A Post- α Gene Function Turns Off the Capacity of a Host Protein to Bind DNA in Cells Infected with Herpes Simplex Virus 1

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HEp-2 cell proteins electrophoretically separated in denaturing polyacrylamide gels and electrically transferred to nitrocellulose sheets contain a polypeptide which efficiently binds linear native DNA end labeled with ^{32}P but not denatured DNA. The polypeptide has an apparent molecular weight of ca. 130,000. The activity of the protein was stable, and no appreciable turnover was observed after exposure of uninfected cells to inhibitory concentrations of cycloheximide for intervals of up to 24 h. However, the activity was absent from lysates of cells harvested 6 h or later postinfection with wild-type viruses. To identify the viral function involved in the loss of DNA-binding activity, we tested the lysates of cells infected with several mutants. Thus, the DNA-binding activity was unaffected in cells infected with a temperature-sensitive mutant (herpes simplex virus 1 *tsLB2*) in the $\alpha 4$ gene and was maintained at a nonpermissive temperature (39°C). Experiments involving (i) temperature shift-down of cells infected with *tsLB2* in the presence of cycloheximide, (ii) withdrawal of cycloheximide in the presence and absence of actinomycin D from cells infected with wild-type virus, (iii) infection of cells at 33 and 39°C with herpes simplex virus 1 *tsHA1* carrying a temperature-sensitive lesion in the $\beta 8$ gene, and (iv) infection of cells in the presence of inhibitory concentrations of phosphonoacetate led to the conclusion that the viral functions responsible for the loss of DNA-binding capacity were specified by either β or γ genes not dependent on viral DNA synthesis for their expression.

Previous studies have shown that in cells infected with herpes simplex viruses 1 and 2 (HSV-1 and HSV-2), host protein and DNA syntheses are turned off, and host RNA synthesis is at least partially diminished (6, 12, 13, 15, 18-21). The available information suggests that HSV turns off host macromolecular metabolism in two steps. The first step occurs soon after infection and appears to be caused by components of the virion (6, 8, 14, 15). HSV-1 and HSV-2 differ in the rate by which the virion component turns off host protein synthesis (8). The location of at least one gene involved in the rapid shutoff of HSV-2-infected cells has been mapped (5).

The second step is an irreversible inhibition of host macromolecular synthesis caused by newly synthesized viral gene products. HSV genes form three groups, α , β , and γ , in which expression is coordinately regulated and sequentially ordered in a cascade fashion (9, 16). Late host shutoff coincides with the expression of β or possibly early γ genes (6, 11, 13). Little is known of the mechanism by which inhibition is effected or of the gene products responsible for inhibition.

Inhibition of host macromolecular metabolism appears to be effected at the levels of transcription and translation. To date, there has been no evidence that viral gene products directly affect the function of one or more host proteins. In this paper, we report evidence that the capacity of a host protein to bind DNA is diminished or lost after infection. Our results show that the loss of the capacity to bind DNA coincides with the expression of β or γ genes.

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MATERIALS AND METHODS

Cells and viruses. HEp-2 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Virus stocks were prepared and titrated in HEp-2 cells (17). The isolation and properties of HSV-1(F), HSV-1(HFEM)*tsLB2*, and HSV-1(mP)*tsHA1* have been described previously (2, 4, 7, 10).

DNA probes. pBR322 and pRB370 DNAs were prepared as previously described (1). pRB370 contains the *Bam*-*Pvu*II fragment representing the left end of *Bam*HI fragment N of HSV-1 fused to HSV-1 *Bgl*III-*Pvu*II fragment containing the thymidine kinase gene (*Bam*HI fragment Q) inserted into the *Pvu*II site of pRB10 which was derived from pBR322 by deletion of the small *Eco*RI-*Pvu*I fragment. The entire construct was cloned in pBR322 by S. Mackem.

