Acta virol. **33** : 401—409, 1989

IN SITU DETECTION OF DNA-BINDING PROTEINS IN HERPES SIMPLEX VIRUS TYPE 1-INFECTED CELLS

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Received June 15, 1989

Summary. — An *in situ* assay for detecting DNA-binding proteins in herpes simplex virus type 1 (HSV-1)-infected cells is described. Seventeen HSV-induced DNA-binding species were visible with nicked, double-stranded DNA as a substrate, while fourteen virus-induced DNA-binding fractions were present in gels containing nuclease-treated, single-stranded DNA. The effects of HSV on cellular DNA-binding protein expression could also be seen. The resolution of DNA-binding fractions was dependent upon the type of DNA substrate utilized, high salt extraction of DNA-binding components and their physical separation from infected cell DNAs, dialysis of the high salt and the length of DNase treatment of gels following electrophoresis.

Key words: HSV-1 DNA-binding protein detection

Introduction

The efforts of this laboratory have been directed toward the development of *in situ* assays to detect herpes simplex virus (HSV)-induced polypeptides that could interact with and alter host cell DNA. We recently described an *in situ* technique, which can distinguish between viral and cellular alkaline nuclease activities that arise as the result of HSV infection (Hafner *et al.*, 1987*a*; Hafner *et al.*, 1987*b*). HSV types 1 and 2 encode at least 70 viral polypeptides. Using DNA-cellulose affinity chromatography, 15—20 infected cell proteins have been reported to bind to DNA (Bayliss *et al.*, 1975; Purifoy *et al.*, 1976; Powell and Purifoy, 1977). The most extensively studied HSV DNA-binding proteins are viral DNA polymerase (Powell and Purifoy, 1977) and a protein designated as ICP8 (Powell *et al.*, 1981; Lee and Knipe, 1983; Littler *et al.*, 1983).

This report describes an *in situ* assay for detecting DNA-binding proteins in HSV-1-infected cells. The principle of the assay is as follows: an infected cell extract containing proteins that have been solubilized and separated from the DNA is subjected to electrophoresis in a polyacrylamide gel impreg-

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nated with a DNA substrate. By definition, DNA-binding proteins would preferentially bind to endogenous DNA. Therefore, a critical aspect of this procedure was to extract and solubilize proteins bound to host cell DNA with high salt, as well as precipitate out viral and cellular DNAs that could compete with *in situ* binding to the DNA substrate. Following electrophoresis, individual protein fractions with DNA-binding ability are visualized by digesting the gel with DNase. Everywhere DNA has been bound to and protected by a DNA-binding protein, DNase digestion will not occur, and the band representing a DNA-binding protein is stainable by ethidium bromide (EtBr).

Materials and Methods

Cells and virus. African green monkey kidney (Vero) cells (4.5×10^6) propagated as described previously (Hafner *et al.*, 1987*a*), were infected with HSV-1 strain KOS (Smith, 1964) at an MOI of 20 PFU/cell, incubated at 39 °C and harvested 18 hr post-infection (p.i.).

Sample preparation. Cell pellets were obtained, processed and stored as previously described (Hafner *et al.*, 1987*a*). Putative DNA-binding proteins were solubilized by a modification of two earlier procedures (Purifoy *et al.*, 1976; Crawford *et al.*, 1982). Frozen cell pellets were suspended in 20 mmol/l Tris-HCl (pH 7.5) containing 0.5 mol/l dithiothreitol (DTT), 1 % Nonidet P-40 (NP-40, Shell Oil Co.), 0.25 mol/l phenyl methyl sulphonyl fluoride (PMSF, Sigma Chemical Co.), and 50 μg/ml of bovine serum albumin (BSA, Sigma). An equal volume of a solution containing KCl and EDTA was added to yield a final concentration of 1.7 mol/l and 5 mmol/l, respectively. After a 40 min incubation at 0 °C, the resulting precipitate was removed by centrifugation for 20 min at 11 600 g at 4 °C in a Beckman microfuge. The supernatant was dialyzed for 1 hr at 4 °C against three changes of buffer containing 50 mmol/l Tris-HCl (pH 7.5), 0.5 mmol/l DTT, 10 % glycerol and 0.2 % NP-40. Dialyzed cell extracts were denatured for 2 min at 95 °C in 2 × sample buffer consisting of 2.3 % SDS, 5 % 2-mercaptoethanol, 69.5 mmol/l Tris-HCl (pH 6.8) and 10 % glycerol.

