JOURNAL OF VIROLOGY, May 1983, p. 661–666
0022-538X/83/050661-06$02.00/0

Vol. 46, No. 2

The Major Herpes Simplex Virus DNA-Binding Protein Holds Single-Stranded DNA in an Extended Configuration

WILLIAM T. RUYECHAN

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

Received 2 December 1982/Accepted 22 February 1983

Properties of the major DNA-binding protein found in herpes simplex virus-infected cells were investigated by using a filter binding assay and electron microscopy. Filter binding indicated that the stoichiometry of binding of the protein with single-stranded DNA is approximately 40 nucleotides per protein molecule at saturation. Strong clustering of the protein in DNA-protein complexes, indicative of cooperative binding, was seen with the electron microscope. Measurements of single-stranded fd DNA molecules saturated with protein and spread for electron microscopy by using both the aqueous and formamide spreading techniques indicated that the DNA is held in an extended configuration with a base spacing of ~0.13 nm per base.

Protein synthesis in herpes simplex virus type 1 (HSV-1)-infected cells is coordinately regulated and sequentially ordered. Three groups of polypeptides have been designated in order of their temporal appearance. They include the α group, which does not require prior viral protein synthesis for expression, the β group, which includes proteins involved in viral DNA metabolism, and the γ group, which is made up primarily of structural proteins found in the intact virion (11). The β polypeptides are synthesized during the peak period of viral DNA synthesis and include the viral DNA polymerase and the major herpesvirus DNA-binding protein, which has been designated infected cell polypeptide 8 (ICP8) based on its electrophoretic mobility relative to other proteins found in infected cells (12).

Estimates of the molecular weight of ICP8 have ranged between 128,000 and 135,000. DNA-cellulose chromatography of radioactively labeled infected-cell extracts has shown that ICP8 binds more tightly to single-stranded than to double-stranded DNA (4, 15). Recent experiments indicate that this protein may be a group-specific antigen for herpesviruses, since antisera against ICP8 crossreact with analogous polypeptides found in other herpesvirus systems (20). Finally, ICP8 has been detected in HSV-transformed cells (10), and antibody to ICP8 has been found in sera obtained from cervical carcinoma patients (3). This report shows that this important herpesvirus-coded protein holds single-stranded DNA in an extended conformation.

^{3}H-labeled HSV-1 DNA was the gift of Michael Bartkoski, as were initial stocks of Vero cells and HSV-1 strain F (9). Bacteriophage fd DNA was the gift of Lucy M. S. Chang. Calf thymus DNA and pancreatic DNase and RNase were purchased from Sigma Chemical Co. (St. Louis, Mo.). Cellulose powder and DEAE-cellulose powder were purchased from Bio-Rad Laboratories (Richmond, Calif.). Phosphocellulose powder (P11) was purchased from Whatman, Inc. (Clifton, N.J.). Preparation of ICP8 was by the procedure of Powell et al. (14). A total of 15 confluent 2.5-liter roller bottles of Vero cells were infected with HSV-1 strain F at an input multiplicity of 20 PFU per cell. The cells were harvested at 18 h postinfection and suspended in buffer containing 20 mM Tris-hydrochloride (pH 7.5) and 0.5 mM dithiothreitol at a cell concentration of ~3×10^7/ml. After sonication, DNase and RNase were added to final concentrations of 20 and 2 μg/ml, respectively. After a 30-min incubation at 4°C, solid NaCl was added to a concentration of 2.0 M, and the resulting precipitate was removed by low-speed centrifugation. Purification of ICP8 through chromatography on DEAE-cellulose and phosphocellulose was carried out exactly as described by Powell et al. (14). Each fraction from these columns was assayed for absorbance at 280 nm and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent silver staining (19). Fractions from the phosphocellulose column containing ICP8 were adjusted to 500 μg/ml in bovine serum albumin, dialyzed against low-salt buffer (50 mM KCl, 20 mM Tris-hydrochloride [pH 7.5], 0.5 mM dithiothreitol, and 20% glycerol) and applied to a 30-ml single-stranded DNA-cellulose column (2) which had been prewashed with buffer containing bovine serum

