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Analysis of the UL36 Open Reading Frame Encoding the Large Tegument Protein (ICP1/2) of Herpes Simplex Virus Type 1

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Using peptide antisera specific for regions within the N terminus and C terminus of the predicted UL36 gene product, immunoblotting experiments were performed to demonstrate definitively that ICP1/2 is encoded by the UL36 gene. These data also suggest that both the cell- and the virion-associated forms of ICP1/2 are colinear with the complete predicted amino acid sequence of the UL36 gene. Computer-assisted analyses of the predicted amino acid sequence of the UL36 gene revealed the presence of two putative leucine zipper-type motifs and a potential ATP-binding domain. The possible functions of these consensus domains will also be discussed.

Morphologically, herpes simplex virus type 1 (HSV-1) virions can be divided into four distinct regions referred to as the core, capsid, tegument, and envelope (24, 30). Our studies have focused on characterizing the largest tegument protein of HSV-1, designated infected-cell protein 1 and 2 (ICP1/2), or virion protein 1/2 (VP1/2). ICP1/2 is a 270-kDa tegument protein which is present at levels of less than 150 copies per virion (9) and appears to interact tightly with the nucleocapsids (17, 24). Studies on the kinetics of ICP1/2 synthesis have revealed that the polypeptide is synthesized late during HSV-1 infection (10, 17) and is expressed as a γ_2 (true late) gene product (17). In vitro studies have demonstrated that ICP1/2 (VP1/2) is phosphorylated by a tegument-associated protein kinase (13), and in vivo studies have also shown that the protein is modified by phosphorylation at serine residues within HSV-1-infected cells (17).

Intertypic recombinant mapping studies have localized the gene encoding ICP1/2 to between 0.430 and 0.580 map units on the HSV-1 genome (14, 19). Examination of the HSV-1 nucleotide sequence within this region of the genome reveals a 9,495-bp open reading frame (ORF), designated UL36, which would encode a protein with a calculated molecular weight of 335,841 (15). On the basis of these results, it seems reasonable to expect that UL36 is the gene encoding ICP1/2; however, this assignment has not been directly confirmed. Furthermore, the large discrepancy between the apparent molecular mass of ICP1/2 (270 kDa) and the predicted molecular mass of the UL36 gene product (336 kDa) raises the question of whether ICP1/2 represents a polypeptide which is colinear with the complete UL36 ORF. Using pulse-chase radiolabeling studies, we have previously shown that ICP1/2 appears to be synthesized as a mature viral gene product (17). In addition, RNA mapping studies (29) suggest that a 9.6-kb mRNA transcript is synthesized from the region of the viral genome containing the UL36 ORF. Thus, the available evidence implies that ICP1/2 may be colinear with the complete UL36 gene.

To directly demonstrate that ICP1/2 is the product of the UL36 ORF and to determine whether ICP1/2 is colinear with UL36, we have generated rabbit antiserum against two synthetic oligopeptides which correspond to amino acid residues 33 to 44 within the N-terminal region and amino acid residues 3048 to 3057 from the C-terminal region of the predicted UL36 polypeptide (Fig. 1). These peptides were chosen on the basis of the high densities of charged residues and their locations near the termini of the predicted UL36 gene product. The synthetic peptides were coupled to horseshoe crab hemocyanin by using 0.2% glutaraldehyde (8), and rabbits were immunized (two rabbits per peptide) with 5 mg of the conjugates in Freund's complete adjuvant followed by boosts with 3 mg of the conjugates in Freund's incomplete adjuvant as previously described (16). Rabbits designated R27 and R28 were immunized with the N-terminal-specific peptide, and rabbits designated R29 and R30 were injected with the C-terminal-specific peptide (Fig. 1). Antisera were subsequently obtained and prepared as previously described (16). To directly confirm that ICP1/2 is encoded by UL36, we utilized a monospecific polyclonal antiserum generated against purified ICP1/2 (17) to determine whether the peptide antisera would cross-react with the 270-kDa polypeptide recognized by the anti-ICP1/2 serum. Thus, HEp-2 cells were mock infected or infected with HSV-1 and then were harvested at 18 h postinfection (16). Following harvesting, the cell lysates were subjected to immunoprecipitation with the anti-ICP1/2 serum as previously described (17). The immunoprecipitated proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a gel containing 5% polyacrylamide (23) and electrophoretically transferred to nitrocellulose as described by Towbin et al. (27), with 1% (vol/vol) methanol in the transfer buffer. Immunoblotting was subsequently performed with each peptide antiserum diluted 1:50 in immunoblotting buffer (3% bovine serum albumin, 0.2% Tween 20, phosphate-buffered saline), and the reactive proteins were visualized by reaction with ^{125}I-labeled protein A (Dupont, NEN Research Products, Boston, Mass.) and autoradiography (Fig. 2). These studies demonstrated that the N-terminal-specific R28 antiserum and the C-terminal-specific R29 antiserum both reacted with the 270-kDa polypeptide, which was immunoprecipitated with the anti-ICP1/2 serum. Furthermore, the preimmune sera did not react with any HSV-