To end label, 1 μg of DNA was cleaved with *Bam*HI in a volume of 20 μl . To the linearized DNA were added dATP, dTTP, and dGTP to a final concentration of 20 μM , and 50 μCi of [α - ^{32}P]dCTP (specific activity, $\sim 3,000$ Ci/mmol; New England Nuclear Corp.). The reaction was carried out with 2 units of the Klenow fragment of *Escherichia coli* polymerase I at room temperature (21°C) for 5 min. Unincorporated [α - ^{32}P]dCTP was removed by chromatography on Sephadex G-50 columns. Typically, the specific activity of the probes prepared by this method was ca. 4×10^6 cpm/ μg of DNA (Cerenkov). To prepare the single-stranded DNA probe, the double-stranded DNA probe was heat denatured and rapidly cooled on wet ice.

Polyacrylamide gel electrophoresis and transfer to nitrocellulose. The cells were solubilized in phosphate-buffered saline (3) containing 1% Nonidet P-40 (NP-40) and 0.5 M NaCl. Insoluble matter was removed by high-speed centrifugation in a Beckman airfuge at 30 lb/in 2 ($160,000 \times g$) for 1 h. The clarified cell lysates were denatured by heating at 80°C for 2 to 3 min in disruption buffer consisting of 2% sodium dodecyl sulfate, 5% β -mercaptoethanol, and 50 mM Tris-

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FIG. 1. Autoradiographic images of DNA end labeled with ^{32}P bound to electrophoretically separated uninfected and infected cell polypeptides: the effect of NaCl concentration and NP-40 on the capacity of the 130,000-molecular-weight host protein to bind DNA. HEp-2 cells were infected with 10 PFU of HSV-1(F) per cell and then harvested at either 0 (immediately after virus adsorption), 6, or 24 h postinfection. The clarified lysates were subjected to electrophoresis, transferred to nitrocellulose, and exposed to 10^6 cpm (Cerenkov) of ^{32}P end-labeled pRB370 DNA (about 0.25 μg of DNA). All lanes are from the same gel and all cell lysates are from the same preparation. Before blotting with the DNA probe, the nitrocellulose-immobilized proteins were soaked in a 1% NP-40 solution, as described in the text, or left untreated. The DNA binding reaction was carried out as described in the text in the presence of either 100 or 250 mM NaCl. The arrow indicates the position of the 130,000-molecular-weight host protein. The numbers to the left and right of the figure identify HSV-1 ICPs.

hydrochloride (pH 7.0) and subjected to electrophoresis in 9.25% polyacrylamide gels crosslinked with N,N,N',N' -tetramethylethylenediamine and containing sodium dodecyl sulfate, as described elsewhere (5). After electrophoresis, the proteins were transferred electrically onto a nitrocellulose sheet in a buffer containing 20% methanol (vol/vol), 25 mM Tris (pH 7.4), and 190 mM glycine (21).

In some experiments, as noted, the nitrocellulose sheet containing the electrically transferred proteins was soaked in a buffer containing 10 mM Tris, 1 mM EDTA, and 1% NP-40 overnight at room temperature (21°C) and then washed extensively in the same buffer without NP-40.

DNA-binding reaction. The nitrocellulose sheets containing the immobilized proteins were reacted with pRB370 DNA end labeled with ^{32}P . Routinely, a nitrocellulose sheet (25 by 25 cm) was exposed to 10^6 cpm (Cerenkov). The nitrocellulose sheet was placed in a plastic bag, and the DNA probe was added in a 10-ml volume of a buffer consisting of 8% glycerol, 10 mM Tris, 1 mM EDTA, and 250 mM NaCl and 1 mM β -mercaptoethanol. The reaction was carried out for 3 h in a 37°C water bath. After extensive washing with the same buffer, the nitrocellulose sheet was dried and exposed to Kodak XS-1 film. The exposure time never exceeded 10 h.

