNA and is required for replication of viral DNA. The sensitivity of the DNA-binding activity of ICP8 to the action of the sulfhydryl reagent *N*-ethylmaleimide has been examined by using nitrocellulose filter-binding and agarose gel electrophoresis assays. Incubation of ICP8 with *N*-ethylmaleimide results in a rapid loss of DNA-binding activity. Preincubation of ICP8 with single-stranded DNA markedly inhibits this loss of binding activity. These results imply that a free sulfhydryl group is involved in the interaction of ICP8 with single-stranded DNA and that this sulfhydryl group becomes less accessible to the environment upon binding. Agarose gel electrophoretic analysis of the binding interaction in the presence and absence of *N*-ethylmaleimide indicates that the cooperative binding exhibited by ICP8 is lost upon treatment with this reagent but that some residual noncooperative binding may remain. This last result was confirmed by equilibrium dialysis experiments with the ^{32}P-labeled oligonucleotide dT_{10} and native and *N*-ethylmaleimide-treated ICP8.

The major herpes simplex virus type 1 (HSV-1) DNA-binding protein, ICP8, is essential for viral DNA replication (5, 18, 20, 35) and also appears to be involved in the regulation of expression of several other viral proteins (11). The gene coding for ICP8 maps in the center of the long unique (U_L) region of the HSV genome opposite the gene for the HSV DNA polymerase and flanking a viral DNA replication origin (3, 5, 26, 35). Quinn and McGeoch (26) have determined the complete nucleotide sequence of the ICP8 gene. The predicted amino acid sequence indicates that the protein is composed of 1,196 amino acids and has a molecular weight of 128,341. This estimate is in excellent agreement with earlier work, which determined the molecular weight of ICP8 to be 125,000 to 130,000 (13, 16, 24, 25, 27). ICP8 contains 124 acidic and 110 basic residues, indicating that it is slightly acidic at or near neutral pH. The presence of 8 tryptophan residues and 27 tyrosine residues suggests that the protein should have a finite fluorescence emission spectrum, and it has been confirmed that ICP8 has a fluorescence emission maximum at approximately 340 nm (W. T. Ruyechan, Fed. Proc. **42**:7, 1983; W. Ruyechan, unpublished data). ICP8 also contains 22 cysteine residues which make up 1.8% of its total composition. Knipe et al. (16) showed that the existence of two conformational forms of ICP8 is due to the presence of intramolecular disulfide bonds.

The interaction of ICP8 with nucleic acids has been the subject of considerable study. ICP8 binds tightly and preferentially to single-stranded DNA with no apparent specificity as to either the sequence or base composition of the DNA (19, 20, 24, 29). ICP8 also binds to duplex DNA and polyriboadenylic acid but with a lesser affinity than that seen with single-stranded DNA as determined either by differential salt elution from DNA-cellulose columns or by competitive filter-binding assays (19, 25, 27). The interaction of ICP8 with single-stranded DNA is cooperative as determined by electron microscopy and Scatchard analysis (19, 27, 28). ICP8 also has the ability to melt duplexes of complementary homopolymers (24), a property which may be related to the increased activity of the HSV DNA polymerase seen in its presence (23, 29).

Vaughn et al. (33), using monoclonal antibodies, have shown that ICP8 appears to be closely associated with several other virus-specified proteins including the viral DNA polymerase and alkaline exonuclease. Earlier work by Littler et al. (20) showed that DNA replication in chromatin isolated from cells infected with a temperature-sensitive mutant of ICP8 ceased upon incubation at the nonpermissive temperature and that levels of DNA synthesis approaching those seen at the permissive temperature could only be achieved by adding back both purified ICP8 and HSV DNA polymerase. Thus ICP8 may be part of a multicomponent replication complex similar to that seen with bacteriophage T4 (1). In support of this idea recent work by Challberg (3) indicates the presence of seven genes which are essential for replication of HSV DNA, two of which are ICP8 and the viral DNA polymerase.

In this article we report on a series of studies aimed at determining whether sulfhydryl groups are involved in the interaction of ICP8 with single-stranded DNA. ICP8 isolated from HeLa cells infected with HSV-1 strain mP (12) was modified by reaction with the sulfhydryl reagent *N*-ethylmaleimide (NEM), and the DNA-binding activities of unmodified and modified protein were compared. The techniques used included nitrocellulose filter-binding assays and an agarose gel electrophoresis assay which can be used to differentiate between cooperative and noncooperative binding. Our results indicate that reaction of ICP8 with NEM results in a significant inhibition of this protein's cooperative binding activity.

MATERIALS AND METHODS

Cells and viruses. Stocks of HSV-1 strain microplaque (mP) were propagated and titered in Vero cell monolayers. An initial stock of the virus was provided by David Knipe. HeLa cells (S600) were obtained from James Reminick (Department of Microbiology, Uniformed Services University of the Health Sciences) and were propagated in suspension culture at 35°C in S-MEM medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum.

Enzymes and reagents. Pancreatic DNase and RNase were

purchased from Sigma Chemical Co., St Louis, Mo. Phosphocellulose powder (P11) was purchased from Whatman, Inc., Clifton, N.J. Heparin Sepharose was purchased from Pharmacia Inc., Piscataway, N.J. Single-stranded DNA agarose was purchased from Bethesda Research Laboratories, Bethesda, Md. Agarose was purchased from FMC Corp. Rockland, Maine. HSV-1 DNA was isolated by the method of Walboomers and ter Schegget (34). Single-stranded M13 DNA was purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Bacteriophage fd DNA and T4 gene 32 protein were gifts of Lucy M. S. Chang, and 3′ ^{32}P-end-labeled dT_{10} was the gift of Samantha Li.