Polyacrylamide gel electrophoresis. Calf thymus DNA (Sigma) was purified as described before (Hafner *et al.*, 1987*a*). To prepare single-stranded DNA, the native form was heated for 5 min at 100 °C and rapidly cooled on ice before incorporation into molten polyacrylamide. Samples were electrophoresed using a Hoefer Model SE 620 vertical slab gel apparatus in a 7—15 % linear polyacrylamide gel (pH 8.8) containing either 100 μg/ml of single-stranded or double-stranded DNA and 10 μg/ml BSA that had been boiled for 10 min to destroy contaminating nuclease. A modified (O'Farrell, 1975) discontinuous SDS buffer system (Laemmli, 1970) was utilized and electrophoresis was carried out at 20 mA constant current.

DNA-binding protein visualization. Following electrophoresis, the gel was washed as previously described (Hafner *et al.*, 1987*a*), rinsed in water and placed in 200 ml of buffer containing 40 mmol/l Tris-HCl (pH 7.5), 5 mmol/l $MgCl_2$ and 20 μg/ml DNase I (Sigma). After DNase digestion for 20 min at room temperature, the gel was washed, stained with EtBr and photographed as described before (Hafner *et al.*, 1987*a*). Molecular weight determinations using comigrating protein standards were performed as previously described (Hafner *et al.*, 1987*a*).

Results

Effects of DNase treatment and centrifugation on DNA-binding protein visualization

Fig. 1 depicts a representative gel of HSV-induced DNA-binding proteins isolated from cells incubated at 39 °C for 18 hr. Single-stranded calf thymus DNA served as the substrate. This temperature was chosen to serve as a base-line for future work on comparative *in situ* assays using wild-type virus

and temperature-sensitive mutants under restrictive conditions (39 °C). Time course experiments (data not shown) demonstrated that the level of expression of infected cell DNA-binding proteins increased with time, and displayed maximal activity 18 hr post-infection.

Following electrophoresis, the gel was cut in the middle of lane 7. Lanes 1-"7.5" received a 40 min DNase treatment, while the remaining lanes, "7.5"-11, were exposed to DNase for 20 min. The right-hand portion of the gel demonstrates that more bands were visible in the lanes that received shorter DNase treatment. See for comparison lane 6, a mock-infected cell sample, which was treated for 40 min with DNase and lane 10, an aliquot of the same mock-infected cell sample that was exposed to DNase for 20 min.

Another feature of this gel is that the number and intensity of DNA-binding fractions were greater in samples that had been subjected to centrifugation at 11 600 g for 20 min versus duplicate samples that were centrifuged for 1 hr at 100 000 g. This is especially evident in lanes 8 and 9, which represent mock- and HSV-infected cell samples, respectively, that have