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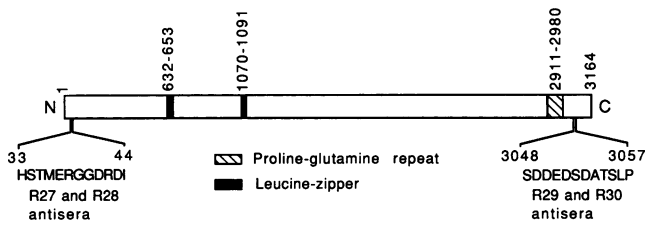

FIG. 1. Schematic representation of the 3,164-amino-acid residue UL36 gene product. The positions of the amino (N) and carboxy (C) termini of the protein are indicated, and the numbers above the diagram correspond to the predicted amino acid residues of the UL36 gene product as described by McGeoch et al. (15). The putative leucine zipper motifs and the proline-glutamine amino acid repeat region are indicated. Below the diagram of the UL36 gene product, the locations and amino acid sequences of the oligopeptides used for the generation of peptide antisera are shown.

1-specific proteins, suggesting that the peptide antisera are reacting specifically with ICP1/2. We have also performed similar immunoblotting studies with the R27 and R30 peptide antisera, and both of these antisera were found to react specifically with ICP1/2 (18).

In addition to confirming that the UL36 ORF encodes ICP1/2, these data imply that ICP1/2 is likely to contain all of the amino acid residues that are present within the predicted UL36 gene product (15). Thus, the apparent molecular mass of ICP1/2 (270 kDa) represents a significant underestimate of the actual molecular mass of the polypeptide (336 kDa). Since the peptide antisera are presumably recognizing epitopes between amino acid residues 33 and 44 and 3048 and 3057, we cannot rule out the possibility that amino acid

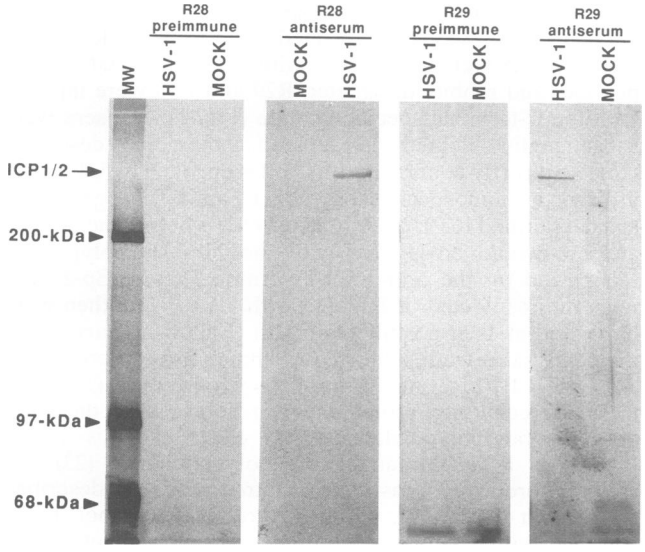

FIG. 2. Reactivity of the peptide antisera with ICP1/2. HEp-2 cells were mock infected or infected with HSV-1 as indicated and harvested at 18 h postinfection. The cell lysates were reacted with the anti-ICP1/2 serum, and the immunoprecipitated proteins were resolved by SDS-PAGE through a 5% polyacrylamide gel. Following electrophoretic transfer to nitrocellulose, the proteins were immunoblotted with either preimmune sera or the corresponding peptide antisera (R28 and R29, the N- and C-terminal-specific peptide antisera, respectively) as indicated above each panel. The molecular mass (MW) markers and the position of ICP1/2 are shown on the left.

