JOURNAL OF VIROLOGY, Apr. 1981, p. 383-388
0022-538X/81/040383-06$02.00/0

Vol. 38, No. 1

Isolation of a Protein Kinase Induced by Herpes Simplex Virus Type 1

WILLIAM T. BLUE* AND DARLENE G. STOBBS

Department of Zoology and Microbiology and the Biomedical Sciences Division, College of Osteopathic Medicine, Ohio University, Athens, Ohio 45701

Received 10 September 1980/Accepted 21 December 1980

We have isolated a new cyclic AMP-independent protein kinase activity induced in HeLa cells by infection with herpes simplex virus type 1. Induction of the enzyme does not occur in cells treated with cycloheximide at the time of infection, or in cells infected with UV-inactivated herpes simplex virus type 1. The amount of enzyme induced in infected cells is dependent upon the multiplicity of infection. An enzyme with identical properties to that appearing in infected HeLa cells is also induced by herpes simplex virus type 1 in BHK cells.

Protein kinases are regulatory enzymes of major import in the control of numerous diverse biological processes (6, 15, 16). Herpes simplex virus type 1 (HSV-1), a DNA virus with oncogenic potential, has been shown to contain an envelope-associated cyclic AMP-independent protein kinase (16a, 23). Other enveloped viruses, as well as adenovirus and vaccinia virus, have also been shown to have virion-associated protein kinases (1, 3, 8, 9, 12, 14, 20, 22, 27-29). With respect to HSV-1, it is not known whether the virion-associated enzyme is virus coded or whether it is a host cell component acquired during either virus assembly or the budding process. Although several HSV-1 structural, as well as nonstructural, proteins are apparently post-translationally phosphorylated (5, 18, 21, 30), the source of the enzyme which carries out this function is unknown. It is also unclear as to whether or not protein phosphorylation is a necessary function in the processes of HSV-1 replication or cell transformation. With respect to the latter process, evidence has been presented which demonstrates that a product of the *src* gene of avian sarcoma viruses is a protein kinase (4, 17, 19, 25) and that the protein products of the transforming genes of both simian virus 40 and polyoma virus have protein kinase activity (2, 7, 24, 26). In this communication we report the isolation of a new protein kinase activity induced by infection of cells with HSV-1 (strain Miyama).

(Preliminary results of this study were presented at the Annual Meeting of the American Society for Microbiology in May 1980.)

Initial experiments consisted of time-course studies aimed at determining whether there was an increase in protein kinase specific activity in HeLa cells after infection with HSV-1. Replicate monolayer cultures were infected at a multiplicity of infection (MOI) of 10 PFU/cell or mock-infected, then harvested and extracted at hourly intervals for 12 h. The crude extracts were then assayed for total protein kinase content. Increases in protein kinase specific activity were found at 1 h, 4 h, and 9 h postinfection (Fig. 1). These results may suggest a role for the involvement of one or more protein kinases in the known sequential synthesis of HSV-1 α, β, and γ proteins (10, 11, 13). Because the greatest rise in specific activity occurred at 4 h postinfection, we selected that time point for our attempts at isolation in all subsequent experiments.

For isolation of the induced protein kinase, carboxymethyl cellulose (CM-52) column chromatography at pH 6.5 was the method chosen. The starting materials consisted of approximate 2-g quantities of mock-infected and 4-h-infected (MOI, 10 PFU) HeLa cells scraped from glass roller bottles. The cell pellets were extracted, dialyzed, and separately chromatographed on columns of CM-52 containing 25-ml bed volumes of ion-exchange resin. The results are shown in Fig. 2. As can be seen, there was only one peak of protein kinase activity eluted from the column charged with the mock-infected sample, whereas two distinct peaks appeared when the HSV-1-infected cell extract was chromatographed. We considered the activity eluting at 0.1 M KPO_4 to be the HSV-induced protein kinase. The individual fractions from each column eluate were assayed with and without the addition of 5 or 10 nmol of cyclic AMP to the standard assay mixture (final concentrations, 50 and 100 μM). No stimulation of protein kinase activity in either of the peaks was seen, nor were there any additional peaks revealed that had a dependence for cyclic AMP. The pooled individual peaks were

