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Herpes Simplex Virus Phosphoproteins

II. Characterization of the Virion Protein Kinase and of the Polypeptides Phosphorylated in the Virion

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The protein kinase associated with purified herpes simplex virus 1 and 2 virions partitioned with the capsid-tegument structures and was not solubilized by non-ionic detergents and low, non-inhibitory concentrations of urea. The enzyme required Mg^{2+} or Mn^{2+} and utilized ATP or GTP. The activity was enhanced by non-ionic detergents and by Na^{+} even in the presence of high concentrations of of Mg^{2+}, but not by cyclic nucleotides. The enzyme associated with capsid-tegument structures phosphorylated virion polypeptides only; exogenously added substrates (acidic and basic histones, casein, phosphovitin, protamine, and bovine serum albumin) were not phosphorylated. The major phosphorylated species were virion polypeptides (VP) 1-2, 4, 11-12, 13-14, 18.7, 18.8, and 23. VP 18.7 and VP 18.8 have not been previously detected, but may be phosphorylated forms of polypeptides co-migrating with VP 19. Of the remainder, only VP 23 has been previously identified as a capsid protein; the others are constituents of the tegument or of the under surface of the virion envelope. The distribution of the phosphate bound to viral polypeptides varied depending on the Mg^{2+} concentration and pH. In the absence of dithiothreitol, in vitro phosphate exchange was demonstrable in VP 23 and to a lesser extent in two other polypeptides on sequential phosphorylation frist with saturating amounts of unlabeled ATP and then with [γ-^{32}P]ATP. Analysis of the virion polypeptides specified by herpes simplex virus 1 × herpes simplex virus 2 recombinants indicates that the genes specifying the polypeptides which serve as a substrate for the protein kinase map in the unique sequences near the left and right reinterated DNA sequences of the L component.

Previous reports have shown that protein kinases are associated with several viruses belonging to the herpesvirus family as well as with other viruses (5, 14, 17, 19, 20, 25, 26). The function of these kinases is not known. In the case of the protein kinase associated with the herpes simplex virus (HSV), the enzyme was reported to be released by non-ionic detergent and to be stimulated by exogenously added substrates (19). Because of the possibility that the enzyme was contained in host-derived contaminants rather than in the virion, the genetic origin of this kinase could not established. In this paper, we report that the protein kinase is associated with subvirion structures and that in situ it phosphorylates virion structural proteins only. Some properties of the enzyme and of the polypeptides which serve as its substrate are also described.

Pertinent to this report are some details concerning the structure of the HSV virion. Architecturally, the virion consists of four structural units. These are the core, consisting of DNA wrapped around protein fibers (3); the icosadeltahedral capsid (28); the tegument, consisting of a layer of fibrous material asymmetrically surrounding the capsid; and the envelope, which surrounds the capsid-tegument structure (18, 28). The polypeptides located on the surface of the envelope, those comprising the enveloped virions, de-enveloped capsid-tegument structures, and full and empty capsids have been described (4, 6, 8, 10, 22, 23, 24). Although by subtraction it is possible to identify the polypeptides internal to the surface of the envelope but external to the capsid, the precise localization of these polypeptides is not known largely because the technique currently available for the fractionation of the virion components does not permit unambiquous differentiation of the structural proteins in the underside of the envelope from those constituting the tegument.

MATERIALS AND METHODS

Radiochemicals. L-[^{35}S]methionine (specific activity, 1,004 Ci/mmol), [γ-^{32}P]ATP (specific activity,

1,000 to 3,000 Ci/mmol), and [γ-^{32}P]GTP (specific activity, 10 to 50 Ci/mmol) were purchased from New England Nuclear Corp. (Cambridge, Mass.).

Cells and viruses. HEp-2 cells were grown in Eagle minimal essential medium supplemented with 10% calf serum, 0.001% ferric nitrate, and 1% sodium pyruvate (23).

The isolation and properties of HSV-1 (MP) and HSV-2 (G) were described elsewhere (2, 9). The isolation of recombinant viruses utilized in this study has been described by Morse et al. (12).

Radiolabeling of infected cells and virions. For the preparation of labeled virions 75 to 80% confluent HEp-2 cell cultures were infected at a multiplicity of 3 PFU/cell and labeled from 5 h postinfection with [^{35}S]methionine (20 μCi/ml) and medium consisting of 1% calf serum and mixture 199 containing 1/25 of the normal methionine concentration. The infected cells were harvested at 40 h postinfection. For the labeling of infected cell polypeptides, HEp-2 cultures were infected at a multiplicity of 20 PFU/cell and labeled as indicated in the figure legends. The cultures were harvested at the end of the labeling interval and suspended in disruption buffer (2% sodium dodecyl sulfate [SDS], 2.5% β-mercaptoethanol, 20% [wt/vol] sucrose, and 0.05 M Tris-hydrochloride, pH 7.0, [Sigma Chemicals Co., St. Louis, Mo.]).

All infected cultures were incubated at 33°C.

Purification of virions and virion capsid-tegument structures. Virions were purified by the method of Spear and Roizman (23) as modified by Heine et al. (7), except that 1 mM EDTA was substituted for sucrose in all solutions, the cell debris were removed by centrifugation at 11,000 rpm for 15 min (12,000 × *g*) in a Sorvall SS34 rotor after disruption of infected cells with a Dounce homogenizer, and urea was not added to virions before pelleting of virions. For preparation of capsid-tegument structures, purified virions were suspended in Tris buffer consisting of 50 mM Tris-hydrochloride (pH 8.0) to which was added Nonidet P-40 (NP40) (BDH Chemicals Ltd., Poole, England) to a final concentration of 1%; the suspension was placed at 24° for 30 min and then centrifuged at 23,000 rpm for 1 h (80,000 × *g*) in a Beckman SW 27.1 rotor through 30% (vol/vol) Tris-buffered glycerol. For extraction with urea, deionized 5 M urea was added to the suspension of capsid-tegument structures to a final concentration of 1 M. The capsid-tegument structures were then pelleted by centrifugation at 27,000 rpm for 1 h in a Beckman SW 50.1 rotor.

The capsid-tegument preparations from cultures infected with HSV-1 × HSV-2 recombinants were prepared as follows. Monolayers of infected cells were swollen in hypotonic buffer and disrupted with a Dounce homogenizer, NP40 was added to a final concentration of 0.001%, and cell suspensions were centrifuged at 11,000 rpm for 15 min in a SS34 Sorval rotor. NP40 was added to the supernatant fluid to a final concentration of 1%, and the suspension was centrifuged through 30% Tris-buffered glycerol at 27,000 rpm for 60 min in a SW 50.1 rotor.

Protein kinase assay. The standard mixture for the assay of protein kinase activity consisted of 50 mM magnesium acetate, 0.1% NP40, 1 mM dithiothreitol (Calbiochem, LaJolla, Ca.), 50 mM Tris-hydrochloride (pH 8.0), and [γ-^{32}P]ATP (1 to 5 μCi/reaction). The reaction volume was 50 μl. All reactions were done at 37°C for 30 min; they were terminated by the addition of either 0.2 ml of EDTA (0.2 M, pH 7.5) or 50 μl of disruption buffer. In several experiments the reaction mixture was modified to test the effect of pH; dithiothreitol; [γ-^{32}P]GTP; Mg^{2+}, Mn^{2+}, and Na^{+} concentrations; and other additives as described in the test. Meaurements of ^{32}P incorporation were done on samples in which the reaction was terminated by the addition of EDTA, and to these samples was added 1 ml of 10% trichloroacetic acid saturated with sodium pyrophosphate. Precipitates were collected on 0.45-μm nitrocellulose filters.

Polyacrylamide gel electrophoresis. The procedures were as described by Morse et al. (13). All autoradiograms shown in this paper were made from SDS-polyacrylamide gels containing 9.25% polyacrylamide cross-linked with *N,N'*-diallyltartardiamide.

