Thermolabile In Vivo DNA-Binding Activity Associated with a Protein Encoded by Mutants of Herpes Simplex Virus Type 1

CYNTHIA K. LEE AND DAVID M. KNIFE*

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

Received 30 December 1982/Accepted 17 March 1983

The major DNA-binding protein encoded by several temperature-sensitive mutants of herpes simplex virus type 1 was thermolabile for binding to intracellular viral DNA. The ability of DNase I to release this protein from isolated nuclei was used as a measure of the amount of protein bound to viral DNA. This assay was based upon our previous observation that the fraction of herpesviral DNA-binding protein which can be eluted from nuclei with DNase I represents proteins associated with progeny viral DNA (D. M. Knipe and A. E. Spang, J. Virol. 43:314–324, 1982). In this study, we found that several temperature-sensitive mutants encoded proteins which rapidly chased from a DNase I-sensitive to a DNase I-resistant nuclear form upon shift to the nonpermissive temperature. We interpret this change in DNase I sensitivity to represent the denaturation of the DNA-binding site at the nonpermissive temperature and the association with the nuclear framework via a second site on the protein. The DNA-binding activity measured by the DNase I sensitivity assay represents an important function of the protein in viral replication because three of five mutants tested were thermolabile for this activity. A fourth mutant encoded a protein which did not associate with the nucleus at the nonpermissive temperature and therefore would not be available for DNA binding in the nucleus. We also present supportive evidence for the binding of the wild-type protein to intracellular viral DNA by showing that a monoclonal antibody coprecipitated virus-specific DNA sequences with the major DNA-binding protein.

The major DNA-binding protein of herpes simplex virus type 1 (HSV-1), infected cell poly-peptide 8 (ICP8), is expressed as a delayed-early or beta viral protein (1, 12, 21, 33). Its role in the replicative cycle of the virus is not completely established, but it has been implicated in the replication of viral DNA (5, 20) and the regulation of viral gene expression (P. J. Godowski and D. M. Knipe, manuscript in preparation). ICP8 from nuclear extracts sediments in deoxyribonuclease complexes (S. S. Leinbach, personal communication). In vitro, the protein binds to single-stranded DNA-cellulose more efficiently than to double-stranded DNA-cellulose (22), shows no preference for viral DNA over cellular DNA in a filter-binding assay, and possesses an unwinding activity for polydeoxycytidylic acid-polydeoxycytidylic acid helices (20).

Temperature-sensitive (ts) mutants of HSV-1 in complementation group 1-1 are candidates for viruses encoding defective ICP8 molecules (32). One member of this complementation group, mP tsHA1, can be converted to a ts+ phenotype by replacement with wild-type viral DNA sequences which map between coordinates 0.372 to 0.388 on the HSV-1 genome. These sequences hybridize to a mRNA species which directs the synthesis of ICP8 in vitro (5). Furthermore, marker rescue experiments with restriction enzyme digests from HSV-2 DNA showed that ts+ recombinants of tsHA1 all encode the donor HSV-2 ICP8 species (5).

It has been shown that there are several discrete steps in the association of ICP8 with the host cell nucleus (16, 23). Immediately after its synthesis, ICP8 associates with the cytoplasmic framework, and subsequently, it is transferred onto the nuclear framework. At later times, a fraction of ICP8 associates with replicating viral DNA, and this association is blocked when viral DNA replication is inhibited. Unlike the earlier nuclear form, the fraction bound to viral DNA can be eluted from nuclei with pancreatic DNase I, and this was proposed as an assay for binding of ICP8 to viral DNA in infected cell nuclei. This present study was conducted to characterize ts mutants belonging to the complementation
group 1-1 which encode major DNA-binding proteins defective in one or more aspects of the maturation process.

MATERIALS AND METHODS

Cells and media. Vero cell monolayers were used for virus propagation, titrations, and cell fractionation experiments. Rabbit skin cell monolayers were used for transfections. Cells were grown in Dulbecco modified Eagle medium containing 10% fetal calf serum (rabbit skin cells) or 10% neonatal calf serum (Vero cells). Virus titrations were performed as described previously (17).