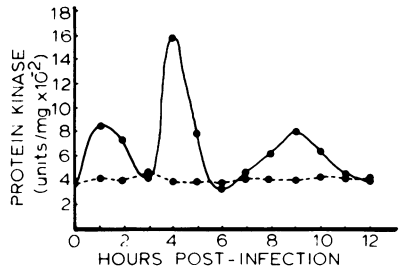

FIG. 1. *Induction of protein kinase activity by HSV-1. Replicate monolayers of HeLa cells were mock-infected (broken line) or infected with HSV-1 (solid line) at an MOI of 10 PFU/cell. At hourly intervals postinfection, the cell sheets were washed with phosphate-buffered saline and scraped into phosphate-buffered saline with a rubber policeman. After low-speed centrifugation (3,000 rpm, 10 min), the cell pellets were suspended in an extraction buffer containing 10 mM Tris-hydrochloride (pH 8.0), 0.1% Nonidet P-40, 0.5 mM dithiothreitol, 0.2 M NaCl, 0.2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N-tetraacetic acid (EGTA), and 10% glycerol. The suspended cells were sonically disrupted for 30 s and centrifuged for 30 min at 15,000 rpm in a Sorvall SS34 rotor. The supernatants were dialyzed overnight against extraction buffer without NaCl and EGTA and used for the measurement of protein kinase activity and total protein. The standard assay mixture for the detection of protein kinase activity, in a final volume of 100 μl, contained 10 mM Tris-hydrochloride (pH 8.0), 0.5 mM dithiothreitol, 10 mM $MgCl_2$, 0.1% Nonidet P-40, 25 μg of dephosphorylated phosvitin, and 3 mM [γ-^{32}P]ATP (2,000 to 5,000 cpm/pmol). After the addition of sample (25 μl), the mixtures were incubated for 30 min at 37°C. Samples (80 μl) were then spotted onto glass fiber filter disks, and the disks were washed three times with 5% trichloroacetic acid, once in denatured alcohol, and once in anhydrous ether. Trichloroacetic acid-precipitable counts were measured in a liquid scintillation counter. One unit of protein kinase specific activity is defined as the amount of enzyme which catalyzes the incorporation of 1 pmol of ^{32}P into a trichloroacetic acid-insoluble product in 30 min at 37°C. Total protein was measured by a Coomassie blue dye-binding assay.*

also tested for stimulation by cyclic AMP over a broad range of cyclic AMP concentration (50 μM to 10 mM). Neither showed an increase in activity due to the presence of cyclic AMP in the assay mixture. The pass-through materials from both columns still retained a considerable amount of protein kinase activity. They were therefore redialyzed and applied to separate columns of CM-52 at pH 5.5. The elution profiles from those columns were identical (data not shown), each revealing three additional peaks of protein kinase activity, although the levels of activity were considerably lower with the HSV-1-infected sample than with the mock-infected sample.

Based on the ease of separation of the virus-induced and host cell protein kinases by CM-52 column chromatography at pH 6.5, a series of experiments were performed which suggest that the induced protein kinase activity is virus coded. First, the induction of protein kinase activity was studied in HeLa cells at various increasing MOIs. As can be seen in Fig. 3, with increasing MOIs, increasing amounts of the virus-induced enzyme were generated. Induction of the protein kinase activity was also studied in cells treated with cycloheximide at the time of infection, as well as with UV-inactivated HSV-1. Uninfected and HSV-1-infected cells were included as controls. The results are presented in Fig. 4. Cycloheximide inhibited the induction of the viral protein kinase, demonstrating that protein synthesis is required for its detection. No virus-specific protein kinase could be detected in cells infected with UV-inactivated HSV-1, demonstrating that a viable virus genome is required for its synthesis. Both results would also tend to rule out the possibility that the intracellular virus-specific protein kinase is carried into the cell by the infecting virus. UV inactivation does not destroy the protein kinase activity associated with the viral particle (S. Lemaster and B. Roizman, personal communication; and our own unpublished data). The data also indicate that the host cell enzyme is probably rapidly turned over since it is greatly reduced in quantity both in the presence of cycloheximide and after virus infection.