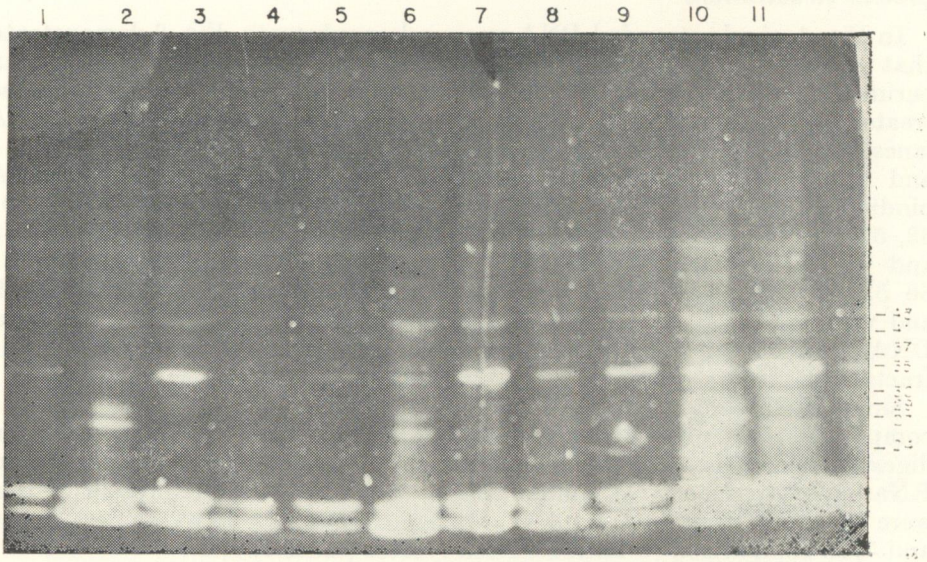

Fig. 1.

Effects of DNase Treatment and Centrifugation on DNA-Binding Protein Visualization
Lanes 1, 3, 5, 7, 9, and 11 contain HSV-infected cell proteins, while lanes 2, 4, 6, 8, and 10 represent mock-infected cell extracts. The gel containing lanes 1-"7.5" received a 40 min DNase treatment, whereas the remaining gel with lanes "7.5"-11 was exposed to DNase for 20 min. Cell extracts run in lanes 1, 4, 5, 8, and 9 were obtained following centrifugation at 100 000 g for 1 hr, while lanes 2, 3, 6, 7, 10, and 11 represent cell extracts obtained following centrifugation at 11 600 g for 20 min. All extracts depicted here were dialyzed as described in *Materials and Methods*. Single-stranded calf-thymus DNA served as the substrate. Molecular weights in kD of cellular DNA-binding proteins are denoted at the right of the figure.

undergone ultracentrifugation at 100 000 g, and in lanes 10 and 11, which are the corresponding mock- and HSV-infected cells subjected to microcentrifugation at 11 600 g. Here, mock-infected cell DNA-binding species with molecular weights of 24, 27, and 30 kD are visible in lane 10, but not in lane 8. Based on the preceding findings, cell extracts were routinely prepared by centrifugation at 11 600 g for 20 min and polyacrylamide gels were exposed to a 20 min DNase treatment following electrophoresis. DNA-binding fractions could not be detected in the absence of enzymatic treatment (data not shown).

Focusing on cellular DNA-binding bands with molecular weights of 35, 29, 27, and 24 kD, which appeared both in mock- and virus-infected cell profiles, it may be observed that HSV stimulated the expression of the 35 kD moiety (lanes 3, 7, and 11), while synthesis of the 29, 27, and 24 kD proteins appeared to be inhibited by the virus [lane 10 (mock-infected sample) versus lane 11 (HSV-infected cells)].

Effect of DNA substrate, RNase treatment and dialysis on DNA-binding protein visualization

In Fig. 1, single-stranded DNA was used as substrate. Fig. 2 demonstrates that more DNA-binding species were resolvable than in the previous experiment when either nicked, double-stranded DNA (Fig. 2A) or nuclease-treated, single-stranded DNA (Fig. 2B) served as substrates. In Fig. 2A, lanes 1, 3, 5, and 7 represent HSV-infected cell extracts, whereas lanes 2, 4, and 6 contain mock-infected cell lysates. Seventeen virus-induced DNA-binding species with molecular weights of 175, 150, 138, 130, 110, 97, 91, 58, 53, 51, 47, 38, 25, 24.5, 21, 18, and 15 kD can be seen in lanes 1, 3, 5, and 7. Cellular DNA-binding proteins with molecular weights of 83, 71, 56, 53, 50, 47, 42, 29, 27, 23.5, 21.5, and 21 kD were apparent in both mock- and virus-infected cell profiles. Moreover, the expression of certain cellular DNA-binding protein bands, i.e. the 42, 31, 29, 27, 25, 23.5, 21.5, and 18 kD fractions, appeared to be enhanced by virus infection.

