Phosphorylation of Animal Virus Proteins by a Virion Protein Kinase

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Compared with several other enveloped viruses, purified virions of frog virus 3 contained a relatively high activity of a protein kinase which catalyzed the phosphorylation of endogenous polypeptides or added substrate proteins. Virions also contained a phosphoprotein phosphatase activity which released phosphate covalently linked to proteins. It was possible to select reaction conditions where turnover of protein phosphoesters was minimal, as the phosphatase required Mn^{2+} ions for activity whereas the protein kinase was active in the presence of Mg^{2+} ions. Electrophoretic studies in polyacrylamide gels containing sodium dodecyl sulfate indicated that at least 10 of the virion polypeptides were phosphorylated in the in vitro protein kinase reaction. Characterization of these phosphoproteins demonstrated that the phosphate was incorporated predominantly in a phosphoester linkage with serine residues. The protein kinase was solubilized by disrupting purified virions with a nonionic detergent in a high-ionic-strength buffer and was separated from many of the virion substrate proteins by zonal centrifugation in glycerol gradients. The partially purified protein kinase would phosphorylate polypeptides of many different animal viruses, and maximal activity was not dependent on added cyclic nucleotides. These properties distinguished the virion protein kinase from a well characterized cyclic AMP-dependent protein kinase which phosphorylated viral proteins only to a small extent.

Protein kinases are enzymes present in a wide variety of organisms which transfer the γ-phosphoryl group of ATP to O-seryl and O-threonyl linkage in proteins (2, 13, 20, 23, 24, 31, 33). A distinguishing characteristic of many protein kinases is their activation by low levels of cyclic AMP (cAMP) (13, 22, 29, 35, 45-48), and most of the available information about the molecular structure and properties of protein kinase has been derived from studies of cyclic nucleotide-dependent enzymes from the soluble fraction of cells (4, 7, 33-35). Protein kinase has also been detected in many subcellular fractions (14, 25), including membranes (35, 48), ribosomes (12, 17, 47), and nuclei (37), and is thought to be associated with specific phosphate acceptor proteins in these fractions (14, 17, 25, 47).

A protein kinase has been detected in purified virions of several enveloped viruses, including both membrane-maturing and intracytoplasmic-maturing species (9, 11, 30, 32, 36, 42, 44). These enzymes were not activated by cAMP and would phosphorylate some polypeptides of the virion, histones, and other basic proteins.

Protein kinase was not detected in several viruses which lacked a membranous envelope (36, 42, 44), and a specific study comparing the protein kinase content of enveloped herpes virus and non-enveloped nucleocapsids led to the suggestion that the enzyme was possibly incorporated in the virion envelope (36). However, other studies suggested that a protein kinase was tightly associated with cores of vaccinia virus (30). Whether the virion protein kinase is a viral-specific or modified host enzyme is presently unknown.

In this communication we describe experiments which show that frog virus 3, and enveloped DNA containing virus which replicates in the cellular cytoplasm (8), contains a high activity of a protein kinase which is active with virion phosphate acceptor proteins as well as exogenous substrates. The enzyme has been solubilized and partially purified, and studies of its substrate specificity suggest that the virion enzyme is distinct from several of the well characterized cAMP-dependent protein kinases. In addition, some experiments are pre-

sented which demonstrate the frog virus also contains an enzyme which catalyzes the dephosphorylation of phosphoproteins. (This work was presented in part at the 1972 meeting of the Fed. Amer. Soc. for Exp. Biol [Fed. Proc. **31**: abstract 1061]).

MATERIALS AND METHODS

Chemicals and media. The following were purchased from the respective companies: salmon sperm protamine sulfate and bovine serum albumin (BSA) from Sigma Chemical Co.; RNase, DNase, and calf thymus histone fractions 1, 2a, 2b and 3 from Worthington Biochemical; Pronase (grade B) and dithiothreitol from Calbiochemicals; Eagle minimal essential medium (MEM), agar, fetal calf serum (heat inactivated), and tryptose phosphate from Gibco; Nonidet P-40 (NP-40) from Shell Oil; ATP from Mann Research Laboratories; cAMP from General Biochemicals; cGMP from P-L Biochemicals; ^{3}H-thymidine from New England Nuclear; and γ-^{32}P-ATP and NCS from Amersham/Searle.

Cells. A continuous line of fathead minnow cells (FHM) (10), obtained from M. Gravell, were grown at 28 C in MEM containing 292 mg of L-glutamine per liter and supplemented with 10% heat-inactivated fetal calf serum. These cells were maintained as monolayer cultures in humidified incubators containing an atmosphere of 5% CO_2. Cells in the 113 to 132 passage were used for these experiments. BHK-21/13 cells (41), obtained from M. Gravell, were grown at 37 C in MEM containing 292 mg of L-glutamine per liter and supplemented with 10% tryptose phosphate, 10% heat-inactivated fetal calf serum, and 10 mM Bes, 10 mM Mops and 15 mM Hepes, final pH 7.4, as described by Eagle (3). These cells were passaged as monolayer cultures in humidified incubators containing an atmosphere of 5% CO_2 and were grown in roller bottles when large numbers of cells were needed for propagation of virus.