residues 1 to 32 or 3057 to 3164 are absent from ICP1/2; however, the removal of these residues would not significantly reduce the molecular weight of ICP1/2. Thus, these data strongly suggest that ICP1/2 is synthesized as a mature polypeptide which is colinear with the complete UL36 gene. In addition, these results confirm our previous pulse-chase radiolabeling studies which suggested that ICP1/2 is synthesized as a mature viral gene product (17).

From the studies described above, the cell-associated ICP1/2 appears to be a full-length UL36 gene product; however, it is conceivable that the ICP1/2 which is associated with HSV-1 virions may be proteolytically processed during or after association with the HSV-1 capsids. Since the proteolytic removal of a portion of the polypeptide may be difficult to distinguish by altered migration in SDS-PAGE, we utilized the peptide antisera as specific probes to determine whether the virion-associated ICP1/2 (VP1/2) molecules are full-length UL36 gene products. Thus, HSV-1 virions were purified by sucrose gradient centrifugation as previously described (4, 16), and the purity of each virion preparation was examined by SDS-PAGE and Coomassie blue staining (18). The structural protein profiles were similar to those previously described by Spear and Roizman (25), suggesting that the virus preparations were relatively free of cellular protein contamination. The HSV-1 virion polypeptides were subsequently resolved by SDS-PAGE and transferred to nitrocellulose, and immunoblot analyses were performed with the R28 and R29 peptide antisera (Fig. 3A). These data indicated that the virion-associated ICP1/2 (VP1/2) reacts with both the R28 and the R29 antisera, suggesting that the ICP1/2 molecules present within purified HSV-1 virions are full-length UL36 gene products.

Although the R28 and R29 antisera clearly react with the virion-associated ICP1/2 as well as the cell-associated ICP1/2, we had not definitively established that the antibodies are reacting with ICP1/2 in a sequence-specific manner. It is conceivable that the antibodies generated against the hapten (hemocyanin) could react with amino acid residues within ICP1/2 and that such an interaction could produce results identical to a sequence-specific reaction with ICP1/2. To demonstrate that the peptide antisera are specifically reacting with amino acid residues within ICP1/2, immunoblotting studies were performed with the R28 and R29 antisera in the presence of excess competing N-terminal-specific peptide and the C-terminal-specific peptide, respectively. The HSV-1 virion polypeptides were resolved by SDS-PAGE and transferred to nitrocellulose, and immunoblotting was performed with the R28 and R29 antisera in the presence of 100 μg of the corresponding synthetic peptide per ml (Fig. 3B). These studies indicated that the interactions of the R28 and R29 antisera with ICP1/2 are inhibited in the presence of excess competing peptide. To determine whether this inhibition was peptide specific, the same immunoblots were subsequently reprobed with the same peptide antiserum in the presence of 100 μg of nonspecific peptide per ml. Thus, the immunoblots were reprobed with R28 antiserum plus the C-terminal-specific peptide, and the R29 antiserum was used in the presence of the N-terminal-specific peptide (Fig. 3C). These results demonstrated that both the R28 and the R29 antisera could react with ICP1/2 in the presence of nonspecific competing peptide, suggesting that the interaction of the R28 and R29 antisera with ICP1/2 is sequence specific. These results further verify that UL36 is the gene encoding ICP1/2 and that the peptide antisera are interacting with the polypeptide in a sequence-specific manner.