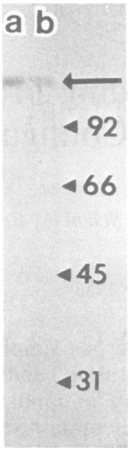

FIG. 1. A 9% sodium dodecyl sulfate-polyacrylamide gel of purified ICP8 peak fractions (a and b) from a single-stranded DNA–cellulose column. The protein was visualized by means of a silver staining procedure (10). Molecular weight markers, in descending order, were phosphorylase B, bovine serum albumin, ovalbumin, and carbonic anhydrase.

albumin. The column was eluted stepwise with 0.05, 0.1, 0.2, 0.4, and 1.0 M KCl. ICP8 was obtained (~50 μg/10^9 cells) in the 0.4 and 1.0 M fractions, depending on the batch of DNA-cellulose. The protein was then dialyzed against 0.01 M Tris-hydrochoride (pH 7.5)–0.001 M EDTA (TE buffer) and quantitated by using a Bio-Rad protein assay kit.

ICP8-fd DNA complexes were prepared for electron microscopy by mixing the two components at a variety of weight ratios. Samples were brought to 80 μl with TE buffer, against which the protein and DNA were also dialyzed. The DNA was present at a concentration of 1 μg/ml in all samples. ICP8 was present at concentrations of 2, 5, and 10 μg/ml. The 80-μl mixtures were incubated at 4°C for 10 min and then fixed with 8% glutaraldehyde (1.25 μl/80 μl) at room temperature for an additional 10 min. The complexes were then spread for electron microscopy by either the aqueous ammonium acetate technique or the formamide technique (7, 17). Ammonium acetate was present in both the hyperphase and the hypophase at a concentration of 100 mM in the aqueous technique. The complexes were observed and photographed at magnifications of 9,800× and 16,000×, using a Zeiss EM 10A high-resolution transmission electron microscope.

A solution of sonicated ^{3}H-labeled HSV-1 DNA (80,000 dpm/μg) was heated to 100°C for 5 min and ice quenced to obtain a stock solution of single-stranded DNA for filter binding assays. A 0.02-μg portion of the DNA was mixed with increasing amounts of ICP8. The samples were brought to 100 μl with TE buffer and incubated at 4°C for 15 min. After incubation, an 80-μl aliquot was withdrawn and adsorbed to an 0.45-μm-pore-size nitrocellulose filter with a Hoefer multichannel filtration manifold. The filtration speed was 4 ml/min. The filters were then washed with 5 ml each of cold TE buffer and 90% ethanol, air dried, and counted.

Sodium dodecyl sulfate-gel electrophoresis of purified ICP8 and subsequent silver staining indicated the presence of a single polypeptide species with a molecular weight of ~128,000 (Fig. 1). Filter binding assays carried out as

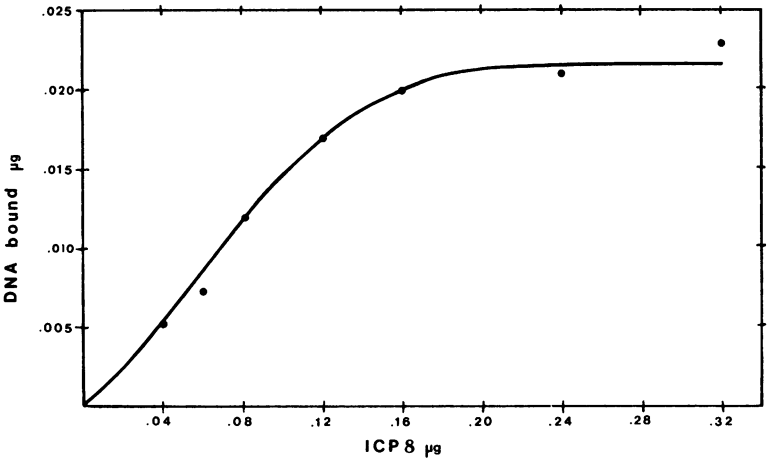

FIG. 2. Typical results from a filter binding assay indicating saturation (maximum retention) of sonicated, heat-denatured, ^{3}H-labeled HSV-1 DNA with ICP8 at a 10:1 protein-to-DNA weight ratio. The HSV-1 DNA had an average length of ~400 nucleotides as determined by electron microscopy (data not shown).