Frog virus 3 growth and purification. The frog virus 3 (FV3) isolate of polyhedral cytoplasmic deoxyvirus (8) was obtained from Brian McAuslan. A stock of this virus was prepared by infecting FHM cells at an input multiplicity of 0.01 PFU per cell and allowing multiple cycles of virus growth for 5 days at 28 C. Media and cells were collected and chilled on ice, and virus was dispersed by sonic oscillation for 1 min at maximum power with a Branson model LS-75 probe sonicator. The resulting stock of virus, 3 × 10^8 PFU per ml, was stored frozen in a liquid nitrogen freezer and samples were used as an inoculum for the production of FV3 for virus purification. Infectious titers were measured by plaque formation on FHM cells (8).

When large quantities of FV3 were required, the virus was propagated in 150-mm-diameter petri dish cultures of FHM cells or roller bottle cultures of BHK cells by infecting at an input multiplicity of 5 to 10 PFU per cell. The virus was added to the cells in a small volume of growth medium (1 ml for petri dishes, 15 ml for roller bottles), and the cultures were rolled or intermittently tilted for 1 h at 28 C to allow adsorp-

tion to take place. The inoculum was then replaced with growth medium (20 ml for petri dishes, 75 ml for roller bottles), and incubation was continued at 28 C. Cells infected for 18 to 30 h were easily detached from the substratum on which they were grown by gentle agitation and were collected by centrifugation at 3,000 × g for 5 min. The cell-associated virus was purified by a modification of the method of Tan and McAuslan (43). All steps were performed at 0 to 4 C. The pellet of infected cells was suspended in hypotonic solution (10 mM Tris-hydrochloride [pH 8.0], 10 mM KCl, 2 mM $MgCl_2$) at a concentration of 5 × 10^7 cells per ml and disrupted by 10 strokes in a Dounce homogenizer. The homogenate was centrifuged at 1,200 × g for 10 min to pellet nuclei, and the supernatant fluid was layered onto linear gradients of 20 to 60% (wt/vol) sucrose in standard virus buffer (SVB) of 10 mM Tris-hydrochloride (pH 8.0), 50 mM KCl, and 2 mM $MgCl_2$. After centrifugation in a Spinco SW25.2 rotor for 30 min at 16,000 rpm, the visible bands of virus, one major and a slower sedimenting minor band, were collected with a needle and syringe, pooled, and diluted with an equal volume of SVB. The virus was concentrated by centrifugation at 200,000 × g for 45 min, and the pellet was suspended in SVB by ultrasonic vibration (ultrasonic device, Heat Systems, Inc.). The virus was further washed and purified by isopycnic centrifugation in gradients of 20 to 40% (wt/wt) of potassium tartrate in SVB (40 ml total volume) on top of which was layered 12 ml of 20% (wt/vol) sucrose in SVB. Virus was layered on the sucrose and, after centrifugation for 4 h at 24,000 rpm in a Spinco SW25.2 rotor, formed two poorly resolved bands at an average density of 1.26 g/cm^3. The virus was collected with a needle and syringe, diluted with an equal volume of SVB, and concentrated by centrifugation at 200,000 × g for 45 min. The pellet was suspended in SVB by ultrasonic vibration to give a protein concentration of 1.8 to 2.2 mg/ml, and had a final titer of 0.5 × 10^{10} to 1.0 × 10^{10} PFU/ml. This purification procedure resulted in a yield of between 20 and 40% of the PFU in the starting homogenate, which represented approximately 100 infectious particles produced per cell. Purified virus stored at 0 C showed no loss of either infectivity or protein kinase activity after 6 months.

When FV3 was labeled during the growth cycle with ^{3}H-thymidine, monolayers of FHM cells were infected as described above, and at 3 h postinfection the media was replaced with normal growth medium supplemented with 10 μCi of ^{3}H-thymidine per ml (20 Ci/mmol). At 7 h postinfection the media was again replaced with normal growth media containing 10 μCi of ^{3}H-thymidine per ml (20 Ci/mmol). Cells were harvested 18 h postinfection, and FV3 was purified as described above.

Other viruses. The Rauscher murine leukemia virus used in these studies was kindly provided by the John L. Smith Memorial for Cancer Research under the auspices of the Special Virus Cancer Program, National Cancer Institute. The virus was grown and purified as described previously (42). Herpes simplex virus (subtype 1) was grown and purified as described by Spear and Roizman (40); the sample of virus used

for these experiments was a generous gift from Bernard Roizman. Vaccinia virus (strain WR) was grown in suspension cultures of HeLa cells and purified as described (15, 16); we are indebted to Sue Horwitz who provided the poxvirus for this study. Reovirus (type 3) was grown in L cells and purified as described previously (6, 39); the sample used in this study was a generous gift from Rise Cross. All viruses were stored at 0 C, although the herpes simplex and vaccinia virus had undergone a single cycle of freezing and thawing prior to being received in this laboratory.

Beef heart protein kinase. The cAMP-dependent protein kinase from beef heart tissue was a generous gift from Jack Erlichman and Charles S. Rubin. The enzyme was the partially purified alumina Cγ fraction as described by Rubin et al. (34).

Escherichia coli basic protein. One of the most active substrates for several protein kinases are a group of histone-like basic proteins from *E. coli* (42). The *E. coli* basic protein used in these studies was purified by the method of Kuo and August (19) and was kindly provided by Ching Hung Kuo.