Since EtBr could conceivably stain both DNA and RNA molecules, a comparison was made of DNA-binding fractions in cell lysates that were digested with RNase versus cell fractions that were electrophoresed without RNase pretreatment. No differences in the DNA-binding protein profiles were observed in cell fractions that were exposed to RNase (lanes 2, 3, 6, and 7) in comparison to untreated cell lysates (lanes 1, 4, 5).

Fig. 2B depicts an assay in which nuclease-treated, single-stranded DNA was used as the substrate. In this experimental situation, fourteen HSV-induced DNA-binding proteins were evident (lanes 2, 4, 6), of which eleven with molecular weights of 175, 150, 138, 130, 110, 97, 91, 58, 53, 51, and 38 kD were analogous to DNA-binding species found in gels containing nicked, double-stranded DNA. However, three other DNA-binding protein fractions with molecular weights of 64, 36, and 19 kD appeared in conjunction with this type of DNA substrate, while the 25, 24.5, 21, 18, and 15 kD virus-induced moieties associated with nicked, double-stranded DNA substrates

were not present. Cellular DNA-binding fractions that appeared in both mock- (lanes 1, 3, 5, and 7) and virus-infected cell profiles with molecular weights of 83, 56, 45, 40, 31, 29, 28, 27, 25.5, 24.5, 24, and 21 kD were more prominent in HSV-infected cells (lanes 2, 4, 6). Interestingly, the 31 kD cellular DNA-binding protein was markedly stimulated in gels containing either the nicked, double-stranded DNA substrate or nuclease-treated single-stranded DNA. Once again, no significant differences could be seen in the DNA-binding protein profile of samples that were treated with RNase (lanes 3, 4, 7) and in those that were not (lanes 1, 2, 5, 6). On the basis of the RNase results, this enzymatic treatment was omitted in subsequent experiments.

Fig. 3A illustrates that the resolution of DNA-binding proteins is considerably less in gels impregnated with native, double-stranded DNA. Although DNA-binding bands of molecular weights 175, 150, 138, 110, 97, 91, 87, 82, 79, and 55 kD were just faintly visible in polaroid negatives (data not shown), only one virus-induced DNA-binding fraction, a 51 kD moiety (lane 3) and eight cellular bands (45, 43, 38, 37, 31, 30, 28.5, and 23 kD) were visible in the Polaroid positive (lane 2). Another feature of Fig. 3A that