We have attempted to determine the molecular weight of the isolated HSV-1-induced protein kinase by glycerol gradient sedimentation. Those results indicate that either the protein kinase associates strongly with other, perhaps substrate, proteins, undergoes self-aggregation, or may perhaps consist of subunits, the smallest of which retains enzymatic activity. In crude extracts, the induced activity sedimented in 10 to 40% glycerol gradients as an apparent complex with a molecular weight of approximately 250,000. After CM-52 column chromatography (resulting in an approximate 100-fold purification), the majority of the isolated peak enzyme sedimented at a molecular weight of approximately 27,000 with some residual activity at a molecular weight of 250,000. Further purification of the CM-52 peak activity over phosphocellulose and DEAE-cellulose (during which the enzyme continued to elute as a single peak) resulted in a species, now approximately 1,000-fold purified, sedimenting in 5 to 20% glycerol gradients at a molecular weight value of approx-

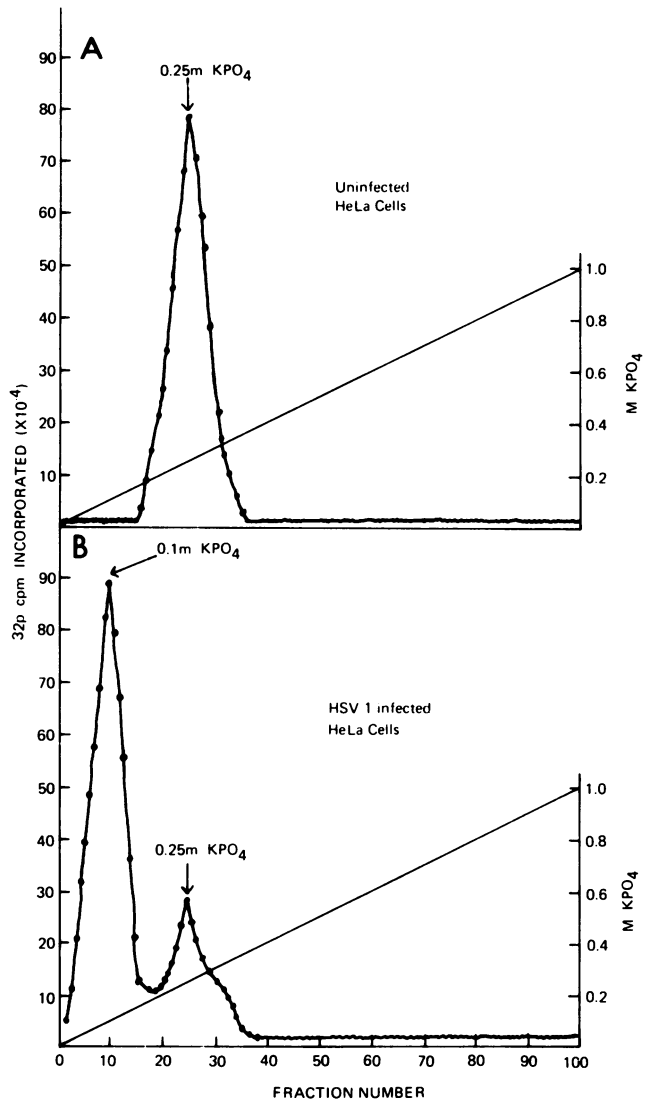

FIG. 2. *CM-52 column chromatography. Uninfected (A) and 4-h HSV-1-infected (B) HeLa cell extracts were dialyzed overnight against 0.01 M potassium phosphate buffer (KPO_4), pH 6.5, containing 0.1% Nonidet P-40, 0.5 mM dithiothreitol, and 10% glycerol (KNDG). The dialyzed extracts were loaded onto pre-equilibrated CM-52 columns, the columns were washed with five bed volumes of KNDG, and elutions were carried out with linear gradients of KPO_4 buffers (pH 6.5), 0.01 to 1.0 M, containing NDG. A 25-μl amount of each fraction was assayed for protein kinase activity by the standard assay. The same elution profiles were obtained with or without the addition of cyclic AMP to the assay mixture (see text).*