Viruses. The wild-type viruses used in this study were mP (11), KOS (obtained from P. A. Schaffer), and KOS 1.1 (obtained from M. Levine). The mutant mP tsHA1 was obtained by in vitro mutagenesis with hydroxylamine (5). KOS 1.1 ts6, ts13, ts18 (L. E. Holland, R. M. Sandri-Goldin, M. Levine, and J. C. Glorioso, manuscript in preparation), and ts656 (13) were isolated by in vivo bromodeoxyuridine mutagenesis. Previous studies showed that tsHA1 (32), ts6, ts13, ts18 (Holland et. al., manuscript in preparation), and ts656 (26) belong to complementation group 1-1. KOS tsD9 is an HSV DNA polymerase mutant (15).

Labeling and fractionation of proteins from infected cells. Vero cells were infected, labeled, and fractionated by methods similar to those described before (17). Briefly, cells were pulse labeled with [35S]methionine (New England Nuclear Corp., Boston, Mass.) 4 h after infection and chased for the times specified by adding cold methionine to a final concentration of 1 mM. The labeled cells were swelled in reticulocyte standard buffer (10 mM Tris-hydrochloride [pH 7.6], 10 mM NaCl, 1.5 mM MgCl2, 0.5 mM phenylmethylsulfonyl fluoride) and fractionated by Dounce homogenization, and the nuclei were collected by centrifugation (1,000 x g for 5 min). The nuclear fractions were further fractionated by either detergent or DNase I treatment. Nuclear fractions that were detergent treated were suspended in reticulocyte standard buffer containing 10% sucrose, and a detergent solution was added gently to a final concentration of 1% Triton X-100 and 0.5% sodium deoxycholate. The suspension was kept on ice for 1 min before fractionating into the detergent-resistant and detergent-soluble nuclear fractions. To assay for viral DNA-bound ICP8 in the nuclei, we suspended nuclei from Dounce homogenization in reticulocyte standard buffer with 10% sucrose and treated them with 40 U of bovine pancreatic DNase I (Millipore Corp. Freehold, N.J.) per ml for 30 min at 20°C. DNA resistant nuclear fraction was recovered in the pellet after centrifugation. Supernatant proteins were precipitated with 9 volumes of acetone. All pellets were dissolved in electrophoresis sample buffer, and the proteins were analyzed by electrophoresis in 9.25% polyacrylamide gels as described before (17).

Immunoprecipitation and analysis of ICP8-DNA complexes. The antibodies used for immunoprecipitation were monoclonal antibodies 395 (27; directed against ICP8) and F4 (3; directed against the reovirus hemagglutinin protein) and hyperimmune rabbit antiserum NC-1 (27; Vero cell monolayers were infected with 20 PFU of KOS 1.1 per cell for 4 h before labeling for 2 h with 5 μCi of [35S]methionine per ml. Nuclei were isolated by Dounce homogenization, and approximate-ly 3 x 106 nuclei were suspended in 400 μl of immunoprecipitation buffer (10 mM Tris-hydrochloride [pH 7.6], 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride). Nuclei were disrupted by 15 s sonication pulses (Sonicator Ultrasonic Cell Disruptor model W-225R fitted with a tapered microtip; Heat System-Ultrasonics, Inc., Plainview, N.Y.). The solution was clarified by centrifugation at 12,800 x g at 4°C for 15 min. Ascites fluid or hyperimmune antisera (one-tenth volume) was added to the nuclear extract and incubated at 0°C for 16 h. The immune complexes were collected with protein A-Sepharose (Sigma Chemical Co., St. Louis, Mo.) at 0°C for 60 min and washed with a solution containing 150 mM NaCl, 50 mM Tris-hydrochloride (pH 7.6), 5 mM EDTA, 0.5% Nonidet P-40, and 0.5 mM phenylmethylsulfonyl fluoride. Samples for protein analysis were dissolved in electrophoresis sample buffer and analyzed by electrophoresis.