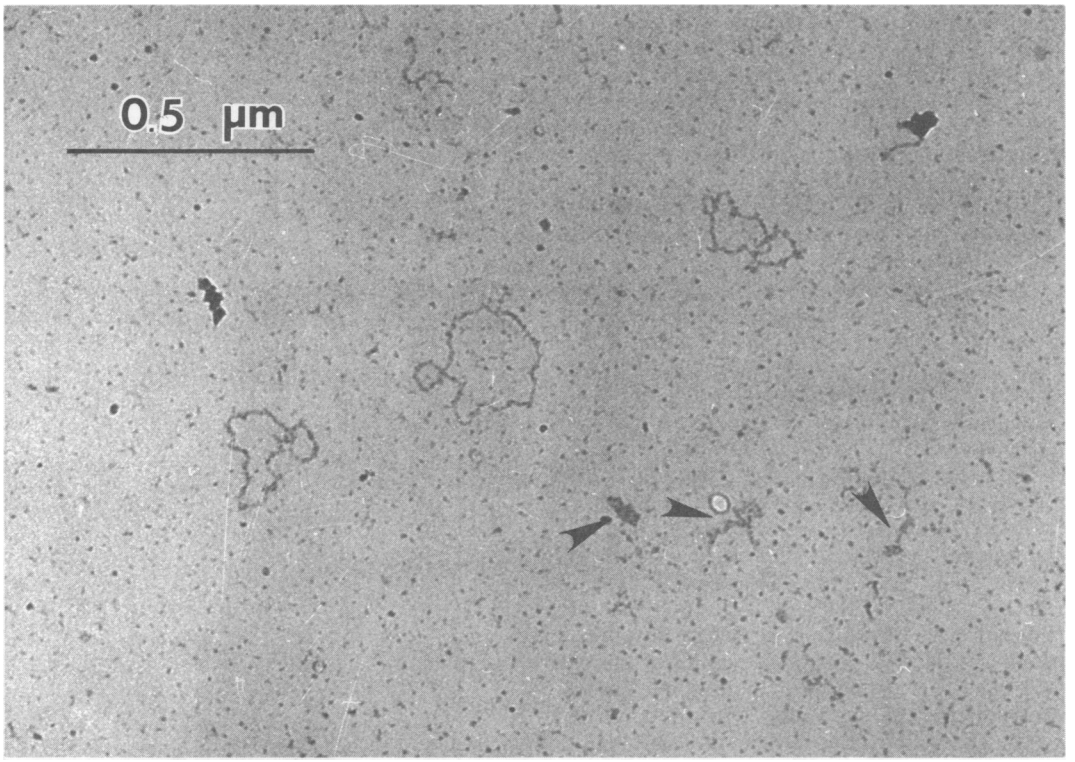

FIG. 3. Electron micrograph of ICP8-fd complexes prepared at a protein/DNA weight ratio of 5:1. The nonrandom distribution of protein is evidenced by the presence of both extended, fully saturated circles and highly collapsed, irregularly shaped molecules (arrows) with little or no protein bound. The complexes were stained with uranyl acetate (12). Glutaraldehyde fixation was required to maintain the integrity of the complexes.

described above indicated that saturation of sonicated, single-stranded HSV-1 DNA by ICP8 occurs at a protein/nucleic acid weight ratio of 10:1 (Fig. 2). This value is in good agreement with results obtained by Powell et al. (14) which indicate that ICP8/nucleic acid weight ratios of 10:1 are sufficient to allow the complete denaturation of polydeoxyadenylic acid-polydeoxythymidilic acid duplexes under thermal and solution conditions where the polynucleotides form a stable double helix. The 10:1 ratio indicates that one molecule of ICP8 covers approximately 40 bases. This stoichiometry is similar to that of the *Escherichia coli* single-strand binding protein (SSB), which covers approximately 32 bases per 80,000-molecular-weight tetramer (18).

Observation of glutaraldehyde-fixed, aqueous-spread ICP8-fd DNA complexes at subsaturating levels of protein shows a clearly nonrandom distribution of the protein (Fig. 3). Some of the DNA molecules appear extended and fully saturated with protein, whereas others are collapsed irregular structures typical of naked single-stranded DNA under the aqueous mounting conditions. The frequency of open circular and collapsed structures at several protein/DNA weight ratios was determined by scoring a minimum of 110 structures seen with the electron microscope at each ratio. The fraction of extended open circular structures increased with increasing amounts of ICP8 (Table 1). The observed increase correlates quite well with the stoichiometry established in the filter binding assay and suggests that the open structures are fully saturated fd DNA-ICP8 complexes whereas the collapsed structures are naked fd DNA. A similar clustering of protein molecules has been observed with T4 gene 32 protein and SSB-DNA