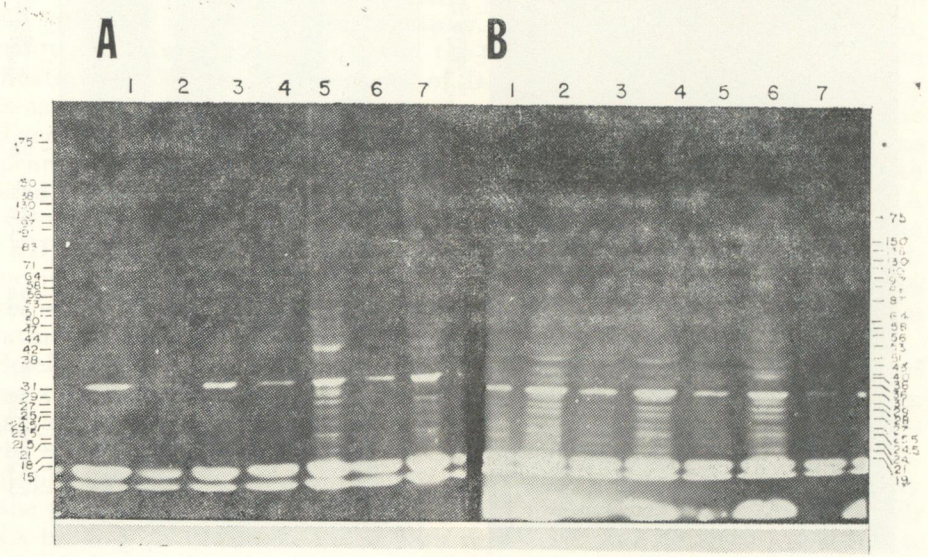

Fig. 2.

Effects of DNA Substrate and RNase Treatment on DNA-Binding Protein Visualization
Samples were divided into equal aliquots and analysed for DNA-binding activity on gels containing either nicked, double-stranded calf-thymus DNA (A) or nuclease-treated, single-stranded DNA (B). Lanes A2, A4, A6, B1, B3, B5, and B7 contain mock-infected cell extracts, while lanes A1, A3, A5, A7, B2, B4, and B6 were run with HSV-1-infected cell extracts. Lanes A2, A3, A6, A7, B3, B4, and B7 represent samples that were treated with RNase. Samples in lanes A1, A4, A5, B1, B2, B5, and B6 received no RNase treatment. All samples were dialyzed and gels received a 20 min DNase treatment following electrophoresis. Molecular weights in kD of all DNA-binding proteins visualized are at the right and left of the gels.

is particularly obvious under experimental conditions such as this is that dialysis to remove high salt concentrations enhanced resolution of the aforementioned proteins. See for comparison lanes 2 and 3 (dialyzed fractions of mock- and HSV-infected cell extracts) and lanes 1 and 4 (undialyzed fractions of mock- and virus-infected cells). It should be noted that all samples depicted in Fig. 1 and Fig. 2A and B were dialyzed prior to electrophoresis.

Fig. 3B represents duplicate samples of those in lanes 1—3 that were run on the same gel but stained with Coomassie Blue. All EtBr-stained proteins present in Fig. 3A were also detectable by Coomassie Blue. However, other proteins stainable by Coomassie Blue could be found in addition to the