Protein kinase assay. The reaction mixtures (0.2 ml total volume) contained 5 μmol of Tris-hydrochloride (pH 8.0), 2 μmol of DTT, 4 μmol of MgCl$_2$, 0.02 ml of 0.2% NP-40, 10 nmol of γ-^{32}P-ATP, and enzyme and acceptor proteins as indicated in the individual legands. Unless otherwise specified, the reaction mixtures were incubated at 30 C for 10 min after which 250 μg of BSA was added as carrier and the reactions were terminated by adding 0.3 ml of cold 10% trichloroacetic acid containing 20 mM NaPPi. After standing for 30 min at 0 C, the precipitate was collected by centrifugation at 10,000 $\times$ *g* for 10 min. The supernatant fluid was discarded, and the precipitate was dissolved in 0.1 ml of 0.1 N NaOH. The protein was again precipitated by adding 5 ml of cold 5% trichloroacetic acid containing 20 mM NaPPi. The acid-insoluble material was collected on a Whatman GF/C glass fiber disk and washed with 25 ml of cold 5% trichloroacetic acid containing 20 mM NaPPi. The filter disk was then dried, and the radioactivity was measured. Reaction mixtures without enzyme served as blanks. For experiments testing the effect of cAMP, the ATP used for the assay was freed from any contaminating cAMP by passage through a column (0.4 by 10 cm) of Dowex AG50X8 resin (100–200 mesh) as described by Krishna et al. (18).

Preparation of phosphorylated FV3. Purified FV3(BHK), 480 μg of protein, was labeled in a 25-fold protein kinase assay mixture of 5 ml as described above, except that NP-40 and DTT were omitted. The reaction was terminated by adding unlabeled ATP to a final concentration of 10 mM and chilling the sample on ice. Subsequent purification of the ^{32}P-labeled proteins was carried out at 0 to 4 C. The sample was layered on 7.5 ml of 10% (wt/vol) sucrose in buffer A (10 mM Tris-hydrochloride [pH 7.6], 100 mM NaCl, and 1 mM ATP), and the virus was pelleted by centrifugation at 200,000 $\times$ *g* for 45 min in a Spinco SW41 rotor. Virus was suspended in 1 ml of buffer B (buffer A, less 1 mM ATP) by ultrasonic vibration, layered on 11.5 ml of 10% (wt/vol) sucrose in buffer B, and again centrifuged as described above

to remove soluble radioactivity. The pellet of virus was suspended in buffer B. Virus prepared in this manner contained 2.1 nmol of acid-precipitable ^{32}P per mg of protein. The product was tested for its contamination by γ-^{32}P-ATP as described by Meisler and Langan (28). Approximately 5% of the total radioactivity was recovered as free Pi following treatment with hot acid.

Preparation of phosphorylated protamine. Protamine was phosphorylated in a reaction catalyzed by a protein kinase prepared from beef heart (34). A typical reaction mixture contained 50 mg of protamine sulfate, 1.4 mg of protein kinase purified through the stage of batch elution from alumina Cγ (34), 20 μmol of γ-^{32}P-ATP, 1 mmol of Tris-hydrochloride (pH 7.2), 400 μmol of MgCl$_2$, 200 μmol of DTT, and 40 nmol of cAMP in a total volume of 20 ml. The reaction was incubated at 35 C for 3 h. Protamine was recovered from the reaction mixture as described by Meisler and Langan (28). The product was checked for its content of alkali-labile phosphate and contamination by ATP as described by Meisler and Langan (28). Phosphorylated protamine contained 200 nmol of alkali-labile phosphate per mg (specific activity 30 counts per min per pmol) which corresponds to approximately 1 mol of phosphate per mol of protamine. ATP contamination accounted for less than 1% of the total radioactivity.

Acrylamide gel electrophoresis. Viral polypeptides were analyzed by high-resolution polyacrylamide gel electrophoresis in the presence of 0.1% SDS as described by Laemmli (21). Samples were prepared by dialyzing in 80 mM Tris-hydrochloride (pH 7.0), and 20% (vol/vol) glycerol. Prior to analysis, sodium dodecyl sulfate, mercaptoethanol and bromophenol blue were added to the samples to final concentrations of 2%, 5%, and 0.005%, respectively, and samples were boiled for 2 min. Electrophoresis was performed at 25 C on 10% polyacrylamide gels at a current of 3 mA per gel. After electrophoresis the gels were removed from the glass tubes and stained by immersion in a solution of 0.2% Coomassie brilliant blue, 7.5% glacial acetic acid, and 50% methanol for 3 h at 37 C. Gels were destained electrophoretically. Autoradiography was performed by the method of Fairbanks et al. (5).

Protein measurement. Protein content of purified virus preparations was measured by the method of Lowry et al. (26) using BSA as a standard.

RESULTS

Protein kinase in FV3. Incubation of purified frog virus with γ-^{32}P-ATP resulted in the incorporation of radioactivity into acid-precipitable material which was subsequently characterized as phosphoprotein, as described below. The incorporation was proportional to the quantity of frog virus present in the reaction mixture over a range of virus concentrations up to as much as 20 μg of protein per assay. The phosphorylation reaction had similar requirements to those described for other virion protein kinases (9, 30, 32, 42). Maximum incorporation

was obtained in the presence of 20 mM $MgCl_2$, 0.02% (vol/vol) NP-40, and 25 mM Tris-hydrochloride, over a range of pH values between 7.6 and 9.2. Addition of 10 mM DTT or 20 μM cAMP did not effect the reaction (data not shown).