Purification of ICP8. The procedure for the isolation of ICP8 used in this study is a modification of that previously described in publications from this laboratory (27, 28, 29). The modifications were made to increase the overall amount of ICP8 which could be isolated during a given preparation. The scheme for purification of the protein was as follows. Four liters of HeLa cell suspension cultures at a density of 10^6 cells per ml was centrifuged at low speed, and the resulting pellet was suspended in 100 ml of S-MEM supplemented with 5% fetal calf serum. The suspended cells were inoculated with HSV strain mP at a multiplicity of infection of 10 to 20. The virus was adsorbed in suspension for 1 h. The cells were then pelleted once again at low speed, the supernatant was discarded, and 500 ml of fresh S-MEM supplemented with 10% fetal calf serum was added. The suspended cells were maintained in spinner culture for 6 to 8 h postinfection and then harvested by pelleting at 500 × *g*. The conditions of infection are essentially those described by Derse et al. (8). The cells were washed twice with ice-cold phosphate-buffered saline, flash frozen in an ethanol-dry ice bath, and stored at −70°C.

The frozen cell pellets were thawed and suspended in 100 ml of lysis buffer (1.7 M KCl, 50 mM Tris hydrochloride [pH 7.5], 5 mM EDTA, 0.5 mM dithiothreitol, and 20 μg of phenylmethylsulfonyl fluoride per ml). The cells were then lysed by sonication as previously described (29). Cell debris was removed from the resulting slurry by centrifugation at 5,000 × *g*. The supernatant was collected, and to it were added 50 ml of the lysis buffer containing 30% (wt/wt) polyethylene glycol. The solution was stirred gently and then incubated on ice for 1 h to allow precipitation of nucleic acids. This precipitate was removed by a second low-speed centrifugation. The supernatant was collected and diluted 1:1 with lysis buffer containing no KCl and supplemented with 20% glycerol and 0.4% Nonidet P-40. This solution was then dialyzed overnight against several 4-liter changes of lysis buffer containing 0.15 M KCl, 10% glycerol, and 0.2% Nonidet P-40 (buffer A).

The dialysate was centrifuged at 100,000 × *g* for 30 min to remove a light precipitate which formed during the dialysis. The supernatant was collected and applied to a single-stranded DNA agarose column (30-ml bed volume) which had been equilibrated in buffer A. The column was washed with 10 column volumes of buffer A and then eluted with a linear 0.15 to 1.50 M KCl gradient. The presence of DNA polymerase and ICP8 was monitored by means of a standard high-salt polymerase assay (29) and sodium dodecyl sulfate-gel electrophoresis, respectively. ICP8 eluted from the single-stranded DNA agarose at salt concentrations between 0.50 and 0.75 M under these conditions. Fractions containing ICP8 were pooled and dialyzed against buffer A overnight.

The dialysate was passed over a phosphocellulose column (Whatman P11; bed volume, 15 ml) preequlibrated with buffer A. ICP8 does not adhere to phosphocellulose at salt concentrations at or above 150 mM; hence it was contained in the flowthrough of the column, whereas the majority of the contaminating proteins remained bound to the phosphocellulose. The flowthrough fraction was then directly adsorbed either to a 3-ml DNA-agarose column or a 3-ml heparin-Sepharose column. The DNA-agarose column was eluted with 0.3, 0.5, and 0.8 M KCl steps, and the heparin-Sepharose column was eluted with 0.3 and 0.5 M KCl steps. ICP8 was present in the 0.5 and 0.8 M KCl fractions obtained from the DNA-agarose column and in the 0.3 M KCl fractions from the heparin-Sepharose column. Partially purified ICP8 fractions obtained from the small DNA agarose column were also passed over heparin-Sepharose to remove minor contaminants. Protein was quantitated with a Bio-Rad protein assay kit. The ICP8 was dialyzed against buffer B (150 mM KCL, 10 mM Tris [pH 7.6], and 1 mM EDTA) containing 50% glycerol and stored at −20°C.

This purification scheme yielded 0.4 to 0.5 mg of ICP8 per cell pellet from 4 liters. We and others have obtained 50 to 100 μg of ICP8 from 10^9 infected cells (24, 27); hence the increased amount of protein obtained in this work is roughly proportional to the increased amount of infected cells utilized. The final ICP8 fractions exhibited a single band migrating at ~128 kilodaltons on sodium dodecyl sulfate-polyacrylamide gels, indicating an overall purity equivalent to that previously described in our preparations of ICP8 (27, 29).

Filter-binding assays. Nitrocellulose filter binding assays for detection of ICP8-DNA interactions were carried out as described in previous reports from this laboratory (27, 29). The DNAs used were either sonicated, heat-denatured HSV-1 DNA labeled to a specific activity of 5×10^6 dpm/μg with [^{3}H]dCTP by nick translation or heat-denatured *Escherichia coli* DNA labeled in vivo to a specific activity of 1.1×10^5 dpm/μg. DNAs (10 to 100 ng) from HSV *E. coli* were used in filter-binding assays designed to determine the effect of NEM on ICP8 binding. In some experiments, protein which had been pretreated with NEM at room temperature for various times was added to the DNA. The samples (100-μl final volume) were incubated for 10 min at room temperature, and 80-μl samples were filtered, washed, and counted as previously described (29). In other experiments, protein and DNA were combined in final volumes of 0.5 to 1.0 ml and incubated at room temperature for 10 min, then NEM (100 mM) was added to the desired concentration. These samples were then incubated at room temperature, and 80-μl samples were withdrawn and filtered at designated times. Under the neutral pH conditions employed, NEM reacts quantitatively and virtually exclusively with free sulfhydryl groups (9). The structural integrity of the NEM-modified protein was spot checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and no significant difference in electrophoretic pattern was observed between control and NEM-treated ICP8.