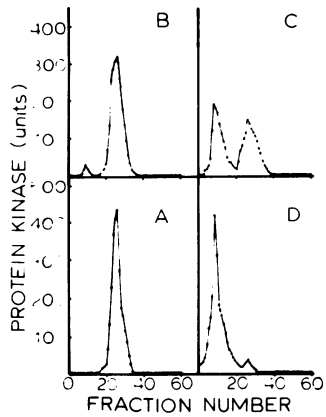

FIG. 3. *Induction of protein kinase activity by HSV-1 at various MOIs. HeLa cell monolayers were infected at MOIs of 0 (A), 1 (B), 10 (C), and 100 (D) PFU/cell. At 4 h postinfection, the cells were extracted, dialyzed, and chromatographed on CM-52 at pH 6.5, eluting with 0.01 to 1.0 M KPO_4 linear gradients containing NDG. One hundred fractions were collected from each column. A 25-μl amount of each fraction was assayed for protein kinase activity.*

imately 18,000 (Fig. 5). The host cell enzyme sedimented throughout various purification procedures at a molecular weight value of approximately 62,500.

We have also detected an apparent ability of the HSV-1-induced protein kinase for self-phosphorylation. We compared our most purified virus-induced and the host cell protein kinase for substrate specificity (Table 1). A control assay without an added phosphate-acceptor protein was included. Although the addition of certain phosphate-acceptor proteins did stimulate the HSV-1-induced protein kinase activity, there was still considerable incorporation in the absence of an added exogenous substrate. Table 1 also shows that the host cell enzyme differs considerably from the HSV-1-induced protein kinase in its specificity for exogenous substrates and, in addition, it does not contain self-phosphorylating ability.

Finally, we looked for the induction of a new protein kinase activity by HSV-1 in a different cell species. Infection of BHK-21 cells at an MOI of 10 PFU/cell induced the production of a new