RESULTS

Requirements for the phosphorylation of viral polypeptides by the virion-associated protein kinase. The experiments described below concern the requirements for the phosphorylation of virion polypeptides by the virion-associated protein kinase and the identification of the polypeptides which become phosphorylated. Standard assay conditions determined from preliminary experiments to be near optimal were 50 mM Mg^{2+}, 0.1% NP40, 1 mM dithiothreitol, and [γ-^{32}P]ATP in 50 mM Tris-hydrochloride buffer, pH 8.0. The experimental basis for the standard assay mixture and the effects of varying the composition of the reaction mixture are described below.

Association of the virion protein kinase with the capsid-tegument structure. In this series of experiments, we assayed the activity of the protein kinase in intact purified virions, in capsid-tegument structures obtained by centrifugation of purified virions extracted with the non-ionic detergent NP40 through 30% Tris-buffered glycerol, and in capsid-tegument structures extracted with both NP40 and urea. The experiments were done as described in Materials and Methods. The results summarized in Table 1 show the following. Intact purified virions yielded 12% of the maximal activity recovered in the course of this experiment. Upon disruption of virions and subsequent centrifugation, 86% of the activity was recovered in the capsid-tegument structures and only 14% of the activity partitioned with the supernatant fluid. A second NP40 extraction of the capsid-tegument structure resulted in some loss of activity, but did not yield an appreciable amount of solubilized en-

TABLE 1. *Distribution of virion-associated protein kinase activity on extraction with NP40 and urea*

Extraction	Portion	cpm[a]	% Activity recovered
None	Virions[b]	109,479	12
1st NP40	Supernatant fluid	120,595	14
	Pellet	758,016	86 (100)[c]
2nd NP40	Supernatant fluid	30,210	4
	Pellet	627,906	83
1 M urea	Supernatant fluid	143,550	23
	Pellet	422,611	67

[a] Standard reaction mixtures contained 1.5 μCi of [γ-^{32}P]ATP. The radioactivity was determined after trichloroacetic acid precipitation as described in Materials and Methods.

[b] 9×10^4 PFU/assay.

[c] The sum of radioactivity recovered in the supernatant fluid and pellet after the first NP40 extraction was taken as 100%. The percents recovery after the second and third extractions were calculated with respect to the amount that should have been present in the material that was extracted.

TABLE 2. *Requirements of the virion-associated protein kinase*

Sample	cpm[b]	% Activity
NP40-extracted Virions[a]	727,073	100
−NP40	205,215	28
+0.2% SDS	8,521	1
$-Mg^{2+}$	23,076	3
+10 mM Mg^{2+}	401,145	55
+100 mM Mg^{2+}	859,577	118
+10 μg of histones[c]	640,858	88
+100 μg of histones[c]	527,906	79
+1,000 μg of histones[c]	532,446	73
+4 mM ATP	3,797	0.5
+1 mM cAMP	58,285	8
+1 mM GTP	17,091	2
+1 mM cGMP	113,007	16
NP40-extracted Virions	3,192	0.4

[a] The reaction volume of 50 μl contained the standard reaction mixture, the equivalent of 9×10^4 PFU, and 10 μCi of [γ-^{32}P]ATP. The standard reaction mixture was modified as indicated above.

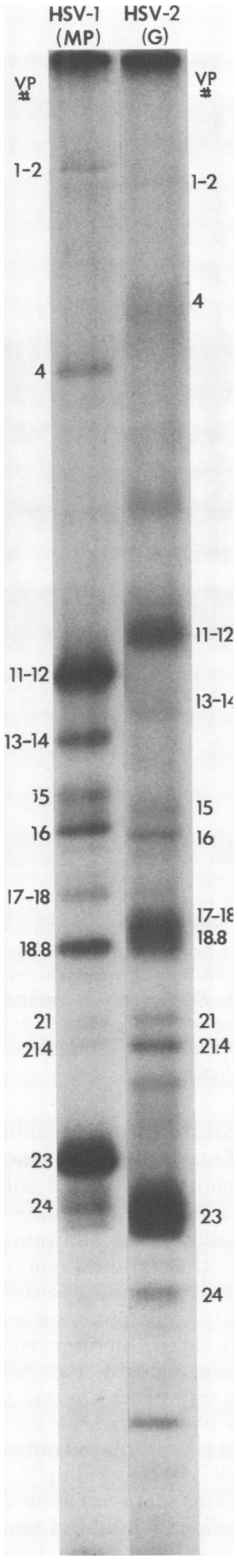
[b] Trichloroacetic acid-precipitable counts per minute.

[c] Micrograms of protein added.

zyme. Reextraction of the capsid-tegument structures with NP40 and with 1 M urea resulted in the recovery of approximately 90% of the activity, of which 67% partitioned with the capsid-tegument structures and 23% with the supernatant fluid. In other experiments (not shown) we found that higher concentrations of urea inactivated the enzymatic activity.

These experiments are consistent with the hypothesis that the protein kinase is tightly associated with the capsid-tegument structures and is not on the surface of the virions. However, additional experiments, especially those summarized in Table 2, indicate that even after disruption of the virions with NP40 the presence of the nonionic detergent enhances the activity of the protein kinase. In this series of experiments, purified virions were disrupted with NP40 and centrifuged through 30% Tris-buffered glycerol. The capsid-tegument structures in the pellet were resuspended in Tris buffer by gentle sonication. In the absence of NP40, activity was reduced to 28% of that observed in the standard mixture. These results are consonant with the observation that the activity of enzymes in a hydrophobic environment is enhanced by presence of nonionic detergents (27). We should note parenthetically, as shown in Table 2, that the cationic detergent SDS (0.2% final concentration) inactivated the enzyme.

Identification of the virion polypeptides phosphorylated by virion-associated protein kinase. Figure 1 shows the HSV-1 and HSV-2 polypeptides which become phosphorylated by the respective virion-associated protein kinases. In this experiment, capsid-tegument structures obtained from purified virions were incubated in the standard reaction mixture and then solubilized in disruption buffer and subjected to electrophoresis in SDS-polyacrylamide gels. Two aspects of the results shown in Fig. 1 are significant. First, with one exception, all of the phosphorylated polypeptides were previously identified in ^{14}C-amino acid preparations of purified virions (1, 23) and reflect the type-specific differences between HSV-1 and HSV-2 polypeptides (4, 23). The exception is VP 18.8, which was not detected in preparation of amino acid-labeled virions. These results indicate that the substrates of the virion protein kinase are virion polypeptides and not contaminating host debris. Second, all phosphorylated virion polypeptides, with one exception, are constituents previously assigned to the tegument or the under surface of the envelope (4, 6, 8, 10, 22, 23, 24). The exception, VP 23, is a constituent of full and empty capsids (4). These results suggest that polypeptides proximal to the protein kinase abut



the tegument, the outer surface capsid, and possibly the inner surface of the envelope.

Requirement for mono- and divalent cations for the virion-associated protein kinase. Several experiments were done to test the requirements for mono- and divalent cations and to determine the effect of the cations on the phosphorylation of individual virion polypeptides. Preliminary experiments summarized in Table 2 indicated that there was little or no phosphorylation in the absence of Mg^{2+} and that the activity doubled when the Mg^{2+} concentration was increased from 10 to 100 mM. Furthermore, Mg^{2+} could be replaced by Mn^{2+}, but not by Na^+ (data not shown).

The results of a more extensive study on the requirement for Mg^{2+}, summarized in Fig. 2, show that the maximum activity was obtained with Mg^{2+} concentrations in the range of 100 to 300 mM. An interesting feature of these results is the leveling off of activity in the presence of 10 to 50 mM Mg^{2+}. The biphasic effect of Mg^{2+} was reproducible and suggested the possibility that this cation may have a dual effect: first as an essential part of the metaloenzyme complex and second as a disruptive agent increasing the accessibility of the substrate to the enzyme. Two additional experiments were done to test these possibilities.