Cell fractionation. Cells were harvested by scraping, washed three times in cold phosphate-buffered saline, and then suspended in a buffer (buffer A) containing 1.6 mM MgCl_2 , 6 mM KCl, 10 mM Tris, 1 mM dithiothreitol, 0.5%

NP-40, and 10^{-5} M N - α - p -tosyl-L-lysine chloromethyl ketone hydrochloride (Sigma Chemical Co.) and L-1-tosylamide-2-phenyl-ethyl-chloromethyl ketone (Sigma). They were then incubated on ice for 15 min followed by blending with a Vortex mixer for 1 min. The nuclei were pelleted by centrifugation at $5,000 \times g$ for 10 min. The supernatant fluid was collected and was designated the cytoplasmic fraction. The nuclei were washed twice in 140 mM NaCl–10 mM Tris–1 mM dithiothreitol and then resuspended in buffer A. NP-40 and NaCl were added to each fraction to final concentrations of 1% and 500 mM, respectively. The fractions were frozen at -80°C until needed.

RESULTS

Effect of NaCl concentration and NP-40 on the capacity of a 130,000-molecular-weight host protein to bind DNA. In preliminary experiments, we observed that a polypeptide contained in HEp-2 cell lysates electrophoretically separated in denaturing polyacrylamide gels and electrically transferred to nitrocellulose sheets bound DNA and that this activity could not be detected after infection with wild-type virus. The purpose of the experiments described below was to determine the effect of the nonionic detergent NP-40 and of the NaCl concentration on the binding of DNA by this protein.

In these experiments, lysates of mock-infected cells and

cells harvested at 6 and 24 h postinfection with HSV-1(F) were solubilized in disruption buffer, subjected to electrophoresis in polyacrylamide gels, and transferred electrically to nitrocellulose sheets as described above. The nitrocellulose sheets were then either soaked overnight in a buffer containing 1% NP-40 or left untreated. The nitrocellulose sheets were then blotted with linearized, double-stranded pRB370 DNA end labeled with [α - 32 P]dCTP as described above. After extensive washing, the nitrocellulose sheets were dried and pressed against Kodak XS-1 film for autoradiography.

The results (Fig. 1) indicate that the DNA binding capacity of the host protein, identified by the arrow, was higher in uninfected than in cell lysates infected for 24 h, under all conditions tested. The differences were largely esthetic; the number of both uninfected and infected DNA-binding protein bands was higher at the lower salt concentrations than at the higher salt concentrations in the absence of the detergent. All subsequent DNA-binding assays were carried out in 250 mM NaCl without the NP-40 treatment.

Characterization of the host DNA-binding protein. The purpose of this series of experiments was to characterize the DNA-binding activity of the host protein identified by the arrow in Fig. 1. In denaturing polyacrylamide gels, the protein migrated faster than infected cell polypeptide 6 (ICP6) but slower than ICP8 and had an apparent molecular weight of 130,000 (Fig. 2).

The DNA-binding activity of the host protein does not appear to be sequence specific. The host protein bound a variety of plasmid vectors without and with DNA sequences derived from various regions of the HSV-1 genome (Fig. 2; some data not shown), and within the limits of the experimental design, no DNA sequence preference was observed. For example, pBR322 DNA and pRB370 DNA bound equally well (Fig. 2, lanes 4 and 6). In all subsequent experiments, only pRB370 DNA was used as a probe.

The host DNA-binding protein strongly prefers double-stranded DNA. This conclusion is based on the results illustrated in Fig. 3. In this experiment, a nitrocellulose sheet containing transferred, electrophoretically separated Hep-2 proteins was cut into identical strips. Lane 3 (Fig. 3) was reacted with 10^6 cpm (Cerenkov) of linearized, native pRB370 DNA end labeled with 32 P, whereas lane 4 (Fig. 3) was reacted with an identical amount of the same DNA, except that it was denatured before blotting.

Figure 4 (lanes 1 and 2) indicates that the DNA-binding activity associated with the ca. 130,000-molecular-weight host protein partitioned with the cytoplasmic fraction.