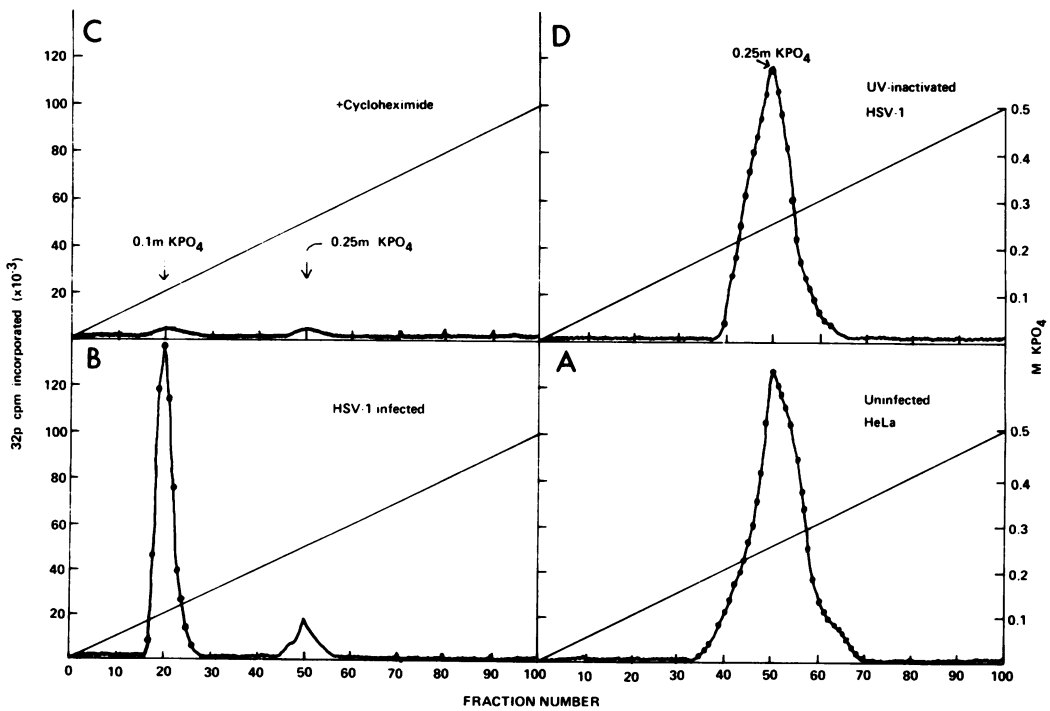

FIG. 4. *The effect of cycloheximide and UV-inactivated HSV-1 on the induction of virus-specific protein kinase activity. HeLa cell monolayers were either uninfected (A), infected with HSV-1 at an MOI of 100 PFU/cell (B), infected at an MOI of 100 PFU/cell but treated with cycloheximide after a 30-min virus absorption period (100 μg per ml of medium; C), or infected with 100 PFU/cell of UV-inactivated HSV-1 (D). At 4 h postinfection, the cells were extracted, dialyzed, and separately chromatographed on CM-52 at pH 6.5. Elutions were carried out with linear KPO_4 (NDG) gradients, from 0.01 to 0.5 M. A 25-μl amount of each fraction was assayed for protein kinase activity by the standard assay.*