When incubated at 30 C, the phosphorylation of frog virus proteins proceeded for about 10 min after which little increase in total incorporation was observed (Fig. 1). The addition of more γ-^{32}P-ATP and protein kinase after 10 min did not increase the yield after 30 min of incubation, suggesting that incorporation ceases as a result of the utilization of all the available sites for phosphorylation on virion proteins.

The apparent specific activity of the phosphorylation of frog virus proteins was between one and two orders of magnitude higher than the equivalent endogenous reaction for purified virions of several other enveloped viruses (Table 1). Moreover, if the effect of the endogenous phosphate acceptor proteins was minimzed by measuring activity in the presence of saturating amounts of added substrate proteins, under which conditions most of the incorporation was due to the phosphorylation of the exogenous substrate (42; H. S., unpublished data), frog virus still demonstrated at least a 10-fold-greater specific activity of protein kinase than any of the other viruses examined. This sug-
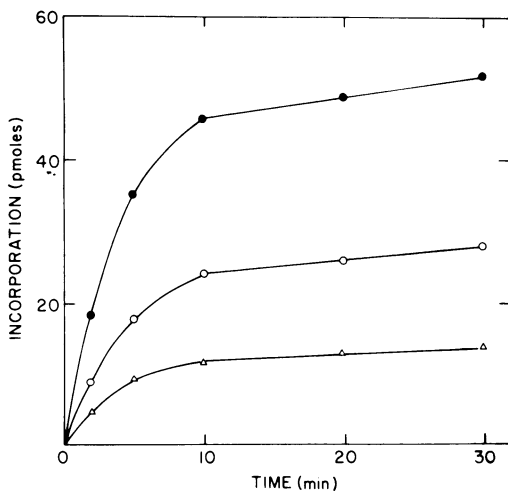
TABLE 1. *Protein kinase in animal viruses*

Virus	^{32}P incorporated[a]		
	Endogenous[b]	+ Histone fraction 3[b]	+ E. coli basic protein[b]
FV3(BHK)	3,620	5,460	7,440
FV3(FHM)	1,380	1,820	2,120
R-MLV	10	32	104
Herpes	38	42	108
Vaccinia	48	62	126
Reovirus[c]	<1	<1	<1

[a] Picomoles of ^{32}P incorporated per milligram of virus protein per minute.

[b] Substrate. The protein kinase activity of purified virions (2-50 μg of viral protein) was measured as described in Materials and Methods either without added substrate, with 50 μg of histone fraction 3, or with 10 μg of E. coli basic protein. Incubations were for 5 min at 30 C. The γ-^{32}P-ATP contained between 19 and 320 counts per min per pmole.

[c] Purified reovirus (3.4 mg of viral protein per ml) was activated by heating at 61 C for 45 s as described by Borsa et al. (1). Unheated virus gave essentially the same results.

gested that virions of FV3 contained a relatively high level of protein kinase enzyme. However, as additional factors may have influenced the activity of this enzyme when assayed with such a complex mixture of proteins as was present in virions, this conclusion should be regarded as tentative until supported by additional evidence such as comparative purification data. Control experiments (not shown) suggested that differences in protein kinase activity among these viruses were not due to the effect of an ATPase on γ-^{32}P-ATP substrate depletion, as the protein phosphorylation reaction for each virus was saturated with respect to ATP with the 50 μM level utilized in these 5-min assays. Consistent with previous observations (36, 42, 44), no protein kinase was detected in a non-enveloped virus such as reovirus. The specific activity of protein kinase in purified frog virus was several-fold higher when virus was propagated in BHK cells rather than FHM cells; however, the enzyme of each had similar ionic requirements and substrate specificities.

Since protein kinase was also present in uninfected host cells (data not shown) and has been found in many subcellular fractions (14, 25), it was essential to establish that the enzyme was not present as a contaminating particulate host cell component in the purified frog virus. For such experiments FV3 was prepared with its DNA labeled with ^{3}H-thymidine so that the isotope served as a marker for particles containing viral DNA. When purified ^{3}H-labeled FV3 was subjected to isopycnic centrifu-

FIG. 1. *Kinetics and effect of frog virus concentration on endogenous protein kinase reaction. At indicated times, 0.2-ml samples were removed from 10-fold protein kinase reaction mixtures of 2 ml containing 4.8 ($\triangle$), 9.6 ($\bigcirc$), or 19.2 ($\bullet$) μg of FV3 (BHK) protein. Samples were analyzed for trichloroacetic acid-precipitable radioactivity as described in Materials and Methods. The γ-^{32}P-ATP contained 43 counts per min per pmol.*

gation in a tartrate density gradient, all of the protein kinase activity and labeled viral particles banded in a sharp coincident peak (Fig. 2). Velocity sedimentation of ^{3}H-labeled FV3 through a sucrose gradient likewise revealed only the coincident migration of protein kinase and FV3 (data not shown). These experiments suggested that the protein kinase was associated as an integral component of the FV3 virion.