In the first, we tested the effect of Na^+ in the presence of varying Mg^{2+} concentrations. The significant finding was that at all concentrations of Mg^{2+} tested, the activity of the protein kinase was twice as high in the presence of 300 mM Na^+ than in the absence of the monovalent cation (data not shown). This result indicates that Na^+ does not substitute for Mg^{2+} even at the high concentrations of Mg^{2+} and, therefore, the enhanced effect of the divalent cation may not be due entirely to its disruptive effect on the capsid-tegument structures. Comparison of the electrophoretic profiles of virion polypeptides phosphorylated under these conditions (Fig. 3) revealed that the extent of phosphorylation of several polypeptides was dependent on the concentration of Mg^{2+} in both the absence and presence of Na^+. Thus, VP 19 was phosphorylated only in the presence of high concentrations of Mg^{2+}. In addition, VP 18.7, VP 18.8, VP 21,

FIG. 1. *Autoradiogram of HSV-1 (MP) and HSV-2 (G) virion polypeptides phosphorylated by the respective virion-associated protein kinases electrophoretically separated in polyacrylamide gels. The standard reaction mixture was incubated with 1.3 μCi of [γ-^{32}P]ATP. The polypeptides were numbered according to the nomenclature of Cassai et al. (1) and Spear and Roizman (23).*

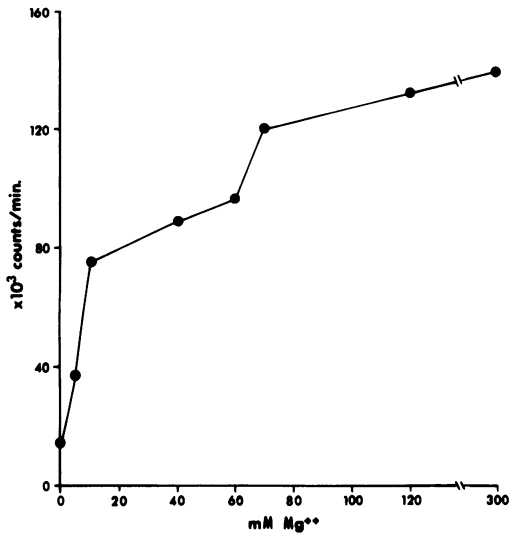


FIG. 2. *Effect of Mg^{2+} on the activity of the virion-associated protein kinase. The standard reaction mixture contained 6 μg of capsid-tegument protein, 2.5 μCi of [γ-^{32}P]ATP, and from 0 to 300 mM magnesium acetate. The protein kinase activity is expressed as ^{32}P trichloroacetic acid-precipitable radioactivity.*

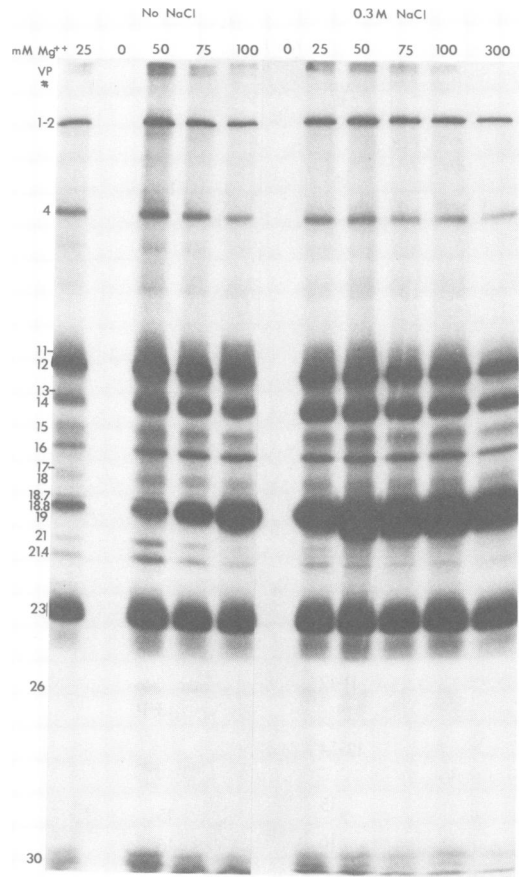


FIG. 3. *Autoradiogram of electrophoretically separated virion polypeptides phosphorylated by the virion-associated protein kinase in the presence of different concentrations of magnesium acetate and sodium chloride. Each reaction mixture contained 8.2 μg of capsid-tegument protein, 2.5 μCi of [γ-^{32}P]ATP, and the appropriate Mg^{2+} and Na^{+} concentrations as shown in the figure.*

and VP 21.4 became maximally phosphorylated at 50 mM Mg^{2+} and were underphosphorylated at higher Mg^{2+} concentrations.

In the second experiment we tested the effect of 50 mM Mg^{2+} at different pH's. As shown in Fig. 4, peak enzyme activities were observed between pH 6 and 7 and again between pH 8 and 9. The decrease in activity observed at pH 7.5 was reproducible in several experiments. Analyses of the polypeptides phosphorylated at various pH's (Fig. 5) indicate that the extent of phosphorylation of VP 1-2 and VP 23 increased with increasing pH. Whereas VP 4 was optimally labeled at pH 7, VP 18.7, VP 19, and to a lesser extent VP 13-14 were more extensively phosphorylated at pH 7.5 and above than at lower pH.

The results of these experiments are consistent with the hypothesis that the cations and pH alter the spatial arrangement between the enzyme and substrate by changing the conformation of the reactants and possibly by causing different kinds of disaggregation of the capsid-tegument structures under the various conditions tested. We should note that the Mg^{2+} effect cannot be explained by changes in pH of the reaction mixture with increasing Mg^{2+} concentrations inasmuch as this possibility was excluded by both experimental design and direct tests of the pH of the assay mixture.

Substrate requirements of the virion-associated protein kinase. Table 2 summarizes the results of experiments designed to determine whether exogeneously added substrates, cyclic nucleotides, and GTP stimulate the incorporation of phosphate. The addition of histones up to 1 mg/ml to the standard reaction mixture did not appreciably stimulate enzymatic activity. Similar results were obtained with protamine, phosphovitin, casein, bovine serum albumin, and basic and acidic histones (data not shown). Furthermore, SDS-polyacrylamide gel electrophoretic analysis of standard reaction mixtures containing added substrates did not reveal phosphorylated bands corresponding to any of these added substrates (data not shown). Inasmuch as partially purified, solubilized enzyme phosphorylates histones (Lemaster and Roizman, unpub-

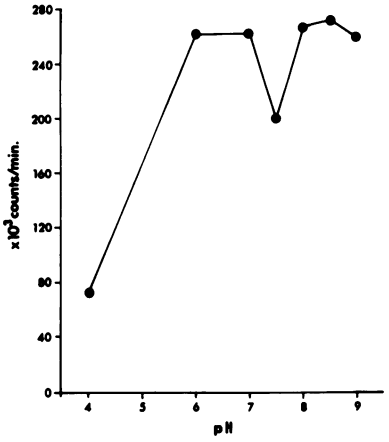


FIG. 4. *Effect of pH on the activity of the virion-associated protein kinase. The standard reaction mixture containing 8.2 μg of capsid-tegument protein and 2.5 μCi of [γ-^{32}P]ATP was adjusted to the pH shown. The enzymatic activity is expressed as ^{32}P trichloroacetic acid-precipitable radioactivity.*

lished data), these results suggest that the enzyme is located within the protein matrix of the capsid tegument structure such that exogenous substrates are not accessible to the enzyme. The decrease in activity observed upon the addition of histones is puzzling and possibly reflects hydrolysis of ATP, the interaction of ATP with protein nonspecifically, or an inhibitory effect due to an impurity present in the substrate preparation.