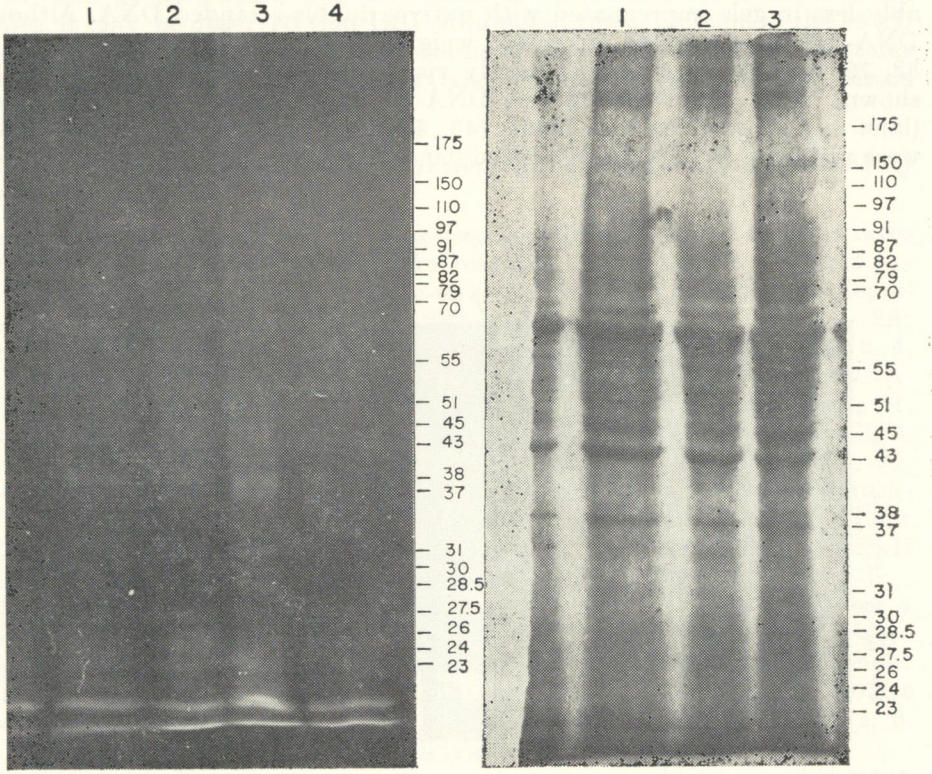

Fig. 3.

Effects of DNA Substrate, Dialysis, and Staining Method on DNA-Binding Protein Visualization The gel, which contained a double-stranded calf-thymus DNA substrate, was exposed to a 20 min DNase treatment before EtBr staining (A) or Coomassie Blue staining (B). Lanes 1 and 3 in A represent HSV-infected cell proteins, while lanes 2 and 3 contain mock-infected cell extracts. Samples in lanes 1 and 4 were undialyzed, whereas cell extracts in lanes 2 and 3 were dialyzed prior to electrophoresis. Lanes 1 and 3 of the Coomassie Blue-stained gel (B) represent HSV-infected cell proteins, while the protein profile in lane 2 is that of mock-infected cells. Numerals to the right of each gel represent the molecular weights in kD of DNA-binding proteins.

corresponding DNA-binding protein fractions. These data indicate that the EtBr-stained bands visualized by the *in situ* assay are DNA-binding proteins.

Another experimental parameter was examined (data not shown), namely, to determine whether non-specific binding between the negatively-charged DNA substrate and positively-charged polypeptides could occur. This was tested by electrophoresing two positively-charged proteins — lysozyme and cytochrome C — in a polyacrylamide DNA-containing gel. After EtBr-staining, no bands corresponding to these polypeptides and their respective molecular weights were observed. This suggests that the DNA-binding proteins visualized by the *in situ* technique are, normally, DNA-binding components.

Discussion

Although it was not tested in the present study, based on earlier investigations, the species of DNA does not seem to play a role in the binding efficiency of HSV DNA-binding components. Both calf-thymus and salmon sperm DNAs yielded analogous HSV-specific DNA-binding proteins in DNA cellulose affinity chromatography experiments (Bayliss *et al.*, 1975; Purifoy and Powell, 1976). However, the findings of the present investigation indicate that the molecular species of DNA-binding proteins resolvable by this assay is dependent on the molecular architecture of the DNA substrate utilized. For example, fewer virus-induced DNA-binding protein fractions appeared in gels containing native, double-stranded DNA than in gels with nicked, double-stranded DNA or nuclease-treated single-stranded DNA as substrates. All high molecular weight DNA-binding species (greater than 64 kD) were relatively faint, regardless of the DNA substrate employed. Conceivably, the gene copy numbers of these fractions were low, which, in turn, could lead to weak interactions with DNA. Upon further DNase treatment of the gel, these proteins might be readily lost.

Solely on the basis of their molecular weights, the presumed functions of some of the HSV-induced DNA-binding proteins detected by the *in situ* assay are as follows: the 175 kD polypeptide could represent the immediate-early transcriptional regulatory protein, ICP4 (Dixon and Schaffer, 1980). This protein has been shown to bind *in vitro* to single-stranded DNA only in the presence of a cellular fraction (Freeman and Powell, 1982). A 130 kD DNA-binding protein, ICP8, has been reported in the HSV-1 system. It is essential for viral DNA synthesis, binds preferentially to single-stranded DNA (Purifoy *et al.*, 1977), which holds it in an extended configuration (Ruechan, 1983) and acts to stabilize viral DNA, DNA polymerase, and alkaline nuclease (Powell *et al.*, 1981). The 130 kD DNA-binding species seen *in situ*, could, therefore, correspond to the 130 kD DNA-binding protein described by others. The 110 kD HSV-induced DNA-binding species detected *in situ* may be equivalent to the immediate-early protein ICP0. It has been suggested that this regulatory polypeptide might be involved in the switch from the latent to the lytic mode of HSV infection (Sacks and Schaffer,