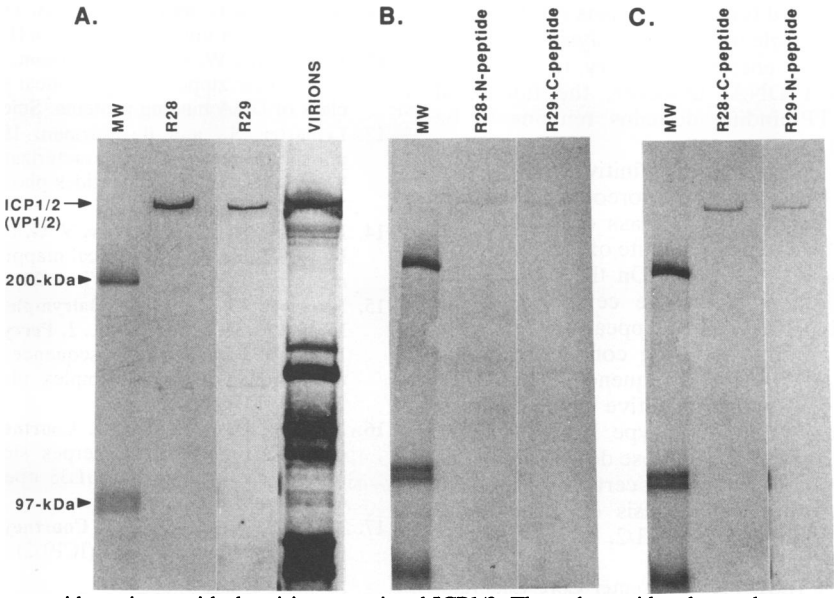

FIG. 3. Reactivity of the peptide antisera with the virion-associated ICP1/2. The polypeptides that make up purified HSV-1 virions were resolved by SDS-PAGE on a 5% polyacrylamide gel and transferred to nitrocellulose, and immunoblot analysis was performed with each peptide antiserum. (A) HSV-1 virion polypeptides probed with the R28 or R29 peptide antiserum as indicated above each lane. ^{14}C-amino-acid-labeled HSV-1 virion polypeptides (virions) are also shown for comparison. (B) HSV-1 virion polypeptides probed with either the R28 or the R29 peptide antiserum in the presence of excess competing N-terminal-specific (R28 + N-peptide) or C-terminal-specific (R29 + C-peptide) synthetic peptide, respectively. (C) Blots in panel B reprobed with the R28 or R29 antiserum in the presence of excess nonspecific competing C-terminal (R28 + C-peptide) or N-terminal (R29 + N-peptide) synthetic peptide, respectively. The positions of the molecular mass (MW) markers and ICP1/2 (VP1/2) are shown to the left of panel A. The figure represents a composite of individual immunoblots performed with each peptide antiserum.

Near the C terminus of the UL36 gene product, 35 tandem repeats of the amino acid sequence proline-glutamine (P-Q repeats) are found between residues 2911 and 2980 (Fig. 1). The DNA sequence which encodes the P-Q repeats of HSV-1 strain 17 (15) consists of 35 tandem reiterations of the nucleotide sequence 5′-CCCCAR-3′, in which R represents A or G. The functional significance of the P-Q repeats and/or the DNA sequence which encodes them is unknown. Since the R29 peptide antiserum reacts specifically with amino acid residues of ICP1/2 which are downstream of the P-Q repeats (Fig. 1), it is likely that the repeated amino acid sequence is present within ICP1/2.

Since ICP1/2 is a DNA-binding protein (3) which is present within the nuclei of HSV-1-infected cells (17), the predicted amino acid sequence of the UL36 gene was analyzed for several common protein sequence motifs which would correlate with these findings. We failed to detect sequences corresponding to metal binding domains or nuclear localization signals, but two potential leucine zipper-type motifs (12) were identified between amino acid residues 632 and 653 and residues 1070 and 1091 (Fig. 1). The functional significance of these leucine repeats is currently unknown, but the presence of such motifs in other nuclear DNA-binding proteins, such as c-*jun* and c-*fos*, has been shown to be responsible for the ability of these proteins to form homo- or heterodimers (7, 11, 22, 28). Interestingly, studies by Chou and Roizman (3) have demonstrated that ICP1/2 forms a heterooligomeric complex with a 140-kDa virus-specific protein, and this protein complex binds in a sequence-specific manner to the *a* sequences of HSV-1. Thus, it is conceivable that the leucine zipper domain(s) within ICP1/2 may mediate the interaction of ICP1/2 with the 140-kDa virus-specific protein.