The amount of ICP8 present in the samples corresponded to binding ratios in which 32 to 65% of the DNA was retained on the filters, thus assuring that the response seen was on the linear portion of the binding isotherm. All of the ICP8 samples used in this study were checked to be certain that this was in fact the case. The solution for all samples was 150 mM KCl–10 mM Tris hydrochloride (pH 7.6)–1 mM EDTA–25% glycerol.

Agarose gel assays. Agarose gel electrophoresis assays designed to determine the nature of the interaction between either ICP8 or T4 gene 32 protein and single-stranded fd or M13 DNA were carried out as described by Lohman et al.

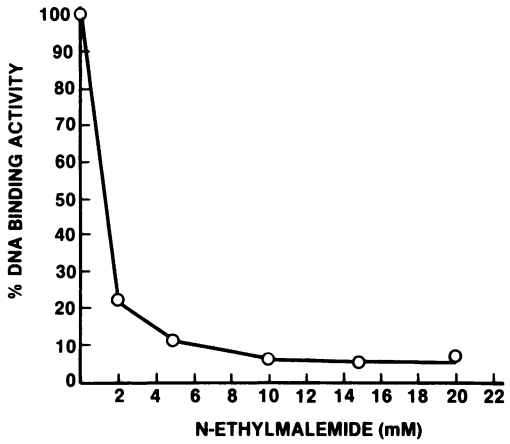

FIG. 1. Data from nitrocellulose filter binding assays showing the loss of ICP8 DNA-binding activity upon preincubation of the protein with increasing amounts of NEM. Assays were carried out on the linear portion of the binding curve. Hence the 100% point represents a maximum possible retention of one-third of the input DNA.

(21). Various amounts of protein were mixed with 0.1 to 0.2 μg of DNA and incubated at room temperature for 15 min. The final volume of each sample was 25 μl. The solutions used in these experiments were identical to those used in the filter-binding experiments. After incubation, 2 μl of tracking dye containing 20% Ficoll and 40 μg each of bromphenol blue and xylene cyanol per ml were added to each sample, and 25 μl was applied to a horizontal, 0.4% agarose gel (15 cm). Gels were run at room temperature in E buffer (40 mM Tris [ph 7.4], 20 mM sodium acetate, 1 mM EDTA) for 4 to 5 h at a constant voltage (3.5 V/cm). The gels were then stained for 10 min by gentle rocking in a solution containing 1.0 M NaCl–10 mM Tris–1 mM EDTA–2 μg of ethidium bromide per ml. Gels were destained for 30 min at room temperature in the same buffer minus ethidium bromide. The high concentration of salt dissociates the protein from the DNA and allows for efficient staining of all of the DNA present on the gel.

Equilibrium dialysis. Equilibrium dialysis experiments were carried out with a motorized equilibrium microvolume dialyzer (Hoefer Scientific, San Francisco, Calif.). Samples of ICP8 at a concentration of 85 nM were incubated at room temperature for 30 min in the presence and absence of 10 mM NEM. The buffer used was buffer B (50% glycerol). ^{32}P-labeled dT_{10} (5×10^6 dpm/μg) was then added to each sample to yield a final concentration of 2.6 nM of oligomer. Duplicate 50-μl samples were then placed opposite 50-μl samples of buffer B. The two halves of the dialysis cell were separated by a dialysis membrane which would allow free passage of molecules with molecular weights less than 10,000. Equilibration took place at 4°C for 6 days with constant rotation of the samples. Control experiments with oligomer alone indicated that equilibrium was reached in 3 to 4 days under these conditions. Samples (25 μl) were then withdrawn from opposite sides of the dialysis chambers and counted by liquid scintillation, and the fraction of oligomer bound in the presence and absence of NEM was determined.

RESULTS

Determination of NEM sensitivity. Initial experiments to determine whether the DNA-binding activity of ICP8 is sensitive to the thiol reagent NEM involved incubation of ICP8 with various amounts of NEM at room temperature for 60 min. The NEM-treated protein was then used in filter-binding assays with single-stranded HSV DNA, and the extent of DNA binding was determined relative to untreated ICP8 which had been incubated at the same concentration for the same length of time. The DNA-binding activity of ICP8 was very sensitive to the action of NEM at the concentration used (Fig. 1). The 100% point corresponded to an initial protein/DNA weight ratio of 3.4:1, which placed the assay on the linear portion of the binding isotherm and should have resulted in approximately 34% of the input DNA being retained. The actual amount of DNA retained in the untreated control sample was actually 20% of the input.

To determine whether ICP8 lost activity upon lengthy incubation at room temperature and to further determine the effect of NEM on this activity, a series of kinetic experiments was done in which ICP8 was incubated at room temperature in the absence of NEM and in the presence of 5 and 10 mM concentrations of the reagent. Samples of ICP8 were removed at times ranging from 0 to 60 min, and standard filter binding assays were performed. The DNA used in these experiments was single-stranded *E. coli* DNA. The protein/DNA ratio was 3.4:1. Typical data (Fig. 2) indicate that ICP8 was in fact somewhat thermolabile upon long-term incubation at room temperature. The drop in binding activity observed in 60 min correlated well with the amount of DNA bound in the control sample in the titration experiment. Figure 2 also shows that the inactivation of ICP8 DNA-binding activity was quite rapid, with over 70 to 90% inactivation being achieved in less than 15 min with 5 and 10 mM NEM, respectively.