Samples for DNA analysis were further treated with 0.5 mg of pronase (Calbiochem-Behring, La Jolla, Calif.) per ml at 37°C for 60 min in 10 mM Tris-hydrochloride (pH7.8)-20 mM EDTA-0.5% sodium dodecyl sulfate. The proteins were removed by phenol-chloroform extractions, and nucleic acid were precipitated with 0.5 μg of carrier yeast tRNA (Sigma Chemical Co.) per ml in ethanol. The DNA was suspended in buffer, digested with restriction enzymes, and separated by electrophoresis in neutral agarose. DNA in the gel was denatured in 0.5 M NaOH and transferred to nitrocellulose filter paper (Schleicher & Schuell, Keene, N.H.) by the method of Southern (28). The nitrocellulose was baked for 2 h at 80°C in vacuo and preincubated in 50% formamide (Pluka Chemical Corp., Hauppauge, N.Y.)-5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0)-0.25% sodium dodecyl sulfate-0.02% Denhardt solution (7; equal concentrations of polyvinylpyrrolidone, Ficoll, and bovine serum albumin)-50 mM potassium phosphate (pH 7.0)-250 μg of yeast tRNA per ml at 42°C for 17 h. Hybridization was carried out under the same conditions with 106 cpm of 32P-labeled DNA. The filter was washed twice in 500 ml of 2× SSC-0.1% sodium dodecyl sulfate for 30 min at 20°C and once in 0.1% SSC-0.1% sodium dodecyl sulfate for 60 min at 50°C. Autoradiography was carried out with Kodak XRP-5 film with a Cronex-Dupont intensifying screen.

Labeling of DNA fragments. The EcoRI F fragment was isolated from the plasmid pSG18 (9) by electroleo-
digested pBR322, and the molecules were used to transform E. coli K-12 strain HB101. Transformants containing insertions into PvuII-SalI-digested pBR322 were selected by screening for tetracycline-sensitive, ampicillin-resistant colonies. Plasmids from these colonies contained the terminal SalI-PvuII fragments of SalI A. Transformants containing insertions into PvuII-digested pBR322 were selected by colony hybridization (10) with 32P-labeled EcoRI F fragment. These colonies carried plasmids which contained the internal PvuII fragments of SalI A. Plasmid DNA for marker rescue experiments was obtained by two methods. Plasmid preparations of pSG18 and the SalI subclones were purified in CsCl density gradients (6). Plasmids from the PvuII subclones were made by an alkaline extraction procedure described previously by Birnboim and Doly (2).

RESULTS
Precipitation of viral DNA with monoclonal antibody against ICP8. We have previously shown that ICP8 can be released from isolated infected cell nuclei by DNase I treatment (17). However, in the absence of viral DNA replication, the association of ICP8 with the infected cell nucleus was resistant to DNase treatment.

On the basis of these data, we concluded that a significant fraction of ICP8 in the infected cell nucleus was capable of binding to progeny viral DNA molecules.

Evidence supporting in vivo binding of ICP8 to viral DNA was obtained by looking for virus-specific DNA sequences which were associated with ICP8 in immunoprecipitates from nuclear extracts. Polyacrylamide gel electrophoresis (Fig. 1A) showed that the monoclonal antibody 39S specifically precipitated ICP8 from sonicated nuclear extracts, whereas a rabbit antiserum (NC-1) precipitated predominantly ICP5, the major capsid protein. Figure 1B shows a HindIII digest of viral DNA associated with ICP8 in infected nuclear extracts. The probe utilized to detect virus-specific sequences was an EcoRI F fragment which is contained within the 40.8-kilobase HindIII A fragment. Immunoprecipitates formed with either 39S or NC-1 antibodies contained viral DNA sequences homologous to EcoRI F. The DNA molecules in the immunoprecipitates homologous to EcoRI F comigrated with the intact HindIII A fragment and with