Since HSV-1 is known to inhibit host macromolecular synthesis soon after infection, and since the host DNA-binding activity was progressively reduced after infection, it seemed necessary to determine whether the decline in binding activity could be explained by the hypothesis that the DNA-binding host protein turns over rapidly and is not replaced in infected cells. To test the hypothesis that the DNA-binding host protein turns over rapidly, HEP-2 cells were treated with cycloheximide (50 μ g/ml of medium). At various intervals thereafter, the cells were harvested, lysed, subjected to electrophoresis in denaturing polyacrylamide gels, transferred to nitrocellulose sheets, and reacted with pRB370 DNA end labeled with 32 P. The results of a representative experiment are shown in Fig. 4 (lanes 3 through 9). The conclusion of this series of experiments is that the DNA-binding activity is not demonstrably affected in uninfected cells treated with cycloheximide for as long as 24 h.

Identification of the viral function responsible for the shutoff

FIG. 2. Autoradiographic images of DNA end labeled with 32 P bound to electrophoretically separated uninfected and infected cell polypeptides: sequence specificity of the DNA-binding, 130,000-molecular-weight host protein. Cell lysates from mock-infected (M) or 24-h, HSV-1(F)-infected (I) HEP-2 cells were denatured in disruption buffer and subjected to electrophoresis in denaturing polyacrylamide gels. The electrophoretically separated polypeptides were then transferred to nitrocellulose sheets. The arrow indicates the positions of the 130,000-molecular-weight host protein. Lanes 1 and 2 show [35 S]methionine profiles serving as molecular weight markers. Lanes 3 and 4 were exposed to 2×10^5 cpm (Cerenkov) of pBR322 DNA end labeled with 32 P. Lanes 5 and 6 were reacted with 2×10^5 cpm (Cerenkov) of pRB370 DNA end labeled with 32 P. The faint background seen in lanes 3 through 6 is due to [35 S]methionine and was used to align the tracks. Lanes 1 and 2 were exposed to Kodak XS-1 X-ray film for 36 h, whereas lanes 3 through 6 were exposed for 2 h. Numbers to the right of lane 2 identify the ICPs.

of the DNA-binding activity of the 130,000-molecular-weight host protein. The studies described in the preceding section suggest that the DNA-binding activity of the host protein is inherently stable and therefore the observed loss in the DNA-binding activity after infection is due to expression of viral functions. In an attempt to define which set of viral functions was responsible for the loss of DNA-binding activity, the following experiments were carried out.

(i) HSV-1(HFEM)/*ts*LB2 specifies a temperature-sensitive ICP4, whereas viral replication is normal at the permissive temperature (33°C); at the nonpermissive temperature (39°C), it makes α -gene products but not β - and γ -gene

FIG. 3. Autoradiographic images of DNA end labeled with ^{32}P bound to electrophoretically separated polypeptides from mock-infected and infected cells: differential binding of single-stranded and double-stranded DNA to the 130,000-molecular-weight host protein. Lanes 1 and 2 contain electrophoretically separated, electrically transferred polypeptides from HSV-1(F) infected for 24 h (I) and mock-infected (M) HEP-2 cells, respectively, labeled with [^{35}S]methionine; they were used for molecular weight markers. Lanes 3 and 4 contain polypeptides from mock-infected cells; they were cut out from the same gel slab. Lane 3 was exposed to 10^6 cpm (Cerenkov) of ^{32}P end-labeled pRB370 double-stranded (d.s.) DNA end labeled with ^{32}P . The polypeptides in lane 4 were exposed to 2.0×10^5 cpm (Cerenkov) of the same pRB370 DNA stock as used in lane 3, but they were rendered single stranded (s.s.) by heating in a boiling water bath for 5 min followed by rapid cooling in wet ice. Lanes 3 and 4 were exposed to Kodak XS-1 X-ray film for 2 h.

products. As shown in Fig. 5 (lanes 1 through 6), no loss in DNA-binding activity was detected in cells infected with this mutant and maintained at 39°C . This experiment suggests that the loss in DNA-binding activity is due to either ICP4 or a β - or γ -gene function.