DNA protection of ICP8. A series of experiments was undertaken to determine whether the presence of single-stranded DNA or single-stranded DNA oligomers was capable of protecting the DNA-binding activity of ICP8 from the action of NEM. This seemed to be a reasonable possibility, since Wickremasinghe et al. (36) found that the presence of activated DNA resulted in the protection of the activity of mammalian DNA polymerase from inactivation by NEM. In one set of experiments, ICP8 at a concentration of 128 μg/ml was incubated at room temperature in the presence of 10 mM NEM. Samples were withdrawn from this mixture at various times, and standard filter-binding assays were done with

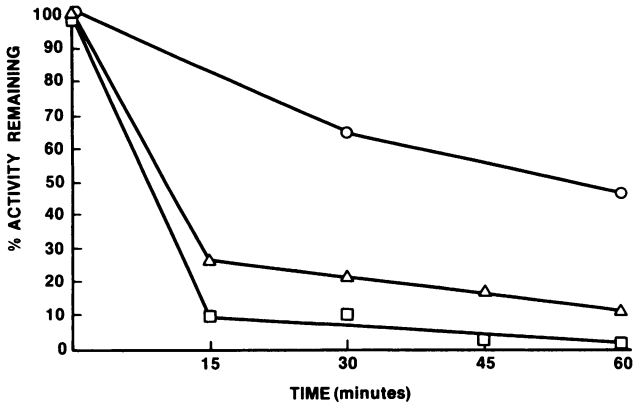

FIG. 2. Kinetics of inactivation of ICP8 by NEM. The protein was incubated in the presence of 0 (○) 5 (△), or 10 (□) mM NEM. All incubations were carried out at room temperature, and protein was withdrawn and used in standard filter binding assays at the designated times.

single-stranded *E. coli* DNA. The final protein/DNA ratio was 3.2:1. In the second set of experiments, ICP8 and single-stranded *E. coli* DNA were mixed at a ratio of 3.2:1 and preincubated for 10 min at room temperature. Concentrated NEM was then added to 10 mM, and incubation was continued. Samples were withdrawn at times corresponding to those in the previous experiments and filtered as described in Materials and Methods.

The results of these experiments (Fig. 3) clearly demonstrate that preincubation of ICP8 with single-stranded DNA protects the DNA-binding activity of ICP8 from the action of NEM. These results indicate that a free sulfhydryl group is required for the interaction of ICP8 with single-stranded DNA. They do not, however, indicate whether the interaction that is inhibited by NEM is the initial intrinsic binding of a single ICP8 molecule to a short stretch of DNA or the cooperative interaction between ICP8 molecules as the DNA is saturated.

A second series of protection experiments was done to gain some insight into this question. In these experiments a short single-stranded DNA oligomer, $d(pCpT)_5$, was used as the protecting nucleic acid. Three sets of experiments were done. In the first set, ICP8 at a concentration of 35 μg/ml was incubated with the oligomer, which was present at a final concentration of 14 μg/ml. This ratio represents a fourfold excess of oligomer to protein. After this incubation NEM was added to a final concentration of 10 mM; samples were withdrawn at 0, 30, 60, and 90 min, combined with single-stranded *E. coli* DNA at a protein/DNA ratio of 6.5:1, incubated for an additional 10 min, and filtered. In the second series of experiments the order of addition of the oligomer and NEM was reversed. Thus the ICP8 was preincubated with NEM before the addition of the oligomer. In the third series of experiments ICP8 was preincubated with the oligomer, but no NEM was added. Incubation and filter binding were then carried out as described above.

The results of these experiments are presented in Fig. 4. No significant protection of ICP8 by $d(pCpT)_5$ was observed. These results suggest but do not necessarily prove that the sulfhydryl residue(s) inactivated by NEM is involved in the interaction of ICP8 with large DNA molecules (cooperative binding) but not necessarily with short DNA molecules (intrinsic binding). The results of the control experiment are consistent with previous results from this laboratory (29) showing that $d(pCpT)_5$ does not compete with large single-stranded DNAs due to the strongly cooperative nature of the ICP8-DNA interaction. The increased stability of ICP8 to thermal inactivation in the presence of $d(pCpT)_5$ was consistently observed in these types of studies and most likely indicates some stabilization of the protein due to interaction with the oligomer (see below).

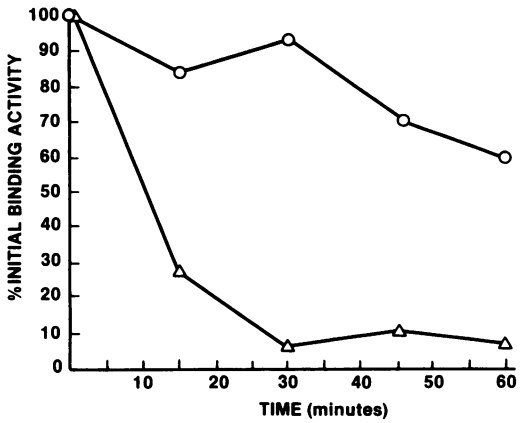

FIG. 3. Protection of the DNA-binding activity of ICP8 by single-stranded DNA. In the first set of experiments (○), ICP8 and ^{3}H-labeled single-stranded *E. coli* DNA were preincubated for 10 min before the addition of NEM to a final concentration of 10 mM. Samples were then withdrawn and filtered. In the second set of experiments (△), ICP8 was incubated in the presence of 10 mM NEM, samples were withdrawn at the indicated times and added to labeled, single-stranded DNA, and these mixtures were filtered.