The addition of excess ATP or GTP to the reaction mixture resulted in a decrease in incorporation of ^{32}P (Table 2). This decrease in ^{32}P incorporation is probably due to isotope dilution by the unlabeled triphosphate since the virion polypeptides labeled with [γ-^{32}P]GTP were found to be identical to polypeptides phosphorylated with [γ-^{32}P]ATP upon electrophoresis in SDS-polyacrylamide gels (data not shown). However, the cyclic nucleotides, cAMP and cGMP were not able to stimulate enzymatic activity as shown in Table 2. This slight decrease in activity in the presence of cyclic nucleotides could be due to competition with the triphosphate. The utilization of ATP or GTP as the phosphate donor and lack of stimulation by cyclic nucleotides suggest that the virion-associated protein kinase is a cyclic nucleotide-independent protein kinase (20).

Characterization of the virion phosphoproteins. The experiments described in this section concern the effect of phosphorylation on the electrophoretic mobility of the virion polypeptides and the stability of the bound phosphate.

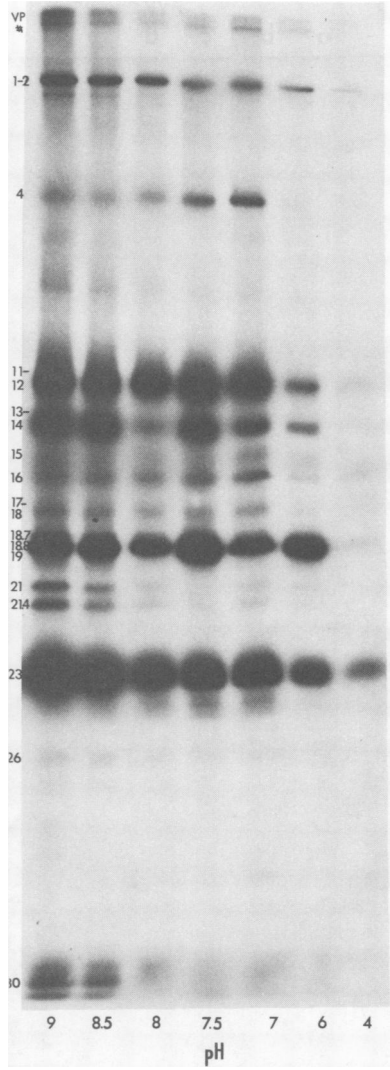


FIG. 5. *Autoradiogram of electrophoretically separated virion polypeptides exemplifying the effect of pH on the phosphorylation of virion polypeptides by the virion-associated protein kinase. Standard reaction mixtures were adjusted to the indicated pH and contained 8.2 μg of capsid-tegument protein and 2.5 μCi of [γ-^{32}P]ATP.*

Effect of phosphorylation on the electrophoretic mobility of the virion polypeptides. Figure 6 allows the comparison of the electrophoretic mobility of (i) [^{35}S]methionine-labeled polypeptides in purified virions (slot a, Fig. 6); (ii) [^{35}S]methionine-labeled virion polypeptides incubated in standard mixture in the absence of ATP (slot c, Fig. 6) and in the presence of 0.2 mM ATP (slot d, Fig. 6) or 0.4 mM ATP (slot e, Fig. 6); (iii) [^{35}S]methionine-labeled capsid-tegument structures (slot f, Fig. 6); and

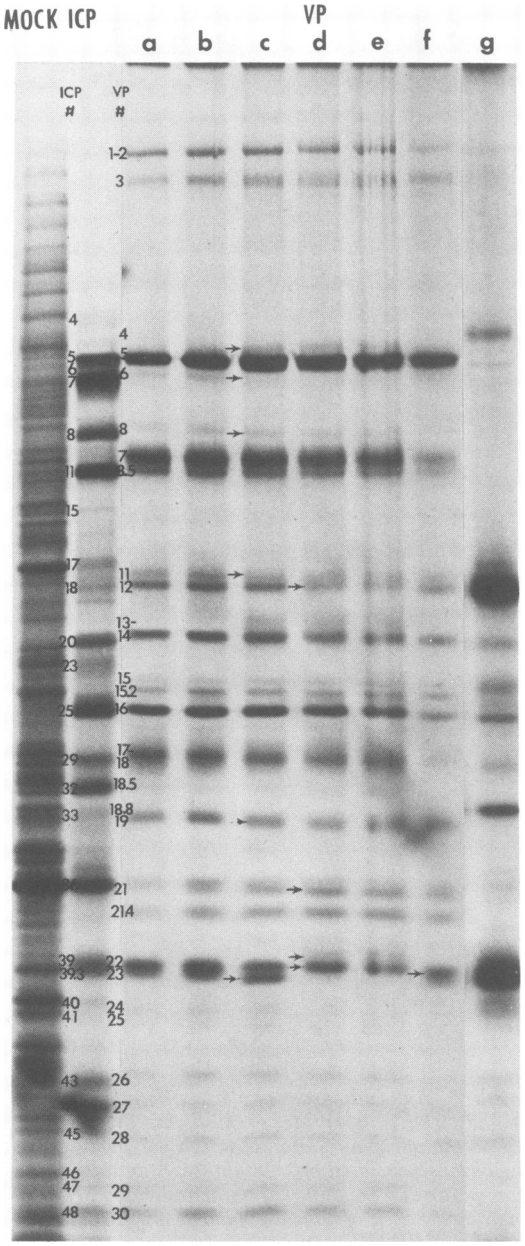


FIG. 6. *Autoradiogram of electrophoretically separated polypeptides exemplifying the effect of Mg^{2+} and of phosphorylation by the virion-associated protein kinase on the electrophoretic mobility of [^{35}S]-methionine-labeled HSV-1 (MP) polypeptides. The two slots on the left side of the figure represent [^{35}S]methionine-labeled polypeptides from mock-infected and HSV-1 (MP)-infected cells, respectively. Infected cell monolayers (20 PFU/cell) were labeled with 20 μCi of [^{35}S]methionine per ml from 7 to 9 h postinfection, whereas mock-infected cultures were simultaneously labeled for 2 h. The infected cell polypeptides (ICP) are numbered and shown to the left of the slot. (a) Virions solubilized directly in disruption buffer; (b) virion polypeptides incubated at 37°C for 30 min with 1 mM EDTA; (c) virions incubated in the standard reaction mixture without ATP; (d) virions incubated in the standard reaction mixture containing 0.2 mM ATP; (e) virions incubated in the standard reaction mixture containing 0.4 mM ATP; (f) capsid-tegument structures obtained as described in the text; (g) capsid-tegument structures labeled with 1.3 μCi of [γ-^{32}P]ATP in the standard reaction mixture. The arrows in slots c, d, and e refer to polypeptides whose electrophoretic mobilities have become altered as a consequence of the indicated conditions of incubation.*

(iv) [^{35}S]methionine-labeled polypeptides in purified virions phosphorylated with [γ-^{32}P]ATP (slot g, Fig. 6) in standard mixture. The salient features of the results were as follows. (i) The capsid-tegument structures differed from intact virions by the absence of VP 22 and by the reduction in the amount of virion glycoproteins VP 8, VP 7, VP 8.5, and VP 17–18 (slots a and f, Fig. 6). (ii) incubation of virions in the presence of Tris buffer alone did not substantially affect the electrophoretic mobility of virion polypeptides (slot b, Fig. 6). (iii) Incubation of virions in standard mixture containing 50 mM Mg^{2+} and NP40 but not ATP produced several striking modifications in the electrophoretic profile of virion polypeptides (slot c Fig. 6); thus, VP 19, VP 23, and VP 8 increased in electrophoretic mobility, VP 6 disappeared concomitantly with the appearance of a band above VP 5, and the band corresponding to VP 11 became more diffuse. (iv) The addition of ATP to the reaction mixture resulted in the disappearance of VP 23 and the appearance of a band above VP 22, the intensity of the VP 11–12 band decreased, and VP 22 resolved into a diffuse upper band and a more intense lower band (slot d, Fig. 6). (v) Doubling the ATP concentration in the reaction mixture did not produce any additional changes (slot e, Fig. 6). Finally, substitution of [γ-^{32}P]ATP for the unlabeled ATP (slot g, Fig. 6) yielded the profile of ^{32}P-labeled polypeptides similar to that shown in Fig. 1.