We have also identified potential type A and type B ATP-binding sites near the C terminus of ICP1/2 which resemble the consensus ATP-binding motifs described by Chin et al. (2). Together, these two structures are thought to form a pocket for ATP binding and hydrolysis (2). The type A and the type B sites of ICP1/2 are located between amino acid residues 2211 and 2219 and residues 2430 and 2443, respectively. The computer-predicted secondary structures of the ICP1/2 domains containing the putative type A and type B ATP-binding sites were similar to the predicted secondary structures surrounding the type A and type B ATP-binding domains of several other known ATP-binding proteins (2). It should be noted that other consensus type A (between amino acid residues 411 and 419, 1018 and 1026, 1034 and 1042, 1532 and 1539, and 1932 and 1940) and type B (between amino acid residues 1369 and 1380, 1700 and 1713, and 2366 and 2378) ATP-binding sites were identified within the predicted amino acid sequence of ICP1/2; however, these consensus sites did not have the appropriate predicted secondary structure (18). Thus, the functional significance of these sites is questionable.

As stated previously, studies have shown that ICP1/2 binds in a sequence-specific manner to the *a* sequences of HSV-1 (3). Since the *a* sequences are the only *cis*-acting DNA sequence elements within the HSV-1 genome which are required for the appropriate cleavage and packaging of viral DNA into capsids (5, 6, 21, 26), it is intriguing to speculate that ICP1/2 may be involved in the packaging of HSV-1 DNA in a manner analogous to that of the well-characterized terminases of bacteriophages (1, 20). The hydrolysis of ATP is thought to provide the energy required for the translocation and/or cleavage of the bacteriophage DNA during the encapsidation process (1). Since ICP1/2

contains potential type A and type B consensus ATP-binding domains (2), it seems plausible that the hydrolysis of ATP by ICP1/2 could provide the energy necessary to drive the encapsidation of HSV-1 DNA; however, the functional relevance of these ATP-binding domains remains to be experimentally verified.

The data presented in this report definitively show that ICP1/2 is encoded by the UL36 gene. Moreover, these data suggest that the apparent molecular mass of ICP1/2 (270 kDa) represents a significant underestimate of the actual size of the UL36 gene product (336 kDa). On the basis of the reactivity of the peptide antisera, the cell- and virion-associated forms of ICP1/2 (VP1/2) appear to represent full-length UL36 gene products. Using computer-assisted analyses of the predicted amino acid sequence of the UL36 gene product, we also identified putative leucine zipper domains and consensus type A and type B ATP-binding motifs. The functional significance of these domains remains to be verified experimentally, but they certainly represent attractive targets for future mutagenesis experiments to further characterize the function of ICP1/2.