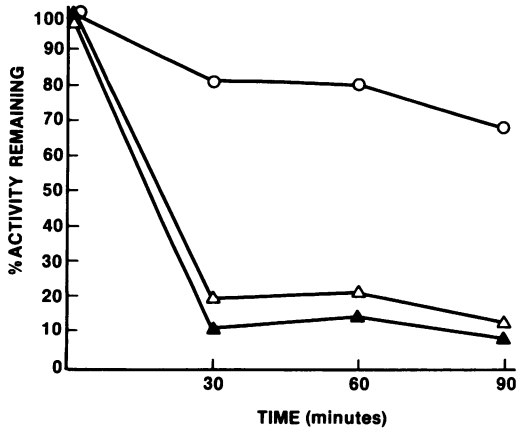

FIG. 4. Loss of ICP8 DNA-binding activity in the presence of a short, single-stranded DNA oligomer. Protection experiments similar to those shown in Fig. 3 were done with the oligomer $d(pCpT)_5$. The results obtained upon preincubation before NEM treatment (△) are not significantly different from those obtained with protein pretreated with 10 mM NEM (▲). The control experiment (○) was done with ICP8 preincubated with the oligomer and then used in filter binding assays in the absence of NEM.

Agarose gel analysis of NEM inhibition. Lohman et al. (21) have recently described an agarose gel electrophoresis assay which is diagnostic for cooperative binding. In studies with the *E. coli* SSB protein these investigators found that at low protein/DNA ratios a very broad band was seen upon electrophoresis of SSB–single-stranded M13 DNA complexes. The broadness of the band reflects a nonrandon distribution of the protein on individual DNA molecules; i.e., some DNA molecules have very little protein bound, whereas others have considerably more. Such a nonrandom distribution would be expected for a cooperatively binding species. As the amount of protein is increased, the differences in migration rate begin to disappear until at relatively high protein/DNA ratios a tight, uniformly migrating band is observed. I used this technique to further examine the nature of the inhibition of ICP8 DNA-binding activity by NEM.

Control experiments were done with T4 gene 32 protein to generate electrophoresis patterns indicative of the cooperative binding of a protein to a uniform species of single-stranded DNA. The cooperative nature of T4 gene 32 binding has been documented and quantitated in numerous studies (7, 14, 15, 17). Figure 5 shows the pattern obtained upon the electrophoresis of complexes of T4 gene 32 protein and single-stranded fd DNA prepared at increasing protein/DNA ratios. The broad smear seen at low protein/DNA ratios is diagnostic for cooperative binding. As the protein/DNA ratio was increased, the migration rate of the complexes slowed, and the band sharpened until a uniformly migrating species was observed at a 10:1 ratio of protein to

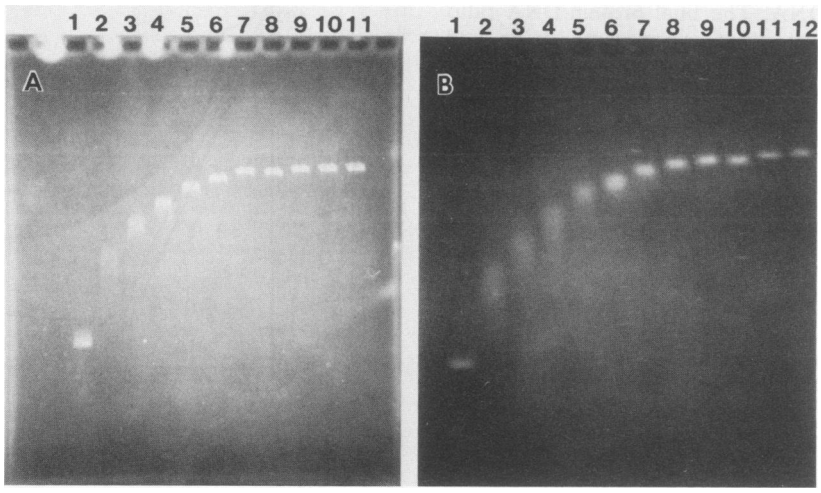

FIG. 5. (A) Electrophoresis of T4 gene 32 protein-fd DNA complexes through an 0.4% agarose gel. The lanes contain complexes prepared at DNA/protein ratios of 0:1, 3.6:1, 5.5:1, 7.3:1, 9.1:1, 10.9:1, 12.7:1, 14.5:1, 16.4:1, and 18.2:1, respectively, reading from left to right. (B) Electrophoresis of ICP8-M13 DNA complexes through an 0.4% agarose gel. The lanes contain complexes prepared at protein/DNA ratios of 0:1, 2.2:1, 4.4:1, 6.5:1, 8.7:1, 10.9:1, 13.1:1, 15.3:1, 17.5:1, 19.6:1, and 28.1:1. The cooperative nature of the interactions between T4 gene 32 protein and ICP8 single-stranded DNA is indicated by the broad smears seen at low protein/DNA ratios.

DNA. This value is in good agreement with the saturating 10:1 to 12:1 ratio for this protein determined by other methods (14, 17). This close agreement may, however, be somewhat fortuitous, since the observed migration rate is a complex function of charge, conformation, and molecular weight. Identical results were obtained with single-stranded M13 DNA, and the two DNAs were used interchangeably in theses studies.

Figure 6 shows a titration of single-stranded M13 DNA with increasing amounts of ICP8. The electrophoresis pattern was strikingly similar to that seen with the T4 gene 32 protein. Broad bands were observed at low protein/DNA ratios, indicating a cooperative mode of binding, whereas at higher ratios the tight bands indicated a more uniform distribution. The curve in this case appeared to flatten between ratios of 13.5:1 and 15.3:1. This simple and direct visualization of cooperative binding thus confirmed earlier results obtained by electron microscopy. The apparent saturation value was somewhat higher than that obtained from nitrocellulose filter-binding and electron microscopy, both of which yielded protein/DNA ratios between 10:1 and 11:1 (27, 28). The significance of this difference is not clear due to the complex nature of the factors influencing the migration rate of the complexes. It should be noted that titration of a fluorescent derivative of polyriboadenylic acid with ICP8 also has yielded a saturating ratio of 10:1 (Ruyechan, unpublished data).