Stability of the phosphate bound to the virion structural proteins. Two series of experiments were done. The first was designed to determine the effect of ATP concentration on the phosphorylation of virion structural proteins and particularly the conditions for saturation of phosphate acceptor sites by the virion-associated protein kinase. In this experiment a fixed amount of [γ-^{32}P]ATP was mixed with varying concentrations of unlabeled ATP. From the amounts of ^{32}P incorporated into virion proteins (Table 3), it was calculated that the maximum

TABLE 3. *Effect of ATP concentration on phosphate incorporation into virion proteins*[a]

ATP in reaction mixture (μmol)	cpm[b]	Incorporated phosphate (pmol/μg of protein)
2,300	0	1.1
670	6,380	2089
67	141,791	4825
0.67	168,416	552
0.00033	164,690	2.7
0.00003	174,634	0.03

[a] The standard reaction mixtures contained 7.42 μg of capsid-tegument protein, 2.5 μCi of [γ-^{32}P]ATP (0.00003/μmol), and the indicated amount of ATP.

[b] The trichloroacetic acid-precipitable radioactivity was measured as described in the text.

phosphorylation was obtained with 67 μmol of ATP in the reaction mixture and resulted in incorporation of 4.8 pmol of phosphate per μg of protein. At higher ATP concentrations, the activity of the enzyme was reduced.

In the second series of experiments, we examined the stability of the phosphate bound to the proteins. Two series of experiments were done which differed only with respect to the presence of dithiothreitol in the standard reaction mixture. The experimental design was as follows. A portion of capsid-tegument structures was phosphorylated in the presence of sufficient unlabeled ATP (0.2 mM) to saturate acceptor sites. The capsid-tegument structures were then pelleted, resuspended in Tris buffer, and phosphorylated again in complete mixtures containing [γ-^{32}P]ATP (slots c and d, Fig. 7). Control experiments included phosphorylation of a portion of the capsid-tegument preparation in a mixture containing [γ-^{32}P]ATP (slots b and d, Fig. 7) as well as phosphorylation of another portion of the capsid-tegument preparation: first in a complete mixture containing [γ-^{32}P]ATP and then after centrifugation in a mixture containing a saturating amount of unlabeled ATP (slots a and e, Fig. 7).

In the absence of dithiothreitol in the standard assay mixture, the results were as follows. The capsid-tegument structures phosphorylated only with [γ-^{32}P]ATP yielded a profile (slot b, Fig. 7) similar to that shown in Fig. 1. Upon rephosphorylation with unlabeled ATP, there was a slight decrease in the labeling of VP 23, VP 11-12, and VP 13-14, as well as a shift in the position of the VP 18.8 (slot a, Fig. 7). The capsid-tegument structures phosphorylated first with saturating amounts of unlabeled ATP and then with labeled ATP (slot c, Fig. 7) showed one major phosphorylated band corresponding to VP 23 and trace amounts of labeled VP 13-14 and VP 18.8. The results of these studies indicate that at least some of the phosphate bound to VP 23 cycles on and off. To a much lesser extent, this may be true for at least some of the phosphate bound to VP 11-12, VP 13-14, and VP 18.8.

Entirely different results were observed in the presence of dithiothreitol in the standard assay mixture. In general, in the presence of dithiothreitol the exchange was less apparent. However, there are changes in electrophoretic mobility of the labeled polypeptides which might be a reflection of processing of these polypeptides concurrent with phosphate labeling. For example, VP 4 phosphorylated with nonsaturating amounts of [γ-^{32}P]ATP (slot f, Fig. 7) migrated more rapidly than the VP 4 phosphorylated first with labeled [γ-^{32}P]ATP and then with saturating amounts of unlabeled ATP (slot e, Fig. 7). However, when the polypeptides were phosphorylated first with saturating amounts of unlabeled ATP and then with [γ-^{32}P]ATP only trace amounts of the rapidly migrating form of VP 4 were phosphorylated (slot d, Fig. 7). Similar arguments may be applied to VP 18.8. In the presence of unsaturating amounts of [γ-^{32}P]-ATP, only a single rapidly migrating form was observed (slot f, Fig. 7). When the polypeptide was phosphorylated with [γ-^{32}P]ATP followed by phosphorylation with saturating amounts of unlabeled ATP, there appeared to be two phosphorylated bands, one co-migrating with VP 18.8 (slot, f, Fig. 7) and the second migrating more slowly (slot e, Fig. 7). Only the more slowly migrating forms are phosphorylated by [γ-^{32}P]ATP in the procedure involving phosphorylation with unlabeled ATP followed by [γ-^{32}P]-ATP (slot d, Fig. 7). VP 23 appeared to be phosphorylated with [γ-^{32}P]ATP under all conditions tested with nearly equal efficiency, except that in the presence of saturating amounts of ATP the electrophoretic mobility of the polypeptides was perceptibly slower.

These observations indicate that dithiothreitol alters the phosphorylation pattern but not the number of polypeptides phosphorylated by the virion-associated protein kinase. The differences in the pattern might be due to the exposure of additional sites for phosphorylation by the kinase. In the absence of dithiothreitol, there is evidence that the phosphate can cycle on and off VP 23 and to a lesser extent in several other polypeptides. The phosphate exchange is less apparent in the presence of dithiothreitol. In this instance, there is a suggestion that if the cycling occurs in other polypeptides, it may be accompanied by changes in electrophoretic mobility of the polypeptides. This is apparent in