FIG. 1. Analysis of 35S-labeled viral proteins (A) and virus-specific DNA sequences (B) in immunoprecipitates from infected cell nuclei. (A) Autoradiogram of proteins separated on a 9.25% polyacrylamide gel. The three lanes contain proteins immunoprecipitated with 39S (a), NC-1 (b), and F4 (c). The molecular weight markers were β-galactosidase (117,000), bovine serum albumin (68,000), and actin (46,000). (B) Southern blot analysis of HindIII digests hybridized with 32P-labeled EcoRI F fragment. The DNA samples were analyzed by electrophoresis in an 0.8% agarose gel, transferred onto a nitrocellulose filter, and incubated with the 32P-labeled probe. The numbers on the right represent the lengths (in kilobases) of a DNA digested with HindIII. The lanes contain KOS 1.1 DNA (a), mock-infected Vero cell DNA (b), sonicated KOS 1.1 DNA (c), and DNA immunoprecipitated from infected cells with 39S (d), NC-1 (e), and F4 (f). kb, Kilobases.
lower-molecular-weight species which were generated during sonic disruption of nuclei. A larger proportion of EcoRI F-specific sequences that precipitated with NC-1 comigrated with the intact HindIII fragment, presumably because DNA molecules within capsids were protected from sonic shearing. The antireovirus monoclonal antibody (F4) did not precipitate HSV DNA, showing that ascites fluid alone was insufficient for precipitation.

These data provide evidence for the binding of ICP8 to viral DNA in infected cell nuclei and support the data obtained by the DNase I release assay. Therefore, we used the DNase I assay to test the DNA-binding activity of ICP8 encoded by a group of ts mutants which are in the complementation group 1-1.

Effect of temperature shift on in vivo binding of wild-type and mutant ICP8 to viral DNA. Cells were infected at the nonpermissive temperature (39°C) and fractionated into cytoplasmic, DNase I-resistant nuclear, and DNase I-sensitive nuclear fractions. The distribution of ICP8 was different in cells infected with the ts+ virus in comparison with cells infected with the mutant tsHA1 (Fig. 2). In cells infected with tsHA1 at 39°C, there was very little ICP8 in the cytoplasm and the DNase I-sensitive nuclear fraction which represents protein bound to viral DNA. Because tsHA1 fails to replicate its DNA at the nonpermissive temperature (5), this observation was consistent with our previous observation that in the absence of viral DNA replication, ICP8 is mostly associated with the nucleus in a form which cannot be eluted with DNase I (17).

We examined the state of ICP8 after a shift of infected cells from 33 to 39°C to determine whether the ts mutants encode ICP8 molecules which have thermolabile in vivo DNA-binding activities. Cells were infected at the permissive temperature to allow viral DNA replication and nuclear transport of ICP8 to occur. The cells were then shifted to the nonpermissive temperature, and the amount of ICP8 bound to viral DNA was assayed. After 60 min at the nonpermissive temperature, the fraction of protein bound to viral DNA in cells infected with tsHA1 was substantially reduced (Fig. 3). The protein profiles from cells shifted from 33 to 39°C resembled the protein distribution obtained from cells infected at the nonpermissive temperature (Fig. 2). No significant changes were observed in the distributions of proteins in the ts+ infection after temperature shift-up (Fig. 3).

The relative amounts of ICP8 were quantitated by densitometry scanning of autoradiograms. The amounts of ICP8 in the three subcellular fractions at various times after temperature shift-up are shown in Fig. 4. In cells infected with tsHA1, the amount of cytoplasmic and DNase-sensitive ICP8 decreased substantially by 30 min; however, the amount of DNase-resistant nuclear protein increased (Fig. 4C). The increase in ICP8 in the DNase-resistant nuclear fraction may be due either to an influx of cytoplasmic protein or to the conversion of the DNase-sensitive to the DNase-resistant form. However, the total amount of cellular ICP8 was reduced, and we favor the explanation that, upon temperature shift, a rapid degradation of cytoplasmic ICP8 occurs concurrent with a conversion of viral DNA-bound ICP8 to a DNase-resistant nuclear form. After conversion to the DNase-resistant form, the mutant protein be-