Phosphoprotein phosphatase in FV3. A question that must be considered in studies of viral protein phosphorylation is whether the phosphate incorporated into protein is subject to turnover, possibly through the coordinate action of a protein kinase and a phosphoprotein phosphatase. Incubation of frog virus and ^{32}P-labeled phosphoproteins resulted in a time-dependent hydrolysis of protein phosphoesters (Fig. 3). The reaction was optimal in 0.1 mM MnCl$_2$, 20 mM DDT, 100 mM NaCl, 0.02% (vol/vol) NP-40, and 5 mM Tris-hydrochloride (pH 7.6). Particularly noteworthy was the requirement this reaction showed for Mn^{2+} ions.
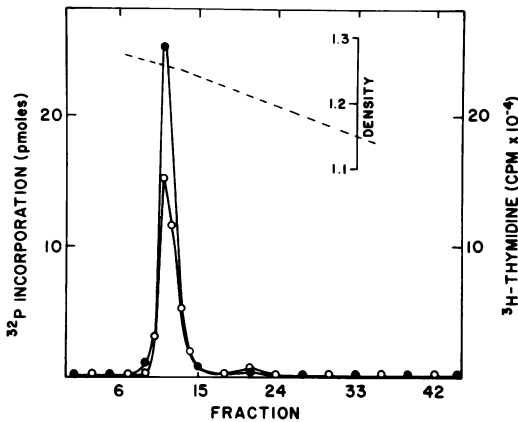
When MnCl$_2$ was replaced with MgCl$_2$ at the concentration used in the protein kinase assay (20 mM), the phosphatase was relatively inactive and ^{32}P-labeled viral phosphoproteins remained phosphorylated even after prolonged incubation (Fig. 3). This suggested that, under the conditions of the protein kinase assay, there was little turnover of phosphoesters and that the incorporation of phosphate occurred at sites on substrate proteins which were vacant of phosphate at the start of the incubation. Preliminary experiments indicated that a phospha-
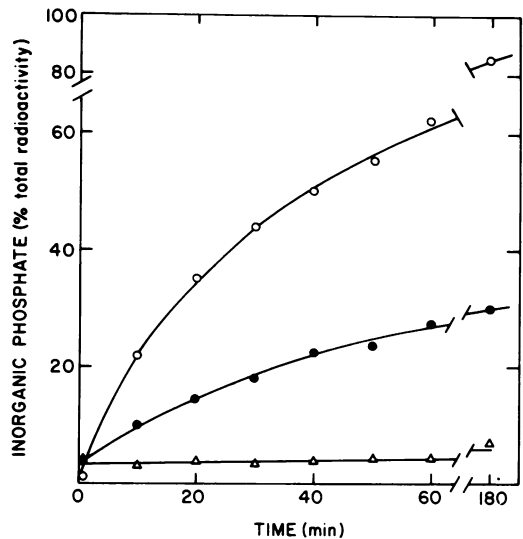

FIG. 3. *Dephosphorylation of phosphoproteins by frog virus. Reaction mixtures, 2.0 ml total volume, contained 10 μmol of Tris-hydrochloride (pH 7.6), 200 nmol of MnCl$_2$, 40 μmol of DTT, 200 μmol of NaCl, 0.02% (vol/vol) NP-40, 180 μg of FV3 (BHK) protein and ^{32}P-labeled phosphoproteins as indicated below. Incubation was at 35 C. At various times 0.2-ml samples were removed and transfered to tubes containing 0.2 ml of 10 mM silicotungstic acid, 5 mM H$_2$SO$_4$, and 0.25 mM KH$_2$PO$_4$. After centrifugation at 2,000 × g for 10 min to pellet insoluble material, orthophosphate was extracted from the supernatant fluid by a modification of the method of Martin and Doty (27). A 0.1-ml amount of 5% ammonium molybdate in 4 N H$_2$SO$_4$ was added to each tube after which the phosphomolybdate complex was extracted with 0.5 ml of isobutyl alcohol-benzene, 1:1. A sample of the isobutyl alcohol-benzene extract was plated on a planchet and dried, and the radioactivity was determined. Symbols: O, reaction mixture contained phosphorylated protamine containing 4 nmol of ^{32}P (16 counts per min per pmol); ●, reaction mixture contained 180 μg of phosphorylated FV3 (BHK) containing 380 pmol of ^{32}P (305 counts per min per pmol) and unlabeled frog virus was omitted; Δ, reaction mixture contained 180 μg of phosphorylated FV3 (BHK) containing 380 pmol of ^{32}P (305 counts per min per pmole), 40 μmol of MgCl$_2$, and both MnCl$_2$ and unlabeled frog virus were omitted.*

FIG. 2. *Co-purification of protein kinase and frog virus 3. FV3 was grown in FHM cells, labeled with ^{3}H-thymidine, and purified as described in Materials and Methods. A 0.20-ml amount of purified FV3 (6 × 10^9 PFU/ml, 2.0 mg protein/ml, 3.0 × 10^7 counts per min of ^{3}H per mg protein) was layered onto a 5.0-ml linear gradient of potassium tartrate (15–35% [wt/wt] in SVB) and centrifuged at 40,000 rpm for 3 h in an SW65 rotor at 4 C. Ten-drop fractions were collected from the bottom of the tube. Protein kinase was measured as described in Materials and Methods, using 0.01 ml of each fraction without added phosphate acceptor proteins. The γ-^{32}P-ATP contained 318 counts per min per pmol. The refractive index of selected fractions was measured using a Bausch and Lomb refractometer, and the density was calculated by comparison to the refractive index of tartrate standards. Symbols: ●, ^{32}P-incorporation; O, ^{3}H-thymidine.*

tase activity of frog virus was capable of dephosphorylating a variety of phosphoproteins such as histones, protamine, and virion polypeptides. Small-molecular-weight, phosphoester-containing compounds such as α-^{32}P-AMP were not attacked. This suggested that the protein dephosphorylation reaction was not due to the action of a nonspecific phosphatase activity.