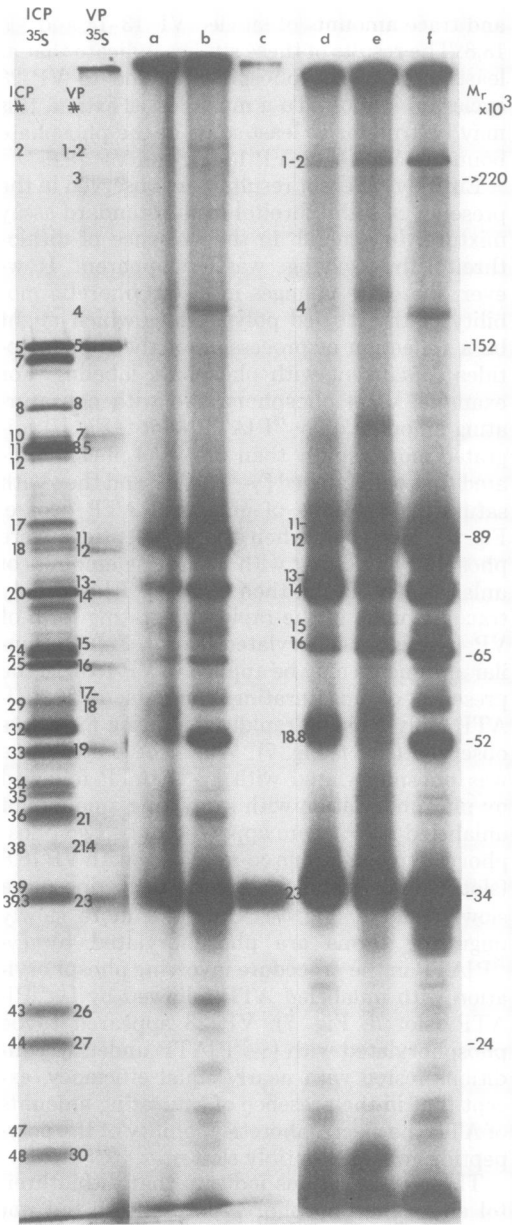


FIG. 7. *Autoradiogram of electrophoretically separated cell polypeptides from capsid-tegument structures phosphorylated sequentially with [γ-^{32}P]ATP and saturating amounts of unlabeled ATP. The two slots on the left show [^{35}S]methionine-labeled polypeptides from HSV-1 (MP)-infected and purified virion polypeptides. Infected cell cultures (5 PFU/cell) were labeled from 18 to 20 h postinfection with 40 μCi of [^{35}S]methionine per ml, whereas mock-infected cultures were labeled similarly for 2 hrs. The ICP and VP numbers are shown to the left of the respective slots. (a and e) Capsid-tegument structures labeled in the standard mixture containing 2.5 μCi of [γ-^{32}P]ATP and after centrifugation through 30% Tris-buffered glycerol were rephosphorylated in the standard mixture containing 0.2 mM ATP; (b and f) capsid-tegument structures labeled in the standard mixture containing 2.5 μCi of [γ-^{32}P]ATP; and (c and d) capsid-tegument structures incubated in the standard mixture containing 0.2 mM ATP and after sedimentation as above were labeled by reincubating in that standard mixture containing 2.5 μCi of [γ-^{32}P]ATP. The experiments represented in slots d, e, and f were done in the presence of 1 mM dithiothreitol. The molecular weights (M_r) shown on the right side of the figure were determined from the unlabeled standards, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme, which were solubilized and subjected to electrophoresis in the same gel.*

the case of VP 18.8 and VP 4; in these instances, the electrophoretic mobility of the polypeptides phosphorylated with saturating amounts of unlabeled ATP should have been identical to those seen in slot e. The observation that after phosphorylation with saturating amounts of unlabeled ATP the only forms which can be phosphorylated are the more rapidly migrating forms suggests that some of these polypeptides became dephosphorylated and migrated more slowly and that the subsaturating amounts of unlabeled ATP on rephosphorylation were insufficient to shift the electrophoretic mobility to rates comparable to those of fully phosphorylated polypeptides.

Mapping of the genes specifying the phosphorylated polypeptides. The purpose of this series of experiments was to map the genes specifying the major phosphorylated polypeptides on the physical map of HSV DNA. The location of the genes was based on the analysis of the phosphorylated polypeptides specified by HSV-1 × HSV-2 recombinants generated by Morse et al. (12, 13). To facilitate analysis of the polypeptides, the capsid-tegument structures were partially purified directly from the cytoplasm of HEp-2 cells infected by HSV-1 × HSV-2 recombinants as described in Materials and Methods. Although such preparations are not as pure as those obtained by the purification of virions, the major structural polypeptides are readily identifiable. Figure 8 shows the electrophoretic profiles of the polypeptides specified by HSV-1 × HSV-2 recombinants phosphorylated in vitro along with the virion polypeptides of HSV-1 (MP) labeled with [^{35}S]methionine and purified as described in Materials and Methods. The relevant physical maps of the recombinants of Morse et al. (12) are shown in Figure 9. The results of these experiments indicate the following.

VP 11–12 maps to the left of map unit 0.15 on the physical map of HSV DNA. This conclusion

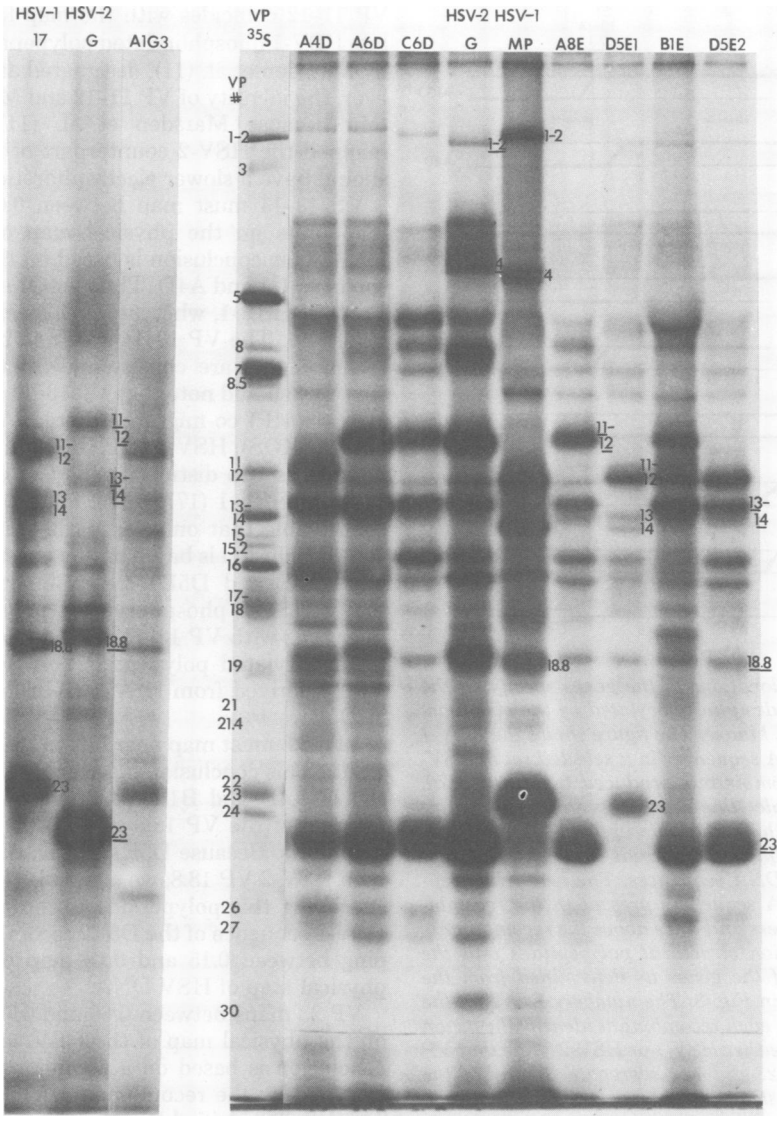


FIG. 8. *Autoradiogram of electrophoretically separated phosphorylated virion polypeptides of HSV-1 × HSV-2 recombinants. Capsid tegument structures, isolated from infected cell cytoplasms, were incubated in the standard reaction mixture containing 1.5 μCi of [γ-^{32}P]ATP as described in the text. The phosphorylated virion polypeptides are numbered to the right of the VP tract and HSV-2 virion polypeptides identified by a line drawn under the VP number. The [^{35}S]methionine-labeled HSV-1 (MP) polypeptides are numbered to the left of the VP tract. Note that [^{35}S]methionine-labeled purified HSV-1 (MP) polypeptides were co-electrophoresed on the same gel, but not immediately adjacent to the slot shown on the right; because of a distortion due to drying the ^{35}S-labeled polypeptides do not coincide precisely with their ^{32}P-labeled counterparts to the right. The polypeptides in the three slots on the left were subjected to electrophoresis separately and therefore include slots of the parental viruses, HSV-1 (17) and HSV (G), for precise reference to the type-specific differences in electrophoretic mobility of the recombinant, A1G3, polypeptides.*

is based on the comparison of recombinants B1E and D5E2. In recombinant B1E, VP 11–12 must map either to the right of 0.65 or to the left of 0.18 map units. Inasmuch as B1E specified HSV-2 VP 11–12 whereas D5E2 specified HSV-1 VP 11–12, the location of the gene specifying this polypeptide must be to the left of 0.15. All other recombinants specify polypeptides consistent with this map location. We should note that some HSV-2 preparations showed another phosphorylated polypeptide migrating slightly faster than HSV-2 VP 11–12. This polypeptide is less