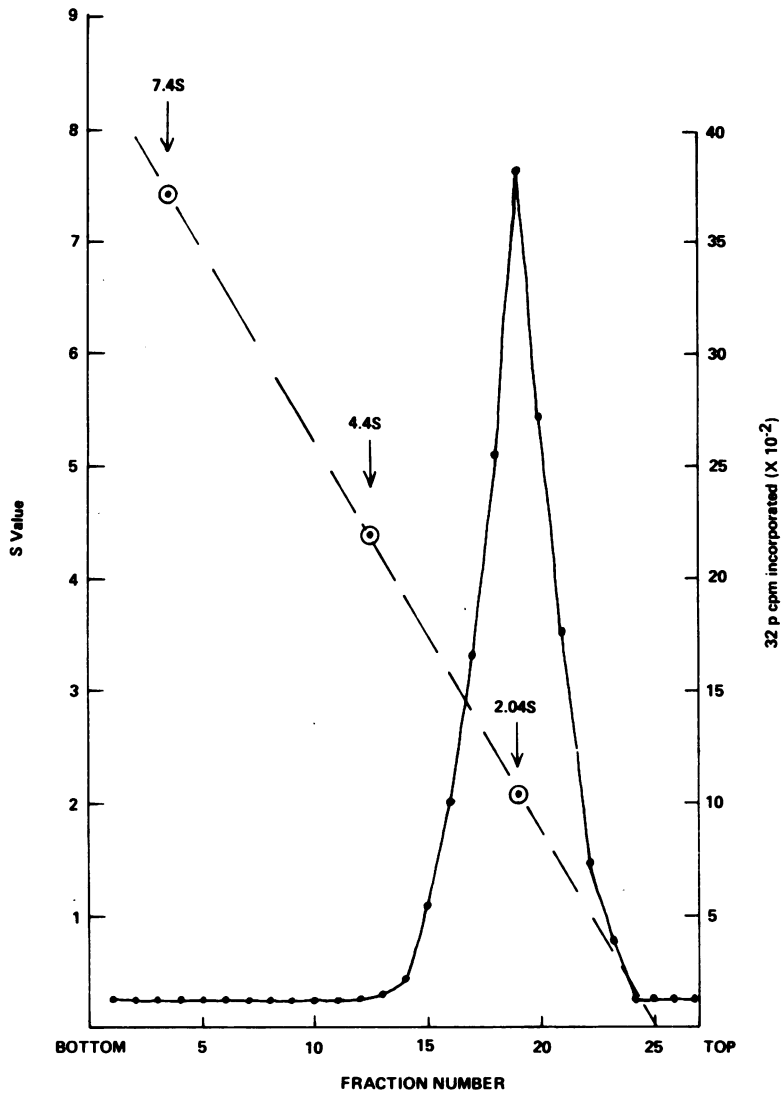

FIG. 5. *Glycerol gradient sedimentation of the HSV-induced protein kinase. The virus-induced enzyme activity, purified through CM-52, phosphocellulose, and DEAE-cellulose, was layered on top of linear 5 to 20% glycerol gradients containing 0.01 M Tris-hydrochloride (pH 8.0), 0.25 M KCl, 0.5 mM dithiothreitol, and 0.1% Nonidet P-40. The gradients were centrifuged at 189,000 × g for 20 h in a Beckman 50.1 swinging bucket rotor. The internal standards lactate dehydrogenase (7.4S), bovine serum albumin (4.4S), and myoglobin (2.04S) were included as markers. Ten-drop (0.2-ml) fractions were collected and assayed for protein kinase activity and for the internal molecular weight markers. The protein kinase activity sedimented at a value of approximately 2.2S.*

TABLE 1. *Use of various protein substrates by HSV-induced and host-cell protein kinases*

Enzyme	^{32}P incorporated (pmol)[a]					
	(−) Protein	Casein	Phosvitin	Protamine sulfate	Lysine-rich histones	Arginine-rich histones
HSV-induced	11.2	7.6	28.2	5.0	16.2	0
HeLa	0	16.2	21.1	0.4	0	0

[a] The assay mixtures contained 25 μg of the protein substrates and equal amounts (units) of the indicated enzymes. Incubation was for 30 min at 37°C.

protein kinase with the chromatographic, molecular weight, and substrate specificity properties identical to those induced in HeLa cells (data not shown).

The possibilities cannot be ruled out that infection of cells with HSV-1 results in either the alteration of a host cell protein kinase or the induction of a host cell enzyme not normally produced in uninfected cells. However, the evidence presented herein is also consistent with the interpretation that the HSV-1 genome codes for its own protein kinase. We are currently investigating these alternative possibilities.

This investigation was supported in part by a grant from the American Osteopathic Association.

The technical assistance of Beth Mickelson and Patrick Dennison was greatly appreciated.

LITERATURE CITED

1. **Akusjarvi, G., L. Philipson, and U. Pettersson.** 1978. A protein kinase associated with adenovirus type 2. Virology **87**:276–286.
2. **Baumann, E. A., and R. Hand.** 1979. Protein kinase activity associated with the D2 hybrid protein related to simian virus 40 T antigen: some characteristics of the reaction products. Proc. Natl. Acad. Sci. U.S.A. **76**: 3688–3692.
3. **Blair, G. E., and W. C. Russell.** 1978. Identification of a protein kinase activity associated with human adenoviruses. Virology **86**:157–166.
4. **Collett, M. S., and R. L. Erikson.** 1978. Protein kinase activity associated with the avian sarcoma virus *src* gene product. Proc. Natl. Acad. Sci. U.S.A. **75**:2021–2024.
5. **Fenwick, M. L., and M. J. Walker.** 1979. Phosphorylation of a ribosomal protein and of virus-specific proteins in cells infected with herpes simplex virus. J. Gen. Virol. **45**:397–405.
6. **Greengard, P.** 1978. Phosphorylated proteins as physiological effectors. Science **199**:146–152.
7. **Griffin, J. D., G. Spangler, and D. M. Livingston.** 1979. Protein kinase activity associated with simian virus 40 T antigen. Proc. Natl. Acad. Sci. U.S.A. **76**: 2610–2614.
8. **Hatanaka, M., E. Twiddy, and R. V. Gilden.** 1972. Protein kinase associated with RNA tumor viruses and other budding RNA viruses. Virology **47**:536–538.
9. **Hizi, A., W. Wunderli, and W. K. Joklik.** 1979. Purification and partial characterization of a protein kinase from the Prague-C strain of Rous sarcoma virus. Virology **93**:146–158.
10. **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.
11. **Honess, R. W., and B. Roizman.** 1975. Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. Proc. Natl. Acad. Sci. U.S.A. **72**:1276–1280.
12. **Houts, G. E., M. Miyagi, C. Ellis, D. Beard, K. F. Watson, and J. W. Beard.** 1978. Protein kinase from avian myeloblastosis virus. J. Virol. **25**:546–552.
13. **Jones, P. C., and B. Roizman.** 1979. Regulation of herpesvirus macromolecular synthesis. VIII. The transcription program consists of three phases during which both extent of transcription and accumulation of RNA in the cytoplasm are regulated. J. Virol. **31**:299–314.
14. **Kleiman, J. H., and B. Moss.** 1975. Purification of a protein kinase and two phosphate acceptor proteins from vaccinia virions. J. Biol. Chem. **250**:2430–2437.
15. **Krebs, E. G.** 1972. Protein kinases, p. 99–133. *In* B. L. Horecker and E. R. Stadtman (ed.), Current topics in cellular regulation. Academic Press, Inc., New York.
16. **Krebs, E. G., and J. Beavo.** 1979. Phosphorylation-dephosphorylation of enzymes. Annu. Rev. Biochem. **48**:923–959.