FIG. 2. Proteins from subcellular fractions of cells infected with either ts+ (mP) or tsHA1 at 39°C. Infected cells were labeled with 6 μCi of [35S]methionine per ml for 15 min and chased with cold methionine for 2 h. They were fractionated into cytoplasmic (C), DNase-resistant nuclear (N), and DNase-sensitive nuclear (D) fractions, and the proteins were analyzed by electrophoresis followed by autoradiography. Numbers on left show molecular weight markers as described in the legend to Fig. 1.
came insoluble after prolonged incubation (greater than 60 min) at the nonpermissive temperature (data not shown). Infection with the mutant virus at the permissive temperature did not show any alterations in the distribution of ICP8 (Fig. 4D). Infections with the ts+ virus also showed no alterations in the distribution of ICP8 at either the permissive (Fig. 4B) or the nonpermissive temperature (Fig. 4A).

The changes in the distribution of ICP8 in cells infected with tsHA1 were due either to a thermolabile DNA-binding function or to the cessation of viral DNA replication. Schaffer et al. (25) have shown that when cells infected with the DNA polymerase mutant tsD9 are shifted to the nonpermissive temperature, DNA replication stops. Therefore, we examined the distribution of ICP8 in cells infected with tsD9. The amount of ICP8 in the DNase-sensitive fraction did not change upon shift to the nonpermissive temperature (Table 1). This shows that the cessation of viral DNA replication alone was insufficient to initiate the changes in DNase sensitivity of ICP8 in cells infected with tsHA1. Therefore, we conclude that the ICP8 encoded by tsHA1 possesses a thermolabile DNA-binding function.

Several other ts mutants in complementation group 1-1 were tested in the in vivo DNA-binding assay (Table 1). The mutants ts13 and ts18 showed the same DNA-binding phenotype as tsHA1, whereas ts6 did now show a thermolabile DNA-binding activity. In cells infected with the ts+ virus, the amount of ICP8 in the cytoplasmic fraction decreased upon a shift to 39°C.

FIG. 3. Effect of temperature shift-up on the subcellular distribution of viral proteins from cells infected with ts+ (mP) or tsHA1 viruses. Cells were infected at 33°C and labeled with 6 μCi of [35S]methionine per ml for 15 min. The cells were chased for 2 h at 33°C, at which time they were either held at 33°C or shifted to 39°C for 60 min. The cytoplasmic (C), DNase-resistant nuclear (N), and DNase-sensitive nuclear (D), fractions were obtained as described in the text, and the proteins in each fraction were analyzed by electrophoresis and autoradiography. Numbers on left show molecular weight markers as described in the legend to Fig. 1.
This was probably due to a faster rate of nuclear localization at the higher temperature. The decrease of ICP8 in the cytoplasmic fraction upon a shift to 39°C was more pronounced in cells infected with mutants of ICP8 (tsHA1, ts6, ts13, ts18). We believe that this is due to degradation of mutant proteins at the nonpermissive temperature. The DNA polymerase mutant tsD9 encodes a normal ICP8, and the amount of this protein in the cytoplasm resembles that seen with the wild-type virus.

**Rate of nuclear association of ICP8** in cells infected with ts+ and ts viruses. We wanted to determine whether any mutants of complementation group 1-1 encode ICP8 molecules which are defective for nuclear transport at the nonpermissive temperature. Because the rate of nuclear association is different in the presence or absence of viral DNA replication (17), we added phosphonoacetic acid (Abbott Laboratories, North Chicago, Ill.), an inhibitor of the viral DNA polymerase, to the infected cultures to ensure that all comparisons were made in the absence of viral DNA replication. Figure 5 shows the rate of nuclear association of ICP8 of two ts mutants and their ts+ parent. ICP8 encoded by the DNA-binding mutant ts13 showed the same rate of nuclear association as that encoded by ts+ virus. There was a transfer of ICP8 from the cytoplasm to the nuclear fraction with a transient increase in the detergent-soluble nuclear fraction. On the other hand, ts656 showed a gradual decrease in the cytoplasmic fraction without any substantial increase in the nuclear fraction after 5 to 10 min of chase. There are two possible explanations for the failure of ICP8 to accumulate in the nuclei of cells infected with ts656. First, the mutant protein molecules were recognized as abnormal proteins and were rapidly degraded in the cytoplasm before nuclear transport could occur. Second, the mutant proteins possessed a defective nuclear transport function, they were not transported into the nucleus, and they were eventually degraded. We have not been able to determine the correct explanation. However, both pathways result in the degradation of the mutant protein and the lack of a nuclear form of ICP8. This also occurs.