TABLE 1. Frequency of open circular structures at various protein/DNA ratios

Protein/DNA ratio (wt/wt)	No. of structures scored	% Open	% Closed
0:1	110	0.0	100.0
2:1	114	18.4	81.6
5:1	130	55.0	45.0
10:1	121	94.2	5.8

complexes and is indicative of coperative binding (1, 17). Measurements of 102 extended ICP8-fd DNA complexes prepared at a protein/DNA ratio of 10:1 yielded an average contour length of 0.85 ± 0.11 μm (Fig. 4). This length corresponds to a nucleotide spacing of 0.132 ± 0.017 nm per nucleotide based on a value of 6,408 nucleotides per fd molecule (5). This value is similar to the base spacing of 0.18 nm per base seen in SSB-fd DNA complexes.

It is possible that the very close DNA base spacing of 0.132 nm measured for the complexes was due to a combination of the action of ICP8 and the aqueous mounting procedure used. First, since the single-stranded DNA is visualized as a highly collapsed structure, it is possible that portions of the putative saturated complexes do not have protein bound to them. Conversely, it is possible that a least some of the collapsed structures are partially saturated complexes. Although the results presented in Table 1 and the tightness of the distribution of contour lengths in Fig. 4 argue against these possibilities, they are nonetheless a source of concern. Second, assuming that all of the open circular structures are fully saturated, the ionic spreading medium used in the aqueous technique could alter the overall conformation of the complex.

To examine these possibilities, ICP8-fd DNA complexes were spread by using the formamide technique, which allows single-stranded DNA to be visualized as an extended smooth filament (7, 17). Procaryotic DNA-binding protein–DNA complexes have been successfully visualized as thicker, denser filaments as compared with naked DNA. Thus, if partially saturated complexes were present in ICP8-fd DNA mixtures, the formamide technique would allow differentiation of protein-bound and protein-free regions. In the second case, i.e., if the extended structures were fully saturated, measurements of their contour lengths would determine whether the apparent geometry of the complexes was altered by the spreading medium. fd DNA and ICP8 were mixed in 80-μl aliquots to yield final concentrations of 1 μg of DNA and ~7 μg of protein per ml. Incubation and fixation of the complexes were carried out as described above. After fixation, an additional 0.1 μg of fd DNA was added to ensure that some naked DNA was present. A 30-μl portion of this mixture was then spread from a 40% formamide hyperphase containing cytochrome *c* (0.3 μg/ml) onto a 15% formamide hypophase. The grids were stained with uranyl acetate, shadowed with gold/palladium, and photographed at a magnification of 16,000×.

An examination of the grids showed that only two types of structure were present: well-extended naked fd DNA circles and much smaller

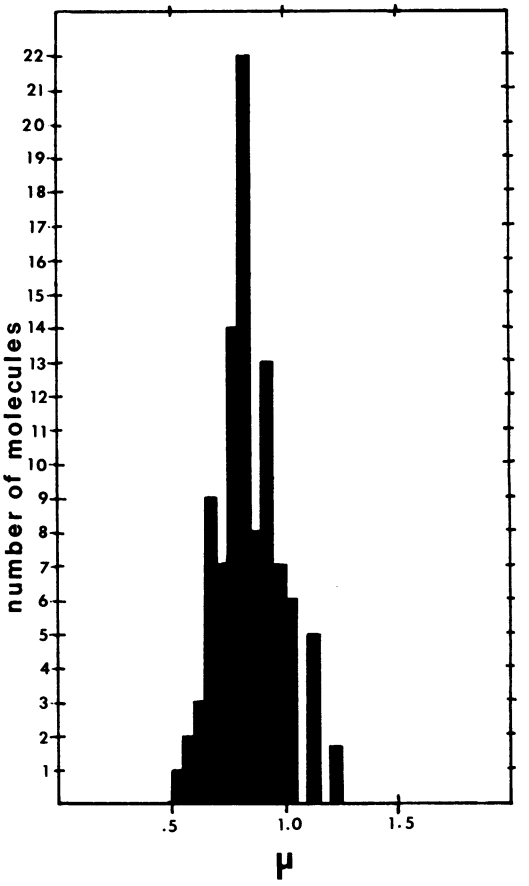

FIG. 4. Histogram of 102 well-extended ICP8-fd complexes prepared at a protein/DNA ratio of 10:1. Photographs were taken at a magnification of 9,800×, using a Zeiss EM 10A high-resolution instrument. Negatives were projected onto a blackboard with a lantern slide projector, and contour measurements of the molecules were taken with a Keuffel and Esser map measuring device.