The ability to visualize the cooperative nature of the interaction of ICP8 with single-stranded DNA was then used in the following experiment. ICP8 was either pretreated with 10 mM NEM and then mixed with single-stranded DNA at a ratio of 1.3:1 or mixed with DNA at this ratio and then treated with 10 mM NEM. The low ratio of protein to DNA was used to maximize the ability to observe cooperative binding. The complexes were then analyzed on an 0.4% agarose gel (Fig. 6). The first two lanes contain untreated M13 DNA and M13 DNA treated with 10 mM NEM for 30 min at room temperature and indicate that there is essentially no difference between the two samples. Lane 3 contains ICP8-M13 complexes prepared in the absence of NEM; the broad smear clearly demonstrates the cooperative nature of the binding. Lane 4 contains DNA mixed with ICP8 which had been pretreated with 10 mM NEM. No cooperative binding is detectable in this lane, although the DNA band observed is slightly broader than those seen in the control lanes. Lane 5 contains DNA which had been mixed with protein before treatment with NEM. Cooperative binding is again observed in this lane, indicating that the reactive sulfhydryl group(s) in ICP8 is not available when the protein is bound cooperatively to single-stranded DNA. These results appear to be in good agreement with those obtained in the filter-binding studies.

Equilibrium dialysis of DNA oligomers in the presence and absence of NEM. The slight broadening of the DNA band in samples where ICP8 had been pretreated with NEM was

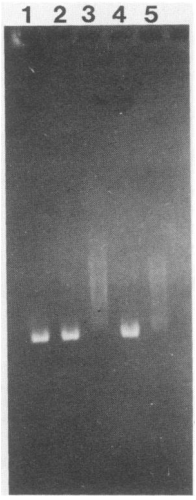

FIG. 6. Agarose gel analysis of ICP8-M13 DNA complexes prepared at a protein/DNA ratio of 1.3:1 in the presence and absence of NEM. Lanes: 1, M13 DNA alone; 2, M13 DNA treated with 10 mM NEM; 3, ICP8 plus M13 DNA; 4, ICP8 pretreated with 10 mM NEM for 30 min before mixing with DNA; 5, ICP8 mixed with DNA and incubated for 10 min before the addition of NEM to 10 mM and subsequent 30-min incubation.

consistently observed in numerous experiments. This broadening hinted that some residual binding activity, possibly the intrinsic noncooperative interaction of single ICP8 molecules with short stretches of DNA, remained after NEM treatment. To determine whether this was indeed the case, equilibrium dialysis experiments with ICP8 and [^{32}P]dT_{10} were done in the presence and absence of NEM as described in Materials and Methods. It should be noted that the ICP8 sample pretreated with 10 mM NEM for 30 min would be expected to contain 5 mM NEM at equilibrium, since the NEM was not removed before dialysis. Thus these samples contained NEM throughout the course of the experiment.

The affinity constants for the interaction of T4 gene 32 protein and the *E. coli* SSB with short DNA oligomers fall into the range of 10^5 to 10^6. These values are 3 to 4 orders of magnitude lower than the estimates of the apparent binding constants characterizing the interaction of these proteins with long single-stranded DNAs due to the absence of cooperative interactions (14, 21, 30–32). The fraction of oligomer bound can be estimated by using the relationship (32) $K = [OP]/[O]_{free}[P]_{free}$, where K is the binding constant, $[P]_{free}$ and $[O]_{free}$ are the concentrations of free protein and free oligomer, respectively, at equilibrium, and $[OP]$ is the concentration of the protein-oligomer complex. At the ratio of protein to oligomer used in these experiments a binding constant of 10^5 would result in approximately 8% of the oligomer being bound at equilibrium, whereas a binding constant of 10^6 would result in approximately 80% of the oligomer being bound. Equilibrium dialysis was carried out at 4°C with 9×10^{-8} M ICP8 and 1.3×10^{-9} M $d(pCpT)_5$. In one case the protein was pretreated with 10 mM NEM for 15 min at room temperature; the control was incubated without NEM. In duplicate assays done in the absence of NEM, 8.2 ± 1.9% of the oligomer was bound; in triplicate assays done in the presence of NEM, 7.4 ± 1.2% of the oligomer was bound. Approximately 8% of the oligomer was bound in the control assay, indicating that the affinity constant of ICP8 for dT_{10} is on the order of 10^5. There was no significant difference between the amount of oligomer bound in the control and NEM-treated samples; this implies that the sulfhydryl group(s) modified by NEM is required for cooperative but not intrinsic binding.

DISCUSSION

The results presented above indicate that at least one free sulfhydryl group present in the ICP8 molecule is susceptible to NEM and that this sulfhydryl group is required for the interaction of the protein with long single-stranded DNA molecules. Filter-binding and agarose gel assays show that interaction of ICP8 with single-stranded DNA before treatment with NEM protects the DNA-binding activity of the protein. This result confirms the fact that the sulfhydryl group is required and argues that this group may have a specific role in binding, as opposed to a modification leading to a general disruption of the tertiary structure of the protein.

The agarose gel electrophoresis results show quite clearly that the cooperative nature of the ICP8-DNA interaction is disrupted by reaction with NEM and that cooperatively is not lost upon NEM treatment of preformed complexes. The question of whether the interaction of ICP8 with a short oligonucleotide is also affected by reaction with NEM was addressed by two different experiments. The first experiment investigated whether $d(pCpT)_5$ was capable of protecting the DNA-binding activity of ICP8. The lack of protection in the presence of the oligomer (Fig. 4) hinted that the free sulfhydryl was required for the cooperative but not the intrinsic interaction of ICP8 with single-stranded DNA. This possibility was confirmed by the results of the equilibrium dialysis experiments, which showed that the binding of ICP8 to dpT_{10} was not affected either by treatment with NEM.