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REFERENCES

1. **Black, L. W.** 1989. DNA packaging in dsDNA bacteriophages. Annu. Rev. Microbiol. **43**:267–292.
2. **Chin, D. T., S. A. Goff, T. Webster, T. Smith, and A. L. Goldberg.** 1988. Sequence of the *lon* gene in *Escherichia coli*. J. Biol. Chem. **263**:11718–11728.
3. **Chou, J., and B. Roizman.** 1989. Characterization of DNA sequence-common and sequence-specific proteins binding to *cis*-acting sites for cleavage of the terminal *a* sequence of the herpes simplex virus 1 genome. J. Virol. **63**:1059–1068.
4. **Compton, T., and R. J. Courtney.** 1984. Virus-specific glycoproteins associated with the nuclear fraction of herpes simplex virus type 1-infected cells. J. Virol. **49**:594–597.
5. **Deiss, L. P., J. Chou, and N. Frenkel.** 1986. Functional domains within the *a* sequence involved in the cleavage-packaging of herpes simplex virus DNA. J. Virol. **59**:605–618.
6. **Deiss, L. P., and N. Frenkel.** 1986. Herpes simplex virus amplicon: cleavage of concatemeric DNA is linked to packaging and involves amplification of the terminally reiterated *a* sequence. J. Virol. **57**:933–941.
7. **Gentz, R., F. J. Rauscher, C. Abate, and T. Curran.** 1989. Parallel association of *fos* and *jun* leucine zippers juxtaposes DNA-binding domains. Science **243**:1695–1699.
8. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Heine, J. W., R. W. Honess, E. Cassai, and B. Roizman.** 1974. Proteins specified by herpes simplex virus. XII. The virion polypeptides of type 1 strains. J. Virol. **14**:640–651.
10. **Honess, R. W., and B. Roizman.** 1973. Proteins specified by herpes simplex virus. XI. Identification and relative molar rates of synthesis of structural and nonstructural herpes virus polypeptides in the infected cell. J. Virol. **12**:1347–1365.
11. **Kouzarides, T., and E. Ziff.** 1988. The role of the leucine zipper in the *fos-jun* interaction. Nature (London) **336**:646–651.
12. **Landschulz, W. H., P. F. Johnson, and S. L. McKnight.** 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science **240**:1759–1764.
13. **Lemaster, S., and B. Roizman.** 1980. Herpes simplex virus phosphoproteins. II. Characterization of the virion protein kinase and of the polypeptides phosphorylated in the virion. J. Virol. **35**:798–811.
14. **Marsden, H. S., N. D. Stow, V. G. Preston, M. C. Timbury, and N. M. Wilkie.** 1978. Physical mapping of herpes simplex virus-induced polypeptides. J. Virol. **28**:624–642.
15. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor.** 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69**:1531–1574.
16. **McNabb, D. S., and R. J. Courtney.** 1992. Identification and characterization of the herpes simplex virus type 1 virion protein encoded by the UL35 open reading frame. J. Virol. **66**:2653–2663.
17. **McNabb, D. S., and R. J. Courtney.** 1992. Characterization of the large tegument protein (ICP1/2) of herpes simplex virus type 1. Virology **190**:221–232.
18. **McNabb, D. S., and R. J. Courtney.** 1992. Unpublished observations.
19. **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.
20. **Murialdo, H.** 1991. Bacteriophage lambda DNA maturation and packaging. Annu. Rev. Biochem. **60**:125–153.
21. **Nasseri, M., and E. S. Mocarski.** 1988. The cleavage recognition signal is contained within sequences surrounding an *a-a* junction in herpes simplex virus DNA. Virology **167**:25–30.
22. **O'Shea, E. K., R. Rutkowski, W. F. Stafford, and P. Kim.** 1989. Preferential heterodimer formation by isolated leucine zippers from *fos* and *jun*. Science **245**:646–648.
23. **Powell, K. L., and R. J. Courtney.** 1975. Polypeptides synthesized in herpes simplex virus type 2-infected HEp-2 cells. Virology **66**:217–228.
24. **Roizman, B., and D. Furlong.** 1974. The replication of herpesviruses, p. 229–403. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 3. Plenum Press, New York.
25. **Spear, P. G., and B. Roizman.** 1972. Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion. J. Virol. **9**:143–159.
26. **Stow, N. D., E. C. McMonagle, and A. J. Davison.** 1983. Fragments from both termini of the herpes simplex virus type 1 genome contain signals required for the encapsidation of viral DNA. Nucleic Acids Res. **11**:8205–8220.
27. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
28. **Turner, R., and R. Tjian.** 1989. Leucine repeats and an adjacent DNA-binding domain mediate the formation of functional *cfos-cjun* heterodimers. Science **243**:1689–1694.
29. **Wagner, E. K.** 1985. Individual HSV transcripts. Characterization of specific genes, p. 45–104. *In* B. Roizman (ed.), The herpesviruses, vol. 3. Plenum Press, New York.
30. **Wildy, P.** 1986. Herpesvirus. Intervirology **25**:117–140.