circular DNA-protein complexes with characteristically thicker, rougher-appearing contours (Fig. 5). No obviously partial complexes were seen. Contour measurements of 87 complexes yielded an average value of 0.79 ± 0.08 μm. This contour length corresponds to a base spacing of 0.125 ± 0.012 nm per base. The naked fd in contrast had a base spacing of 0.27 nm per base, in good agreement with the previously published value of 0.28 nm per base with the formamide technique (17).

The contour length and base spacing values obtained by the two spreading techniques are the same within experimental error, indicating that (i) partial saturation of the fd DNA with ICP8 does not occur under the conditions used, thus implying a high degree of cooperativity in

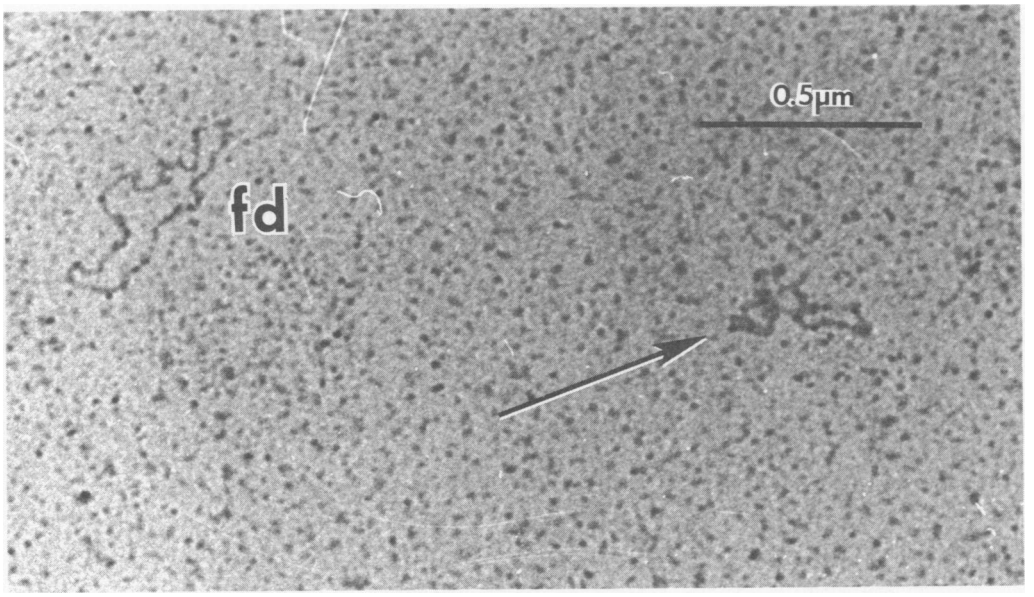

FIG. 5. An electron micrograph showing an ICP8-fd DNA complex (arrow) and an extended protein-free fd DNA molecule mounted by using the formamide technique and shadowed with gold/palladium. Note the thicker, "grainy" appearance and reduced contour length of the complex.

the binding reaction, and (ii) the tight base spacing is imposed on the DNA by ICP8, and the complexes are stable under widely varying solution conditions. These results imply that single-stranded DNA in the aqueous environment of the infected-cell nucleus can be held in an extended configuration by ICP8 with a characteristic base spacing of ~0.13 nm per base.

The results described above show that the interaction of ICP8 with single-stranded DNA is similar to that observed with the procaryotic single-strand binding proteins T4 gene 32 protein and SSB. The highly cooperative nature of the interaction seen with the electron microscope would indicate an apparent association constant of $\geq 10^7$ by analogy with studies on these procaryotic proteins (13, 16). Both SSB and T4 gene 32 protein are involved in DNA replication and recombination. Recently isolated temperature-sensitive mutants with lesions in ICP8 have a DNA-negative phenotype (6; D. Knipe personal communication), indicating an essential role for ICP8 in HSV DNA replication. Thus, in the HSV system, ICP8 may play a role similar to that of T4 gene 32 protein and SSB in the bacteriophage T4 and *E. coli* systems.