(ii) To determine whether the loss in DNA-binding activity is caused by ICP4, a series of cycloheximide reversal experiments was carried out (Fig. 6A). Thus, the DNA binding activity was lost when cycloheximide inhibition was reversed in the absence of additional treatment of the infected cells (Fig. 6A, lane 3) but not when the cycloheximide was withdrawn in the presence of actinomycin D (Fig. 6A, lane 4); this procedure allowed the synthesis of ICP4 by the preformed ICP4 mRNA but not the synthesis of later gene products (e.g., β or γ). The conclusions derived from the cycloheximide reversal experiments were further sup-

ported by the results of a series of temperature shift-down experiments with mutant HSV-1(HFEM)*ts*LB2. When the cells were shifted down from 39 to 33°C , the DNA-binding activity was lost (Fig. 6B, lane 3). However, when the shift down was carried out in the presence of cycloheximide, the activity was not lost (Fig. 6B, lane 4), thus indicating that the shutoff is not due to α -gene expression. These results indicate that the loss in the DNA-binding activity is due to β - or γ -gene products but not α -gene products, including ICP4.

(iii) HSV-1(mP)*ts*HA1 carries a lesion in the gene specifying ICP8, a β -gene product essential for viral DNA synthesis. At the nonpermissive temperature (39°C), the cells infected with this mutant accumulate α and β proteins and only some of the γ -gene products (2). The DNA-binding activity shuts off normally in *ts*HA1-infected cells maintained at the nonpermissive temperature (39°C) (Fig. 5, lanes 7 through 12). These results suggest that the loss of the DNA-binding activity of the host protein was not related to ICP8, viral DNA synthesis, or viral proteins stringently dependent on viral DNA replication for their synthesis.

(iv) The conclusion that the loss of the capacity to bind DNA by the host protein is not due to viral DNA synthesis or γ -gene products dependent on viral DNA replication for their synthesis is reinforced by the observation that the loss of DNA-binding capacity is unaffected by inhibiting doses of phosphonacetate (Fig. 6A, lanes 5 and 6).

FIG. 4. Autoradiographic images of DNA end labeled with ^{32}P bound to electrophoretically separated proteins from nuclei, cytoplasm, and whole HEP-2 cells treated with cycloheximide. HEP-2 cells were treated with $50 \mu\text{g}$ of cycloheximide per ml of medium for 0, 1, 2, 4, 8, 16, and 24 h (lanes 3, 4, 5, 6, 7, 8, and 9, respectively). The cells were then harvested. The electrophoretically separated, transferred polypeptides were tested for the reactivity of the host protein. In lanes 1 and 2, HEP-2 cells were fractionated into cytoplasmic (lane 1) and nuclear (lane 2) fractions. The arrow indicates the position of the 130,000-molecular-weight host protein.

DISCUSSION

In this paper, we report that a Hep-2 cell protein lost its capacity to bind DNA after infection of the cells with HSV and that this loss could not be explained by rapid turnover of the host protein. The host protein has an electrophoretic mobility in denaturing polyacrylamide gels which is characteristic of proteins with molecular weights of 130,000; it accumulates in the cytoplasm and has an affinity for double-stranded DNA. The function of this protein in uninfected cells is unknown.