**TABLE 1. Effect of temperature shift-up on the amount of ICP8 in subfractions of cells infected with ts+ and several ts viruses**

<table>
<thead>
<tr>
<th>Virus</th>
<th>33°C&lt;sup&gt;a&lt;/sup&gt;</th>
<th>33–39°C&lt;sup&gt;b&lt;/sup&gt;</th>
<th>39°C&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amt ICP8 at:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C&lt;sup&gt;d&lt;/sup&gt;</td>
<td>N&lt;sup&gt;d&lt;/sup&gt;</td>
<td>D&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>ts+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tsHA1</td>
<td>1.11 9 35</td>
<td>50 24 40</td>
<td>93 163 158</td>
</tr>
<tr>
<td>ts6</td>
<td>125 18 73</td>
<td>4 76 28</td>
<td>42 280 17</td>
</tr>
<tr>
<td>ts13</td>
<td>140 21 44</td>
<td>48 64 53</td>
<td>83 274 42</td>
</tr>
<tr>
<td>ts18</td>
<td>106 6 27</td>
<td>17 130 14</td>
<td>100 296 38</td>
</tr>
<tr>
<td>tsD9</td>
<td>30 0 17</td>
<td>4 18 0</td>
<td>46 52 0</td>
</tr>
</tbody>
</table>

<sup>a</sup> At 4 h postinfection, cells were labeled with 6 µCi of [35S]methionine per ml for 15 min and chased with cold methionine for 2 h. They were then either shifted to the nonpermissive temperature for 30 min or held at the same temperature for another 30 min. Arbitrary units of protein were obtained from densitometry and planimetry quantitation of 35S-labeled ICP8 from autoradiograms.

<sup>b</sup> Cells were held at 33°C for the duration of the infection.

<sup>c</sup> Cells were infected and pulse chased at 33°C before being shifted to 39°C for 30 min.

<sup>d</sup> Cells were held at 39°C for the duration of the infection.
to a more limited extent in cells infected at the permissive temperature (data not shown). For this reason, it was difficult to determine the in vivo DNA-binding activity of ICP8 encoded by ts656.

The mutants listed in Table 1 were also tested for the rate of nuclear association of ICP8. No other mutants, including the DNA polymerase mutant tsD9, showed defective nuclear association of ICP8.

Marker rescue of ts mutants with cloned viral DNA fragments. The ts lesions of the mutants were mapped to identify the portions of the ICP8 molecule which are required for the DNA-binding function. Subclones of the recombinant plasmid pSG18 (9) were used in marker rescue experiments. pSG18 contains the EcoRI F fragment which is located from 0.314 to 0.420 map units on the HSV-1 genome. All of the ts mutants described in this study mapped within a single Sall fragment, Sall A, between map units 0.386 and 0.417 (Table 2). In addition, PvuII subclones of Sall A were used to rescue the ts lesions of these mutants (Table 2). The map position of the ts lesions are shown in Fig. 6. The mutant ts656 could not be rescued by any of the PvuII subclones, suggesting that there may be multiple lesions or that the lesion(s) may be near or span a PvuII restriction site. The mutant ts6 was rescued most efficiently by Sall A. The low level of plaques at 39°C in the Sall D, Sall E, and Pvu II B fragment transfections may be due to leakiness of the mutant. The three DNA-binding mutants, tsHA1, ts13, and ts18, were all rescued by sequences between 0.395 and 0.410

![Graph](image)

**Fig. 5.** Rate of nuclear association of ICP8 in cells infected with ts+ (KOS 1.1), ts13, or ts656 virus at 39°C. Phosphinoacetic acid (a gift from Abbott Laboratories) was added to infected cultures to a concentration of 400 μg/ml. At 4 h postinfection, the cells were pulse labeled with 12 μCi of [35S]methionine per ml for 5 min and chased for 0 to 60 min. Cytoplasmic (●), detergent-resistant nuclear (▲), and detergent wash (■) fractions were obtained, and the amount of ICP8 in each fraction was quantitated.