Phosphate acceptor proteins of FV3. The endogenous phosphate acceptor molecules of FV3 were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 4). With 130 μg of FV3 protein, as many as 18 separate classes of polypeptides could be distinguished after staining with Coomassie blue. At least 10 polypeptides were phosphorylated by the in vitro kinase reaction, and the specific activity of these labeled phosphoproteins varied widely. The major phosphorylated species migrated with apparent molecular weights of 23,000 and 12,000 to 15,000 and appeared to be phosphorylated to a very high specific activity as they were minor protein components of the virion. The capsid polypeptide, molecular weight 50,000, was not labeled in the in vitro reaction.

To verify that all of the molecules containing ^{32}P were proteins, the endogenous acceptors were labeled in vitro, samples were individually treated with enzymes of defined specificity, and the product, was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 5). Treatment of the phosphorylated product with RNase A or DNase I had no effect on the radioactive molecules, whereas Pronase treatment converted all of the radioactive species into rapidly migrating components of low molecular weight.

The nature of the chemical bond linking the phosphate to the protein was also investigated. More than 75% of the phosphorylated product was sensitive to *E. coli* alkaline phosphatase, which would be expected if the phosphate was incorporated as a phosphomonoester possibly of serine or threonine. Chemical characterization as described by Strand and August (42) indicated that approximately 90% of the acid-precipitable radioactivity was stable to treatment with hot acid, whereas 99% was sensitive to hot alkali. This was consistent with the properties expected of protein phosphoesters. Acylphosphate sensitive to hydroxylamine at pH 5.5 was not detected, and moreover only 10% of the radioactivity was lost after washing the acid-precipitable product with chloroform-methanol. Analysis of the product by thin-layer electrophoresis after partial acid hydrolysis demonstrated that phosphoserine was the predominant phosphorylated amino acid (Fig. 6).
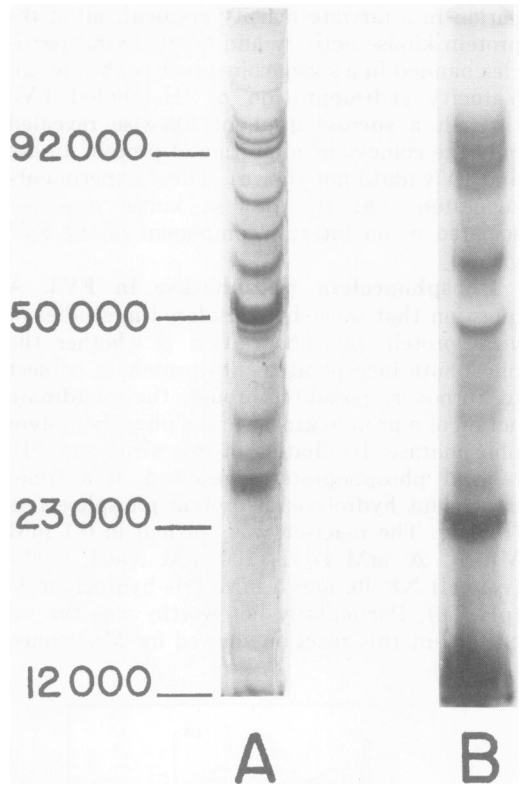

FIG. 4. *Phosphate acceptor proteins of frog virus. A 55-μg amount of FV3 (FHM) protein was phosphorylated in a two-fold protein kinase reaction mixture of 0.4 ml as described in Materials and Methods. The γ-^{32}P-ATP contained 1,250 counts per min per pmol. The reaction was stopped by the addition of 1 M EDTA, pH 7.0, to a final concentration of 20 mM and chilling on ice. Purified FV3 (FHM), 0.2 ml (380 μg of protein), was added, and the entire mixture was dialyzed at 4 C overnight in 80 mM Tris-hydrochloride (pH 7.0), and 20% glycerol. A 130-μg amount of FV3 protein (0.1 ml of dialyzed reaction mixture) was analyzed by high resolution SDS-polyacrylamide gel electrophoresis and an autoradiogram prepared as described in Materials and Methods. The molecular weight values were determined by including known proteins (phosphorylase A, BSA, aldolase, and lysozyme) with the virus. A, Gel stained for protein; B, autoradiogram of A.*