1987). The molecular weight of HSV DNA polymerase has been reported to range from 145—150 kD (Powell and Purifoy, 1977). Therefore, the 150 kD fraction appearing in the present study may represent viral DNA polymerase.

An *in situ* assay developed for HSV alkaline nuclease yielded virus alkaline nuclease-induced species of 90, 85, and 65 kD (Hafner *et al.*, 1987*a*). Consequently, the 91, 85, and 65 kD virus-induced DNA-binding proteins visualized *in situ* could be equated with polypeptides possessing nuclease activity. It is noteworthy that studies from this and another laboratory demonstrated the existence of cooperative interactions between viral alkaline nuclease, ICP8, and DNA polymerase (Littler *et al.*, 1983; Hafner *et al.*, 1987*b*), which provided evidence for the existence of a DNA replication complex in the HSV system. Two virus-induced DNA-binding fraction with molecular weights of 55 and 51 kD observed *in situ* could correspond to the 55 and 51 kD polypeptides that have been characterized as HSV-1 structural proteins (Wagner, 1985). The 55 kD DNA-binding protein which appeared in both double-stranded and single-stranded DNA-containing gels, is of particular interest since it has been reported to be an internal capsid component that may function in packaging or anchoring viral DNA (Braun *et al.*, 1984).

Using DNA cellulose chromatography, sixteen HSV-1 (Bayliss *et al.*, 1975) and seventeen HSV-2 (Purifoy and Powell, 1976) DNA-binding proteins have been isolated from infected cells. In the present study, seventeen HSV-induced DNA-binding species were visible with nicked, double-stranded DNA, and fourteen were apparent with nuclease-treated single-stranded DNA. Four of the HSV-induced DNA-binding proteins with molecular weights of 150, 138, 130, and 64 kD detected by the *in situ* assay may be analogous to those of comparable molecular weights obtained by DNA cellulose chromatography of HSV-1-infected cell extracts (Bayliss *et al.*, 1975). The differences in molecular weights between the remaining virus-induced DNA-binding proteins visualized here and those reported previously, could be explained by the fact that different experimental systems were utilized.

Certain advantages are inherent in this type of methodology. An *in situ* approach obviates the necessity for extensive purification of the proteins in question and SDS-polyacrylamide gel electrophoresis provides the rapid separation of proteins into discrete fractions. Moreover, unlike sequential extraction procedures, proteins would not tend to be lost by the *in situ* technique. Additional resolution of protein moieties was afforded by the use of linear gradient polyacrylamide gels rather than fixed polyacrylamide concentrations employed in previous studies. Another powerful advantage of the *in situ* method is that viral and cellular gene products can be detected simultaneously and independently of one another. Thus, the temporal sequence and level of expression of all DNA-binding species — both viral and cellular — can be discerned at a given moment during the replicative cycle. This would not be possible with other contemporary protein purification techniques. The stimulatory, as well as inhibitory effects of HSV on the expression of cellular DNA-binding proteins were clearly evident in this

investigation. Once they become available, monoclonal antibodies to individual HSV DNA-binding proteins will be useful for unequivocally establishing the identity of the virus-induced DNA-binding proteins visualized *in situ*.

Acknowledgments. The authors gratefully acknowledge the financial support of Hussain Makki Al Juma, helpful discussions of W. McGrew and manuscript preparation by L. DiBernardo.

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