16a. **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. **35**:798–811.

17. **Levinson, A. D., H. Oppermann, L. Levintow, H. E. Varmus, and J. M. Bishop.** 1978. Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein. Cell **15**:561–572.
18. **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.
19. **Owada, M., and K. Moelling.** 1980. Temperature-sensitive kinase activity associated with various mutants of avian sarcoma viruses which are temperature sensitive for transformation. Virology **101**:157–168.
20. **Paoletti, E., and B. Moss.** 1972. Protein kinase and specific phosphate acceptor proteins associated with vaccinia virus cores. J. Virol. **10**:417–424.
21. **Pereira, L., M. H. Wolff, M. Fenwick, and B. Roizman.** 1977. Regulation of herpesvirus macromolecular synthesis. V. Properties of α polypeptides made in HSV-1 and HSV-2 infected cells. Virology **77**:733–749.
22. **Rosok, M. J., and K. F. Watson.** 1979. Fractionation of two protein kinases from avian myeloblastosis virus and characterization of the protein kinase activity preferring basic phosphoacceptor proteins. J. Virol. **29**:872–880.
23. **Rubenstein, A. S., M. Gravell, and R. Darlington.** 1972. Protein kinase in enveloped herpes simplex virions. Virology **50**:287–290.
24. **Schaffhausen, B. S., and T. L. Benjamin.** 1979. Phosphorylation of polyoma T antigens. Cell **18**:935–946.
25. **Sefton, B. M., T. Hunter, and K. Beemon.** 1979. Product of in vitro translation of the Rous sarcoma virus *src* gene has protein kinase activity. J. Virol. **30**:311–318.
26. **Smith, A. E., R. Smith, B. Griffin, and M. Fried.** 1979. Protein kinase activity associated with polyoma virus middle T antigen in vitro. Cell **18**:915–924.
27. **Strand, M., and J. T. August.** 1971. Protein kinase and phosphate acceptor proteins in Rauscher murine leukemia virus. Nature (London) New Biol. **233**:137–140.
28. **Tan, K. B.** 1975. Comparative study of the protein kinase associated with animal viruses. Virology **64**:566–570.
29. **Tan, K. B., and F. Sokol.** 1974. Virion-bound protein kinase in Semliki forest and Sindbis viruses. J. Virol. **13**:1245–1253.
30. **Wilcox, K. W., A. Kohn, E. Skylanskaya, and B. Roizman.** 1980. Herpes simplex virus phosphoproteins. I. Phosphate cycles on and off some viral polypeptides and can alter their affinity for DNA. J. Virol. **33**:167–182.