The equilibrium dialysis results are important for two other reasons. First, they are the first demonstration of the ability of ICP8 to interact with single-stranded DNA of such a short chain length. Second, they allow an estimate of the binding constant of the interaction of ICP8 with such an oligonucleotide. The value of $\sim 10^5$ inferred from the observed extent of binding is similar to that determined for the intrinsic binding constants of T4 gene 32 protein and *E. coli* SSB (14, 21, 31, 32). An exact determination of the value of the intrinsic binding constant will require an extensive series of equilibrium dialysis experiments with oligomers of several different chain lengths. Such studies are currently underway.

The question then arises as to which of the potential free sulfhydryl groups in ICP8 is being modified and how these modifications impair the ability of the protein to interact cooperatively with DNA. Investigation of the sequence encoding the ICP8 gene (26) indicates that cysteine residues occur throughout the predicted amino acid sequence; two major clusters are found near the middle and near the carboxy terminus of the sequence, respectively. The central cluster occurs between residues 455 and 546 and encompasses six cysteines. The carboxy-terminal cluster occurs between residues 966 and 1148 and also encompasses six cysteines.

The central cluster falls in a region of the ICP8 molecule that has been implicated as being involved in DNA binding based on rescue and mapping of temperature-sensitive mutants which are negative for DNA binding at the nonpermissive temperature (5, 18, 35; D. Knipe, personal communication). This region also contains a putative divalent metal binding site of the form: Cys-X_{2-4}-Cys-X_{2-15}-A-X_{2-4}-A, where X is any residue and *A* may be either Cys or His (2, 22). The Cys and A residues presumably coordinate to the metal ion, whereas the intervening amino acids loop out to form a metal-binding domain. The putative metal binding site in ICP8 is of the form Cys-X_2-Cys-X_2-His-X_4-His and encompasses residues 499 through 512. The two cysteine residues which fit the concensus sequence are residues 499 and 502. A third cysteine, residue 510, is present in the sequence but does not fit the spacing required for a ligand position. Berg (2) has shown that such sequences occur in numerous nucleic acid-binding proteins including the T4 gene 32 protein. Giedroc et al. (10) have confirmed that the T4 gene 32 protein contains a Zn atom which is coordinated to cysteine residues and whose presence is required for DNA binding.

Experiments aimed at determining whether ICP8 requires a metal ion for activity have not yet yielded definitive results (Ruyechan et al., unpublished data), but the presence of such a domain both in the sequence of this HSV protein and at essentially the identical place in the sequence of the varicella zoster virus equivalent of ICP8 (6) strongly suggests that this may be the case. The possibility that NEM modifies one of the cysteines in the metal-binding domain therefore must be considered. Such a modification does not seem likely to be based on results obtained with the T4 gene 32 protein. This procaryotic DNA-binding protein is modified by reaction with NEM, but its DNA-binding activity is not affected (9). Only the strongly dissociating sulfhydryl reagent *p*-hydroxymercuriphenylsulfonate has been shown to be capable of removing the associated Zn atom efficiently

and, as a result, markedly diminishing the ability of T4 gene 32 protein to interact with DNA (10). Thus treatment of ICP8 with NEM is probably not sufficiently stringent to disrupt any metal-sulfhydryl coordination which may exist in the protein.

A more likely site of action for NEM based upon the protection data presented above would be a site involved in the cooperative interaction between ICP8 molecules occurring on a long stretch of DNA. Free sulfhydryls could be involved in intermolecular hydrogen bonding interactions. Modification with NEM would preclude the occurrence of such interactions, resulting in a loss of cooperativity. The protection of binding activity seen in the presence of DNA could then be explained by the assumption that once these interactions have occurred the residues involved are no longer accessible for modification. It has been clearly demonstrated that the amino-terminal portion of the T4 gene 32 protein is involved in cooperative protein-protein interactions (32). Whether a similar situation exists in ICP8 and involves the carboxy-terminal cluster of cysteines or a free sulfhydryl in another part of the protein is not known. Experiments with radioactively labeled NEM and protease digestion of ICP8 are currently underway in an effort to quantitate and localize the required sulfhydryls. Such experiments coupled with the results presented in this communication should increase our understanding of the interaction of ICP8 with DNA at the molecular level.

ACKNOWLEDGMENTS

The expert assistance of Christopher Fisher in developing the ICP8 purification procedure and the excellent technical skills of Steven Mares throughout this work are gratefully acknowledged. I also thank David Knipe for discussions on the functions of various domains of ICP8.

This work was supported by Public Health Service grant AI22468 from the National Institute for Allergy and Infectious Diseases.