Practically no phosphothreonine was detected. A significant amount of ^{32}Pi was formed during the acid hydrolysis, much of which could be accounted for by the breakdown of phosphoserine in the presence of 6 N HCl at elevated temperatures (17). As was also evident in Fig. 6B, radioactivity was associated with three additional compounds labeled P-peptides 1, 2, and 3. In experiments not shown, phosphoserine
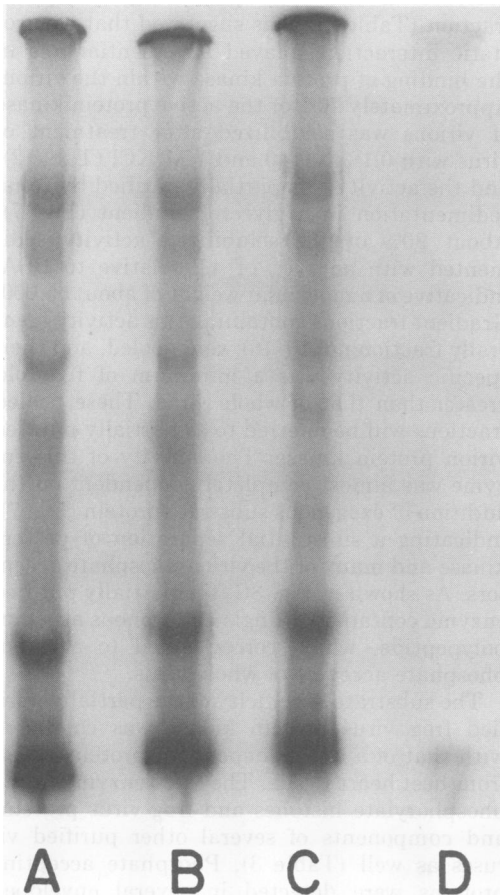
FIG. 5. Characterization of endogenous phosphorylated product. A 150-µg amount of FV3 (FHM) protein was phosphorylated in an eight-fold protein kinase reaction mixture of 1.6 ml as described in Materials and Methods. The γ-^{32}P-ATP contained 250 counts per min per pmol. The reaction was terminated by removing 0.3-ml samples and boiling them for 3 min. DNase (20 µg), RNase (20 µg), and Pronase (20 µg) were added individually to the samples and these were incubated together with a control sample (no additions) at 37 C for 1 h. Each sample was then dialyzed at 4 C overnight in 80 mM Tris-hydrochloride (pH 7.0), and 20% glycerol, and analyzed by SDS polyacrylamide gel electrophoresis; an autoradiogram of each gel was prepared as described in Materials and Methods. A, control; B, RNase treated; C, DNase treated; D, Pronase treated. Bovine pancreatic RNase A, dissolved in water, had been previously boiled for 3 min at 100 C; Pronase, dissolved in 0.3 M potassium phosphate (pH 6.7), had been previously incubated for 2 h at 37 C, and the insoluble material was removed by centrifugation.

was isolated from each of these compounds, demonstrating that they were phosphopeptides yielded from the partial hydrolysis of virion

proteins. From the above experiments, as well as considerations of the chemical properties of phosphorylated virus, it was concluded that at least 75% of the radioactive product of the endogenous protein kinase reaction of FV3 was phosphoprotein containing O-phosphoserine residues.

Properties of the FV3 protein kinase. Initial attempts to solubilize the protein kinase from FV3 virions indicated that most of the activity was tightly associated with the viral particles. For example, after disruption of the virion envelope with a nonionic detergent, a procedure
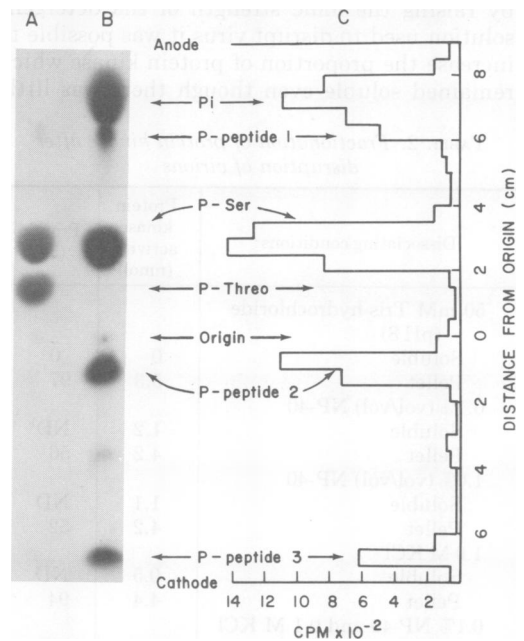

FIG. 6. Identification of phosphoserine from the phosphorylated product. A 24-µg amount of phosphorylated FV3, prepared as described in Materials and Methods, containing 50 pmol of ^{32}P (1,090 counts per min per pmol) were suspended in 0.5 ml of 6 N HCl and subjected to hydrolysis at 110 C for 60 min in an evacuated, sealed glass ampoule. After hydrolysis, 200 nmol of unlabeled phosphoserine and phosphothreonine were added as markers, and the aqueous HCl was removed in vacuo. The sample was suspended in H_2O, again dried in vacuo, and suspended in 0.05 ml of electrophoresis buffer consisting of 2.5% (wt/vol) formic acid and 7.8% (wt/vol) acetic acid, pH 1.9. A sample containing 9.8 pmol of ^{32}P was analyzed by electrophoresis for 110 min at 500 V on a thin-layer plate of cellulose. Marker phosphoserine and phosphothreonine were located by spraying with ninhydrin and following the preparation of an autoradiograph, the cellulose was fractionated into 0.5-cm sections, and the radioactivity was measured. A, Ninhydrin stain; B, autoradiograph; C, quantitation of radioactivity on thin-layer plate.

which solubilized about half of the virion protein, most of the protein kinase activity was found in a particulate fraction which was possibly the viral core (Table 2). Washing the native virus in a high-ionic-strength buffer such as 1.0 M KCl solubilized only a small fraction of the total protein kinase activity, the remainder of which was recovered with isolated viral particles. Clearly the fractionation behavior of the protein kinase under these conditions which would remove loosely adsorbed surface proteins indicated that the enzyme was an integral component of the virion.