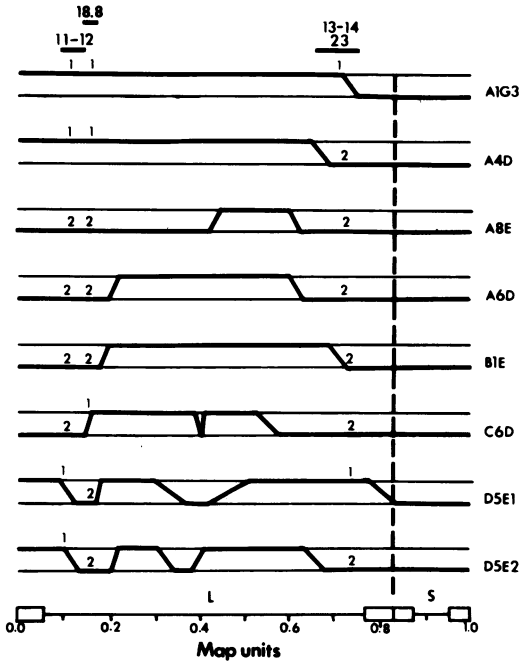


FIG. 9. *Map locations of the genes specifying the major polypeptides phosphorylated by the virion associated protein kinase. The figure shows the HSV-1 and HSV-2 DNA sequences in a selected set of HSV-1 × HSV-2 recombinants produced by Morse et al. (12). Each doublet line represents the recombinant designated on the right. The upper line represents HSV-1 DNA sequences, whereas the lower line represents HSV-2 DNA sequences. The heavy line represents the DNA sequences present in the recombinant. The numbers and lines above the recombinants identify the virion structural polypeptides and the map location of the genes as determined from the data presented in Fig. 8. The numbers 1 or 2 on the doublet lines for each recombinant identify the virion polypeptides as either HSV-1 or HSV-2. Note that VP 13-14 and VP 23 did not segregate in any of the recombinants tested.*

extensively phosphorylated than VP 11-12. It is particularly prominent in recombinant B1E. It is conceivable that only one of the polypeptides in the VP 11-12 complex is being mapped. This problem arises from the observation that the HSV-1 VP 11-12 migrate close together, whereas HSV-2 VP 11-12 on phosphorylation form two bands, i.e., a more intensively phosphorylated band migrating slower than VP 11-12 of HSV-1 and a minor phosphorylated band migrating slightly slower than HSV-1 VP 11-12. In none of the recombinants did the two bands segregate. Inasmuch as both bands co-map, the unresolved question is which of the two bands is more extensively phosphorylated in HSV-2 capsid-tegument structures. The map location of VP 11-12 coincides with the map location of a late HSV-1 phosphorylated polypeptide mapped by Marsden et al. (11), designated as M85; however, the identity of VP 11-12 and M85 is uncertain because Marsden et al. (11) have not mapped the HSV-2 counterpart of M85, which should have a slower electrophoretic mobility.

VP 13-14 must map between 0.66 and 0.76 map units on the physical map of the HSV DNA. This conclusion is based on the comparison of A1G3 and A4D. Thus, A1G3 specified VP 13-14 of HSV-1, whereas A4D specified HSV-2 VP 13-14. The VP 13-14 specified by the other recombinants are consistent with this conclusion. We should note that VP 13-14 specified by HSV-1 (MP) co-migrate, whereas VP 13-14 of HSV-1 (KOS), HSV-1 (HFEM), and HSV-1 (17) migrate as two distinct bands (for example VP 13-14 of HSV-1 (17) in Fig. 8, and (7, 15). It is conceivable that only VP 14 is being mapped. This conclusion is based on the observation that both A8E and D5E2 recombinants specify a major HSV-2 phosphorylated polypeptide co-migrating with VP 13-14 of HSV-2 and a minor phosphorylated polypeptide co-migrating with VP 13 derived from HSV-1 (KOS) and present in D5E1.

VP 18.8 must map near but to the right of VP 11-12. This conclusion is based on the comparison of A4D and B1E, which restrict the gene location of the VP 18.8 gene to the left of 0.18 map units. Because D5E1 and D5E2 both specify a HSV-2 VP 18.8, we conclude that the gene specifying this polypeptide is contained in the HSV-2 sequence of the D5E2 recombinant mapping between 0.15 and 0.18 map units on the physical map of HSV DNA.

VP 23 maps between 0.66 and 0.76 map units on the physical map of the HSV genome. This conclusion is based on a comparison of VP 23 specified by the recombinants A1G3 and A4D. The VP 23 specified by the other recombinants is consistent with these map locations. The map location of VP 23 (ICP 39.3) coincides with the map location of a late phosphorylated polypeptide mapped by Marsden et al. (11) designated as M36 for HSV-2 and M38 for HSV-1.

DISCUSSION

This report describes some of the properties of the protein kinase associated with HSV virions, its localization in the virion, and the properties of the virion structural polypeptides which serve as its substrate in situ. It is convenient to discuss each of these topics separately.

Localization and substrate specificity of the virion-associated protein kinase. The pertinent observations are as follows. (i) In all of

the experiments described in this report, the virion-associated protein kinase partitioned with capsid-tegument structures. (ii) The capsid-tegument-associated enzyme phosphorylated predominantly VP 1-2, 4, 11-12, 13-14, 18.7, 18.8, and 23. With the exception of VP 18.7 and VP 18.8, all of these polypeptides have been previously identified as virion polypeptides (23). Moreover, whereas VP 23 is a constituent of the capsid, the remainder of the previously identified virion polypeptides are either in the underside of the envelope or are constituents of the virion tegument. VP 18.7 and VP 18.8 have not been previously detected, either because they are present in few copies per virion or because they co-migrate with another virion polypeptides (e.g., VP 19), but change their electrophoretic mobility after phosphorylation. The relationship between VP 18.7 and VP 18.8 is not known; it is conceivable that VP 18.7 is a more extensively phosphorylated from of VP 18.8. (iii) The virion-associated protein kinase does not phosphorylate exogenous substrates such as histones, etc., even though the solubilized kinase phosphorylates these substrates (Lemaster and Roizman, unpublished data). We conclude from these observations the following. (i) The protein kinase is a structural component of the virion and not a host contaminant co-purifying with the virion inasmuch as host proteins and added substrates are not phosphorylated. (ii) The protein kinase is probably localized in the interface between the capsid and tegument inasmuch as in the experiments described in this report that the enzyme was not solubilized and its substrates were polypeptides localized in these structures. (iii) Whereas the substrate was accesible to ATP and other constituents of the assay mixture, it was not accessible to exogenously added substrates.

Requirements and properties of the capsid-tegument-associated protein kinase. The properties of the protein kinase appear to be defined by two sets of characteristics. First, the kinase requires a divalent cation, Mg^{2+} or Mn^{2+}, and utilizes ATP or GTP. The activity of the enzyme is enhanced by non-ionic detergents, but not by cyclic nucleotides. These properties suggest that the HSV protein kinase is in a hydrophobic environment and is a cyclic nucleotide-independent enzyme (20, 27).

Second, the kinase is stimulated by Na^+ and responds to increasing concentrations of Mg^{2+} and to variations in pH in a biphasic fashion. It is of special interest that the activtiy of the enzyme increases in the presence of Mg^{2+} in concentrations as high as 300 mM and that the distribution of the ^{32}P on labeled virion proteins varies depending on pH. Inasmuch as preliminary studies with solubilized enzyme indicate that its Mg^{2+} optimum is nearer to 5 mM (Lemaster and Roizman, unpublished data), the effects of Mg^{2+} and pH could reflect changes in the juxtaposition of substrate proteins to the insoluble enzyme rather than the intrinsic properties of the enzyme. The results of the experiments obtained after sequential phosphorylation with [γ-^{32}P]ATP and unlabeled ATP in the presence and absence of dithiothreitol are subject to the same interpretation. In this instance the reduction of disulfide bonds may progressively alter the secondary structure of both substrate and enzyme such as to make accessible new phosphate acceptor sites as well as render inaccessible the sites phosphorylated initially or in the absence of the reducing agents.