### Table 2. Marker rescue of ts mutants with cloned HSV DNA fragments

<table>
<thead>
<tr>
<th>Plasmid DNA</th>
<th>tsHA1%</th>
<th>ts6</th>
<th>ts13</th>
<th>ts18</th>
<th>ts656</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0.0015</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EcoRI F</td>
<td>5.4</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
<td>33.5</td>
</tr>
<tr>
<td>Sall A</td>
<td>1.3</td>
<td>46</td>
<td>80.5</td>
<td>12.7</td>
<td>31.4</td>
</tr>
<tr>
<td>Sall D</td>
<td>0</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sall E</td>
<td>0</td>
<td>0.06</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PvuII A</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>PvuII B</td>
<td>0.4</td>
<td>0.04</td>
<td>0.014</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PvuII C</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PvuII D</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PvuII E</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Rabbit skin cell monolayers were cotransfected with 0.5 μg each of intact viral DNA and linearized plasmid DNA by methods described elsewhere (16). The transfected cells were incubated at 37°C for 4 to 7 days, and the progeny virus was titrated at 33 and 39°C.

* Plasmid DNA containing ts+ (KOS 1.1) DNA fragments. Positions of the DNA fragments of the HSV-1 genome are shown in Fig. 6.

* (Virus titer at 39°C/virus titer at 33°C) × 100.

* Intact viral DNA was purified from infected cells by centrifugation through NaI equilibrium density gradients.

* ND, Not done.
map units, suggesting that some or all of these sequences are required for the DNA-binding function. The map positions of the ts18 and tsHA1 lesions are separated by approximately 1 kilobase of viral DNA, which indicates that defects in different portions of the protein molecule can result in a thermolabile DNA-binding activity.

**DISCUSSION**

**Association of ICP8 with intracellular viral DNA.** This study was undertaken to define the in vivo functions of the major DNA-binding protein encoded by HSV-1, ICP8, by analyzing ts mutants defective in this gene product. We used an in vivo DNA binding assay to identify a group of HSV-1 ts mutants which encode proteins with thermolabile DNA-binding activities. Our assay for in vivo DNA binding is based on previous evidence (17) that ICP8 associates with different subcellular compartments during its maturation process and that the final association represents binding to progeny viral DNA. The final, DNase-sensitive association with the infected cell nucleus is inhibited by drugs or a ts mutation which block viral DNA replication. In this study, we present additional evidence for the in vivo binding of ICP8 to viral DNA by showing that HSV-specific DNA sequences were associated with ICP8 immunoprecipitated with a monoclonal antibody directed against that protein. The association was stable under wash conditions which removed all other viral proteins from the immunoprecipitate. Additional experiments will be conducted to determine whether any host DNA or specific viral DNA sequences are associated with ICP8 in the immune precipitates.

The in vivo DNA-binding activity reflects a critical function of the protein in the replicative cycle of the virus because, among the ts mutants from complementation group 1-1 that we examined, three of five (tsHA1, ts13, and ts18) exhibited a thermolabile DNA-binding function, whereas a fourth, ts656, produced a relatively
FIG. 7. Model for interaction of ICP8 with structures in the nucleus. ICP8 (8) associates with the nuclear framework (n) upon entering the nucleus (A). When viral DNA replication occurs, the protein binds to progeny viral DNA (d) which is attached to other structures (a) in the nucleus (B). Protein bound to viral DNA can be eluted from the nucleus with DNase I (C). When cells infected with tsHA1 are shifted to 39°C, the DNA-binding site denatures and the protein binds to the nuclear framework (D).

The increased rates of protein synthesis were due to higher levels of mRNA. Therefore, there may be a correlation between the DNA-binding activity and the levels of expression of viral genes as reflected by viral mRNA accumulation.