I thank Anna C. Weir for excellent technical assistance and Christa Stanoyevitch for typing the manuscript.

This work was supported by grant CO7114 from the Uniformed Services University of the Health Sciences.

LITERATURE CITED

1. **Alberts, B., and L. Frey.** 1970. T4 bacteriophage gene 32—a structural protein in the replication and recombination of DNA. Nature (London) **227**:1313–1318.
2. **Alberts, B. M., and G. Herrick.** 1971. DNA-cellulose chromatography. Methods Enzymol. **21**:198–217.
3. **Anzai, T., G. R. Dreesman, R. J. Courtney, E. Adam, W. E. Rawls, and M. Benyesh-Melnick.** 1975. Antibody to herpes simplex virus type 2-induced non-structural proteins in women with cervical cancer and control groups. J. Natl. Cancer Inst. **54**:1051–1059.
4. **Bayliss, G. J., H. S. Marsden, and J. Hay.** 1975. Herpes simplex virus proteins: DNA binding proteins in infected cells and in the virus structure. Virology **68**:124–134.
5. **Beck, E., R. Sommer, E. Auerswald, C. Kurz, B. Zink, G. Osterburg, H. Schaller, K. Sugimoto, H. Sugiaski, T. Okamoto, and M. Takanami.** 1978. Nucleotide sequence of bacteriophage fd DNA. Nucleic Acids Res. **5**:4495–4503.
6. **Conley, A. J., D. M. Knipe, P. C. Jones, and B. Roizman.** 1981. Molecular genetics of herpes simplex virus. VII. Characterization of a temperature-sensitive mutant produced by in vitro mutagenesis and defective in DNA synthesis and accumulation of γ polypeptides. J. Virol. **37**:191–206.
7. **Davis, R. W., M. Simon, and N. Davidson.** 1971. Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids. Methods Enzymol. **21**:413–428.
8. **Dreesman, G. R., J. Burek, E. Adam, R. H. Kaufman, J. L. Melnick, K. L. Powell, and D. J. Purifoy.** 1980. Expression of herpesvirus induced antigens in human cervical cancer. Nature (London) **285**:591–593.
9. **Ejercito, P. M., E. D. Kieff, and B. Roizman.** 1968. Characterization of herpes simplex virus strains differing in their effect on social behavior of infected cells. J. Gen. Virol. **3**:357–364.
10. **Flannery, V. L., R. J. Courtney, and P. A. Schaffer.** 1977. Expression of an early, nonstructural antigen of herpes simplex virus in cells transformed in vitro by herpes simplex virus. J. Virol. **21**:284–291.
11. **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.

12. **Morse, L. S., L. Pereira, B. Roizman, and P. A. Schaffer.** 1978. Anatomy of herpes simplex virus (HSV) DNA. X. Mapping of viral genes by analysis of polypeptides and functions specified by HSV-1 × HSV-2 recombinants. J. Virol. **26**:389–410.
13. **Newport, J. W., N. Lonberg, S. C. Kowalczykowski, and P. H. von Hippel.** 1981. Interactions of bacteriophage T4-coded gene 32 protein with nucleic acids. II. Specificity of binding to DNA and RNA. J. Mol. Biol. **145**:105–121.
14. **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.
15. **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.
16. **Schneider, R. J., and J. G. Wetmur.** 1982. Kinetics of transfer of *Escherichia coli* single strand deoxyribonucleic acid binding protein between single-stranded deoxyribonucleic acid molecules. Biochemistry **21**:608–615.
17. **Sigal, N., H. Delius, T. Kornberg, M. L. Gefter, and B. M. Alberts.** 1972. A DNA-unwinding protein isolated from *Escherichia coli*: its interaction with DNA and DNA polymerases. Proc. Natl. Acad. Sci. U.S.A. **69**:3537–3541.
18. **Weiner, J. H., L. L. Bertsch, and A. Kornberg.** 1975. The deoxyribonucleic acid unwinding protein of *Escherichia coli*: properties and functions in replication. J. Biol. Chem. **250**:1972–1980.
19. **Wray, W., T. Boulikas, V. P. Wray, and R. Hancock.** 1981. Silver staining of proteins in polyacrylamide gels. Anal. Biochem. **118**:197–203.
20. **Yeo, J., R. A. Killington, D. H. Watson, and K. L. Powell.** 1981. Studies on cross-reactive antigens in the herpesviruses. Virology **108**:256–266.