LITERATURE CITED

1. **Alberts, B. M., J. Barry, P. Bedinger, T. Formosa, C. V. Jogeneel, and K. N. Kreuzer.** 1982. Studies on DNA replication in the bacteriophage T4 *in vitro* system. Cold Spring Harbor Symp. Quant. Biol. **47**:655–688.
2. **Berg, J. M.** 1986. Potential metal-binding domains in nucleic acid binding proteins. Science **232**:485–487.
3. **Challberg, M. D.** 1986. A method for identifying the viral genes required for herpesvirus DNA replication. Proc. Natl. Acad. Sci. USA **83**:9094–9098.
4. **Chase, J. W., J. J. L. Italien, J. B. Murphy, E. K. Spicer, and K. R. Williams.** 1984. Characterization of the *Escherichia coli* SSB-113 mutant single-stranded DNA-binding protein. Cloning of the gene, DNA and protein sequence analysis, high pressure liquid chromatography peptide mapping, and DNA-binding studies. J. Biol. Chem. **259**:805–814.
5. **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 polypeptides. J. Virol. **37**:413–428.
6. **Davison, A. J., and J. E. Scott.** 1986. The complete sequence of varicella zoster virus. J. Gen. Virol. **67**:1759–1816.
7. **Delius, H., N. J. Mantell, and B. M. Alberts.** 1976. Characterization by electron microscopy of the complex formed between T4 bacteriophage gene-32 protein and DNA. J. Mol. Biol. **67**: 341–350.
8. **Derse, D., R. F. Bastow, and Y.-C. Cheng.** 1982. Characterization of the DNA polymerase induced by a group of herpes simplex virus type 1 variants selected for growth in the presence of phosphonoformic acid. J. Biol. Chem. **257**:10251–10260.
9. **Doan, T. L., J.-J. Toulme, and C. Helene.** 1984. Involvement of tryptophyl residues in the binding of model peptides and gene 32 protein from phage T4 to single-stranded polynucleotides. A spectroscopic method for detection of tryptophan in the vicinity of nucleic acid bases. Biochemistry **23**:1202–1207.
10. **Giedroc, D. P., K. M. Keating, K. B. Williamms, W. H. Konigsberg, and J. E. Coleman.** 1986. Gene 32 protein, the single-stranded DNA binding protein from bacteriophage T4, is a zinc metalloprotein. Proc. Natl. Acad. Sci. USA **83**: 8452–8456.
11. **Godowski, P., and D. M. Knipe.** 1983. Mutations in the major DNA-binding protein gene of herpes simplex virus result in increased levels of gene expression. J. Virol. **47**:478–486.
12. **Hoggan, M. D., and B. Roizman.** 1959. The isolation and properties of a variant of herpes simplex producing multinucleated giant cells in monolayer cultures in the presence of antibody. Am. J. Hyg. **70**:208–219.
13. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. **14**:8–19.
14. **Kelly, R. C., D. E. Jensen, and P. H. von Hippel.** 1976. DNA "melting" proteins. IV. Fluorescence measurements binding parameters for bacteriophage T4 gene 32-protein to mono-, oligo-, and polynucleotides. J. Biol. Chem. **251**:7240–7250.
15. **Kelly, R. C., and P. H. von Hippel.** 1976. DNA "melting" proteins. III. Fluorescence "mapping" of the nucleic acid binding site of bacteriophage T4 gene 32-protein. J. Biol. Chem. **251**:7229–7239.
16. **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.
17. **Kowalczykowski, S. C., N. Lonberg, J. W. Newport, and P. H. von Hippel.** 1981. Interactions of bacteriophage T4-coded gene 32 protein with nucleic acids. I. Characterization of the binding interactions. J. Mol. Biol. **145**:75–104.
18. **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.
19. **Lee, C. K., and D. M. Knipe.** 1985. An immunoassay for the study of DNA-binding activities of herpes simplex protein ICP8. J. Virol. **54**:731–738.
20. **Littler, E., D. Purifoy, A. Minson, and K. L. Powell.** 1983. Herpes simplex virus nonstructural proteins. III. Function of the major DNA-binding protein. J. Gen. Virol. **64**:983–995.
21. **Lohman, T. M., L. B. Overman, and S. Datta.** 1986. Salt-dependent changes in the DNA binding cooperativity of *Escherichia coli* single strand binding protein. J. Mol. Biol. **187**:603–615.
22. **Miller, J., A. D. McLachlan, and A. Klug.** 1985. Repetitive zinc-binding domains in the protein transcription factor IIIA from *Xenopus* oocytes. EMBO J. **4**:1609–1614.
23. **O'Donnel, M. E., P. Elias, B. E. Funnell, and I. R. Lehman.** 1987. Interaction between the DNA polymerase and single-stranded DNA-binding protein (infected cell protein 8) of herpes simplex virus 1. J. Biol. Chem. **262**:4260–4266.
24. **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.
25. **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.
26. **Quinn, J. P., and D. J. McGeoch.** 1985. DNA sequence in the genome of herpes simplex virus type 1 containing the genes for DNA polymerase and the major DNA binding protein. Nucleic Acids Res. **13**:8143–8163.
27. **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.
28. **Ruyechan, W. T., A. Chytil, and C. M. Fisher.** 1986. *In vitro* characterization of a thermolabile herpes simplex virus DNA-binding protein. J. Virol. **59**:31–36.
29. **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.

30. **Ruyechan, W. T., and J. G. Wetmur.** 1975. Studies on the cooperative binding of the *Escherichia coli* DNA unwinding protein to single-stranded DNA. Biochemistry **14**:5529–5534.
31. **Ruyechan, W. T., and J. G. Wetmur.** 1976. Studies on the noncooperative binding of the *Escherichia coli* DNA unwinding protein to single-stranded nucleic acids. Biochemistry **15**:5057–5064.
32. **Spicer, E. K., K. R. Williams, and W. H. Konigsberg.** 1979. T4 gene 32 protein trypsin-generated fragments. Fluorescence measurement of DNA-binding parameters. J. Biol. Chem. **254**:6433–6436.
33. **Vaughn, P. J., L. M. Banks, D. J. M. Purifoy, and K. L. Powell.** 1984. Interactions between herpes simplex virus DNA-binding proteins. J. Gen. Virol. **65**:2033–2041.
34. **Walboomers, J. M. M., and J. ter Schegget.** 1976. A new method for the isolation of herpes simplex virus type 2 DNA. Virology **74**:256–258.
35. **Weller, S. K., K. J. Lee, D. J. Sabourin, and P. A. Schaffer.** 1983. Genetic analysis of temperature sensitive mutants which define the gene for the major herpes simplex virus type 1 DNA binding protein. J. Virol. **45**:354–366.
36. **Wickremasinghe, R. G., I. P. Hesselwood, A. M. Holmes, and I. R. Johnston.** 1977. Differential *N*-ethylmaleimide inhibition of two enzymes of the α-polymerase-fraction from calf thymus. FEBS Lett. **78**:139–142.