Properties of the polypeptides phosphorylated by the virion-associated protein kinase. We have already noted that with one exception the virion polypeptides phosphorylated by the protein kinase are localized within the domain of the virion defined by the tegument and undersurface of the envelope. The VP 18.7 and VP 18.8 are probably in this structure as well, and as noted above, both may be the processed forms of other polypeptides. It is of interest to note that the band defined as VP 19 in the virion consists of many more copies per virion than could be predicted from the concentration of VP 19 in the purified capsid (7). VP 19 present in the virion is therefore heterogeneous and probably contains other polypeptides in addition to the capsid VP 19. The function of the polypeptides phosphorylated by the protein kinase is not known and it is uncertain whether these polypeptides become phosphorylated in the course of infection. Of particular interest are the observations that several polypeptides became altered with respect to their electrophoretic mobility after exposure to Mg^{2+} and especially after phosphorylation with saturating amounts of ATP. Our data suggest that VP 23 might be cleaved by an enzyme dependent on Mg^{2+} for activation. G.H. Cohen and associates independently found that VP 23 undergoes cleavage in preparations of purified virions (G. H. Cohen, personal communication). In addition, VP 23 as well as several other polypeptides (e.g., VP 4, VP 18.8) are characterized by slower electrophoretic mobility after phosphorylation.

The experiments concerning the stability of the phosphate on the virion polypeptides suggest that the phosphate bound to some of the polypeptides can be exchanged in vitro. The exchange of phosphate in VP 23 is readily mani-

fested when disulfide bonds are not reduced. More extensive exchange and possibly more extensive phosphorylation were observed in the presence of dithiothreitol, possibly as a consequence of changes in the highly ordered structure of the proteins in juxtaposition with the enzyme. The results suggest that phosphorylation occurs in stages and is reflected in the electrophoretic mobility of the phosphorylated polypeptides.

The phosphorylation of proteins in the absence of dithiothreitol probably reflects more accurately the topology of the enzyme and its interaction with its substrate. The apparent exchange of phosphate in VP 23 suggests either that VP 23 is a component of the enzyme or the enzymatically active polypeptide or that the enzyme can both phosphorylate and dephosphorylated the juxtaposed substrate (20). We cannot presently discern between these alternative hypotheses.

Physical mapping of the genes specifying the polypeptides phosphorylated by the virion protein kinase. The results of the mapping studies indicate that the genes specifying the virion polypeptides phosphorylated in situ by the protein kinase cluster in two regions of the genome, i.e., within the unique sequences of the L component adjacent to the left and right inverted repeats. These observations are consistent with the conclusions that genes specifying architecturally related structural polypeptides are arranged in clusters which are within noncontiguous regions of the genome (12, 16, 21). The significance of this observation, particularly as it relates to the regulation of the expression of genes specifying structural polypeptides, remains obscure.

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LITERATURE CITED

1. **Cassai, E. N., M. Sarmiento, and P. G. Spear.** 1975. Comparison of the virions proteins specified by herpes simplex virus types 1 and 2. J. Virol. **16**:1327–1331.
2. **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. **2**:357–364.
3. **Furlong, D., H. Swift, and B. Roizman.** 1972. Arrangement of herpesvirus deoxyribonucleic acid in the core. J. Virol. **10**:1071–1074.
4. **Gibson, W., and B. Roizman.** 1972. Protein specified by herpes simplex virus. VII. Characterization and composition of multiple capsid forms of subtypes 1 and 2. J. Virol. **10**:1044–1052.
5. **Hatanaka, M., E. Twiddy, and W. V. Gilden.** 1972. Protein kinase associated with RNA tumor viruses and other budding RNA viruses. Virology **47**:536–538.
6. **Heilman, C. J., Jr., M. Zweig, J. R. Stephenson, and B. Hampar.** 1979. Isolation of a nucleocapsid polypeptide of herpes simplex virus types 1 and 2 possessing immunologically type-specific cross-reactive determinants. J. Virol. **29**:34–42.
7. **Heine, J. W., R. W. Honess, E. N. Cassai, and B. Roizman.** 1974. Proteins specified by herpes simplex virus. XII. The virion polypeptides of type 1 strains. J. Virol. **14**:640–651.
8. **Heine, J. W., P. G. Spear, and B. Roizman.** 1972. Proteins specified by herpes simplex virus. VII. Viral proteins in the plasma membrane. J. Virol. **9**:431–439.
9. **Hoggan, M. D., and B. Roizman.** 1959. The isolation and properties of a variant of herpes simplex producing multinucleated giant cells in monolayer cultures in the presence of antibody. Am. J. Hyg. **70**:208–219.
10. **Honess, R. W., and B. Roizman.** 1975. Proteins specified by herpes simplex virus. XIII. Glycosylation of viral polypeptides. J. Virol. **16**:1308–1326.
11. **Marsden, H. S., N. D. Stow, V. G. Preston, M. C. Timbury, and N. M. Wilke.** 1978. Physical mapping of herpes simplex virus-induced polypeptides. J. Virol. **28**:624–642.
12. **Morse, L. S., T. G. Buchman, B. Roizman, and P. A. Schaffer.** 1977. Anatomy of herpes simplex virus DNA. IX. Apparent exclusion of some parental DNA arrangements in the generation of intertypic (HSV-1 × HSV-2) recombinants. J. Virol. **24**:231–248.
13. **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.
14. **Paoletti, E., and B. Moss.** 1972. Protein kinase and specific phosphate acceptor proteins associated with vaccinia virus cores. J. Virol. **10**:417–424.
15. **Pereira, L., M. H. Wolff, M. Fenwick, and B. Roizman.** 1977. Regulation of herpesvirus macromolecular synthesis. V. Properties of polypeptides made in HSV-1 and HSV-2 infected cells. Virology **77**:733–749.
16. **Preston, V. G., A. J. Davison, H. S. Marsden, M. C. Timbury, J. H. Subak-Sharpe, and N. M. Wilkie.** 1978. Recombinants between herpes simplex virus types 1 and 2. Analyses of genome structures and expression of immediate early polypeptides. J. Virol. **28**:499–517.
17. **Randall, C. C., H. W. Rodgers, D. N. Downer, and G. A. Gentry.** 1972. Protein kinase activity in equine herpesvirus. J. Virol. **9**:216–222.
18. **Roizman, B., and D. Furlong.** 1974. The replication of herpes viruses, p. 299–403. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 3. Plenum Publishing Corp., New York.
19. **Rubenstein, A. S., M. Gravell, and R. Darlington.** 1972. Protein kinase in enveloped herpes simplex virions. Virology **50**:287–290.
20. **Rubin, C. S., and O. M. Rosen.** 1975. Protein phosphorylation. Annu. Rev. Biochem. **44**:831–877.
21. **Ruyechan, W. T., L. S. Morse, D. M. Knipe, and B. Roizman.** 1979. Molecular genetics of herpes simplex virus. II. Mapping of the major viral glycoproteins and of the genetic loci specifying the social behavior of infected cells. J. Virol. **29**:677–697.
22. **Spear, P. G.** 1976. Membrane proteins specified by herpes simplex viruses. I. Identification of four glycoprotein precursors and their products in type 1-infected cells. J. Virol. **17**:991–1008.
23. **Spear, P. G., and B. Roizman.** 1972. Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpes virion. J. Virol. **9**:143–159.

24. **Spring, S. B., B. Roizman, and J. Schwartz.** 1968. Herpes simplex virus products in productively and abortive infection. II. Electron microscopic and immunological evidence for the failure of virus envelopment as a cause of abortive infection. J. Virol. **2**:384–392.
25. **Strand, M., and J. T. August.** 1971. Protein kinase and phosphate acceptor proteins in Rauscher murine leukemia virus. Nature (London) N. Biol. **233**:137–140.
26. **Tan, K. B., and F. Sokol.** 1974. Bound protein kinase in Semliki Forest and Sindbis viruses. J. Virol. **13**:1245–1253.
27. **Tanford, C., and J. A. Reynolds.** 1976. Characterization of membrane proteins in detergent solutions. Biochim. Biophys. Acta **457**:133–170.
28. **Wildy, P., W. C. Russell, and R. W. Horne.** 1960. The morphology of herpesvirus. Virology **12**:204–222.