Model for interaction of ICP8 with the cell nucleus. We propose a model which explains the effect of a nonfunctional DNA-binding site on the nuclear association of ICP8 (Fig. 7). In this model, we assume that the viral DNA-binding site on the protein is partially or entirely distinct from the site required for binding to the nuclear framework. This assumption is supported by the observation that the DNA-binding activity can be inactivated without inactivating the ability of the protein to bind to the nucleus. In the absence of viral DNA replication, the protein is bound to the nuclear framework (Fig. 7A) and cannot be eluted by DNase treatment (17, 23). However, protein bound to newly synthesized viral DNA can be released from nuclei with DNase I be-

minute amount of nuclear ICP8 that would be available for DNA binding. The last mutant, ts6, may either define an unknown function or encode a protein which is stabilized by binding to DNA. Furthermore, a group of ICP8 mutants of HSV-2 has been found to encode thermolabile DNA-binding proteins. Like the HSV-1 DNA-binding mutants, these HSV-2 proteins also associate normally with the nucleus (D. M. Knipe and A. E. Spang, unpublished data). The function of ICP8 in the replicative cycle of the virus as represented by the in vivo DNA-binding activity may reflect the role of the protein in DNA replication or gene expression. A separate study from our laboratory (P. J. Godowski and D. M. Knipe, manuscript in preparation) showed that two of the DNA-binding mutants, tsHA1 and ts13, overproduced ICP8 and several other viral proteins upon shift of infected cells to the nonpermissive temperature. However, cells infected with ts6 did not exhibit this phenotype.
cause allosteric constraints prevent the protein from binding to the nuclear framework when the DNA binding site is occupied (Fig. 7C). When cells infected with a ts mutant encoding a thermolabile DNA-binding function are shifted to 39°C, ICP8 is released from DNA but is still associated with the cell nucleus, possibly by binding to the nuclear framework (Fig. 7D). An alternative model postulates that only one site is used for binding to viral DNA or to the nuclear framework. In this situation, the site would have a higher affinity for viral DNA than for the nuclear framework. Our current model also assumes that binding to either site involves a monomeric form of the protein. Additional work will be needed to show whether this protein assumes multimeric forms.

Comparison with other mutant DNA-binding proteins. Thermolabile DNA-binding proteins have been reported in other animal virus systems. For example, the tsA mutants of simian virus 40 show thermolabile binding of the large T antigen to the simian virus 40 replication origin in vitro (14, 30, 34) and to minichromosomes in vivo (18). These mutant proteins are usually degraded rapidly at the nonpermissive temperature (29), and therefore, it has not been possible to study their subcellular associations at the nonpermissive temperature. Temperature-sensitive mutants in the 72-kilodalton DNA-binding protein encoded by adenovirus have also been identified (8). The protein encoded by one of these mutants is thermolabile for binding to DNA-cellulose (31). In our HSV system, it was not possible for us to test whether mutant ICP8 molecules synthesized at 39°C could bind to DNA-cellulose because these proteins were insoluble after a long labeling period at the nonpermissive temperature (unpublished data). Proteins synthesized in cells infected with tsHAl and ts13 at 33°C bind to single-stranded DNA-cellulose with the same affinity as ts" proteins, and we observed no differences in their elution profiles at temperatures up to 40°C (unpublished data). We believe that this in vitro assay does not reflect the requirement for binding to viral DNA in the infected cell. We are currently investigating the relationship between the in vivo DNA-binding function and the DNA-binding and -unwinding activities observed in vitro.

ACKNOWLEDGMENTS

We thank L. F. Rafieff for advice and assistance in the subcloning and Southern blot experiments and D. Zantos for isolation of Sall subclones. We also thank M. Zweig, G. H. Cohen, and D. R. Spriggs for their gifts of antibodies. The technical assistance of M. E. Levin and A. E. Spang is gratefully acknowledged.

This work was supported by Public Health Service grant CA26345 from the National Cancer Institute and a postdoctoral fellowship award from the Damon Runyon-Walter Winchell Cancer Fund (to C.K.L.). D.M.K. is a Cancer Research Scholar of the American Cancer Society, Massachusetts Division.

LITERATURE CITED