Previous studies have shown that the inhibition of host macromolecular synthesis in cells infected with HSV takes place in at least two stages. The earliest effect on host DNA and protein synthesis occurs immediately after exposure of cells to virus and does not require de novo synthesis of viral proteins (6, 8, 14). The second and more extensive shutoff of host macromolecular metabolism coincides with the onset of synthesis of viral proteins specified by β genes or possibly early γ genes (6, 11, 13). The studies reported in this paper indicate that the loss of the capacity to bind double-stranded

FIG. 5. Autoradiographic images of DNA bound to electrophoretically separated, transferred proteins from Hep-2 cells infected with temperature-sensitive mutants and maintained at permissive or nonpermissive temperatures. Hep-2 cells were infected with 10 PFU of HSV-1(HFEM)*tsLB2* per cell (lanes 1 through 6) or with HSV-1 (mP)*tsHA1* (lanes 7 through 12) at the nonpermissive temperature (39°C; lanes 1, 2, 3, 7, 8, and 9) or at the permissive temperature (33°C; lanes 4, 5, 6, 10, 11, and 12). The cells were harvested and processed for electrophoresis at 0 (immediately after virus adsorption), 6, and 24 h postinfection. The electrophoretically separated polypeptides were transferred to a nitrocellulose sheet and then exposed to 10^6 cpm (Cerenkov) of pRB370 DNA end labeled with 32 P. The arrow indicates the position of the 130,000-molecular-weight host protein.

FIG. 6. Autoradiographic images of DNA bound to electrophoretically separated, transferred proteins from Hep-2 cells that were mock-infected, infected with HSV-1(F), or infected with HSV-1(HFEM)*tsLB2*: evidence that the loss of DNA-binding capacity is not caused by an α -gene function. (A) Lanes 1 through 6 results of cycloheximide reversals. Lane 1: Mock-infected Hep-2 cells maintained in the presence of cycloheximide (50 μ g/ml) for 6 h and then reversed for 15 h; lane 2, Hep-2 cells infected with HSV-1(F) (10 PFU per cell) for 21 h without inhibitors; lane 3, Hep-2 cells infected with 10 PFU of HSV-1(F) per cell and maintained for 6 h in the presence of cycloheximide (50 μ g/ml), the drug was then replaced with fresh medium and maintained for 15 h; lane 4, same as in lane 3, except that cycloheximide was removed in the presence of actinomycin D (10 μ g/ml); lane 5, Hep-2 cells infected with HSV-1(F) (10 PFU per cell) and maintained in the presence of 300 μ g of phosphonoacetate per ml for 21 h; lane 6, mock-infected Hep-2 cells maintained in 300 μ g of phosphonoacetate per ml for 21 h. (B) Lane 1, Mock-infected Hep-2 cells; lane 2, Hep-2 cells infected with HSV-1(HFEM)*tsLB2* (10 PFU per cell) and maintained at the permissive temperature for 21 h; lane 3, same as in lane 2, except that the infected cells were maintained at the nonpermissive temperature (39°C) for 8 h and then shifted to the permissive temperature (33°C) for 13 h; lane 4, same as lane 3, except that at the time of the shift down from 39 to 33°C, cycloheximide (50 μ g/ml) was added, and the cultures were maintained until 21 h postinfection. The cells were harvested and processed for electrophoresis in denaturing gels. The electrophoretically separated polypeptides were then transferred to nitrocellulose and were exposed to 10^6 cpm (Cerenkov) of pRB370 DNA end labeled with 32 P. The arrow indicates the position of the 130,000-molecular-weight host protein.

DNA by the 130,000-molecular-weight host protein is associated with products of β or γ genes whose expression is not stringently dependent on viral DNA synthesis. Additional studies with mutants in specific viral genes will be necessary to identify the viral function responsible for the observed

loss in the capacity to bind DNA and to determine whether the host protein is degraded or merely modified in the course of HSV infection.

The turnoff of host macromolecular metabolism observed in cells infected with HSV includes the shutoff of transcription and translation of host nucleic acids (15, 18–20). The shutoff could occur either by modification of the templates as a result of binding of viral gene products to cellular DNA and mRNA or as a consequence of specific effects of viral gene products on host proteins. Although it is not clear whether one or both of these potential mechanisms prevails, it is likely, nevertheless, that viral gene products do interact with and modify the function of host proteins. It remains to be seen whether the observed loss in the capacity to bind DNA is an example of a specific modification of a host protein function by viral gene products. If this is the case, it would be the first such instance.

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