Upon further analysis, it was observed that by raising the ionic strength of the detergent solution used to disrupt virus it was possible to increase the proportion of protein kinase which remained soluble even though there was little

TABLE 2. *Fractionation of protein kinase after disruption of virions*

Dissociating conditions[a]	Protein kinase activity (nmol)	Protein (μg)
50 mM Tris-hydrochloride (pH 8)		
Soluble	0	0
Pellet	5.3	97
0.1% (vol/vol) NP-40		
Soluble	1.2	ND[b]
Pellet	4.2	56
1.0% (vol/vol) NP-40		
Soluble	1.1	ND
Pellet	4.2	52
1.0 M KCl		
Soluble	0.5	ND
Pellet	4.4	94
0.1% NP-40 and 0.1 M KCl		
Soluble	2.3	ND
Pellet	2.7	51
0.1% NP-40 and 1.0 M KCl		
Soluble	3.7	ND
Pellet	1.5	46

[a] A 50-μliter amount of purified FV3(BHK), 100 μg of protein, was suspended in 1.0 ml of detergent and/or salt solution in 50 mM Tris-hydrochloride (pH 8) as indicated, and the samples were incubated at 30 C for 15 min. Each sample was then chilled on ice, layered on 0.5 ml of 40% (wt/vol) sucrose in SVB in a screw-capped polyallomer tube for a Spinco SW 50 rotor, and centrifuged at 38,000 rpm for 90 min at 4 C. The soluble material (total volume 1.5 ml) was removed, and the pellet was suspended in 0.5 ml of SVB by ultrasonic vibration (ultrasonic device, Heat Systems, Inc.). A sample of each fraction was tested for protein kinase activity as described in Materials and Methods by using 50 μg of histone fraction 3 as phosphate acceptor, and from this information the total activity of the soluble and pellet fractions was calculated. The γ-³²P-ATP contained 105 counts per min per pmol.

[b] ND, Not determined.

decrease in the total protein of the pellet fraction (Table 2). This suggested that electrostatic interactions played an essential role in the binding of protein kinase within the virion. Approximately 70% of the active protein kinase of virions was solubilized after treatment of virus with 0.1% NP-40 and 1 M KCl (Table 2), and the activity was partially purified by zonal sedimentation in a glycerol gradient (Fig. 7). About 90% of the solubilized activity sedimented with an $s_{20,w}$ of 4.2 relative to BSA, indicative of a molecular weight of about 65,000. Gradient fractions containing this activity (generally fraction no. 10–16) were pooled, and their specific activity was a minimum of five-fold greater than that of whole virus. These pooled fractions will be referred to as partially purified virion protein kinase. The activity of this enzyme was almost completely dependent on the addition of exogenous substrate protein (Fig. 7), indicating a substantial separation of protein kinase and many of the virion phosphate acceptors. As shown in Fig. 8C, the partially purified enzyme contained a single endogenous acceptor polypeptide, which corresponded to a minor phosphate acceptor of whole virus.

The substrate specificity of the partially purified frog virus protein kinase was compared with that of a cAMP-dependent protein kinase from beef heart tissue. The viral enzyme would phosphorylate histones and frog virus proteins and components of several other purified viruses as well (Table 3). Phosphate accepting proteins were detected in several enveloped viruses and in reovirus, a non-enveloped virus for which an endogenous protein kinase has not been detected. In contrast, the muscle enzyme would readily phosphorylate histones but not virion phosphate acceptors. Even with a 25-fold greater concentration of beef heart kinase than indicated in Table 3 and prolonged incubation at 30 C, no phosphorylation of frog virus polypeptides was observed. On the other hand, a small amount of phosphorylation of Rauscher murine leukemia virus (R-MLV) protein was detected when high levels of the beef heart enzyme were employed; however with saturating amounts of R-MLV as substrate in the assay this incorporation proceeded at a rate of less than 1% than the reaction with histone fraction 2b as substrate. Moreover, the yield of phosphorylation of R-MLV was only 9% of that obtained when the partially purified frog virus protein kinase catalyzed the reaction (data not shown).

A clear distinction in the substrate specificity of these protein kinases was demonstrated with protamine as phosphate acceptor (Table 3). Furthermore, the frog virus enzyme phosphoryl-
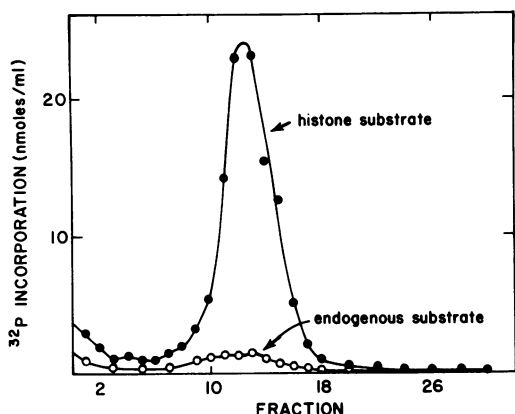
FIG. 7. *Solubilization and sedimentation of protein kinase. A 0.2-ml amount (300 µg of protein) of purified FV3 (BHK) was adjusted to 0.1% NP-40 and 1 M KCl. The virus suspension was incubated at 30 C for 15 min, chilled on ice, layered onto a 5.0-ml linear gradient of glycerol (5–20% [vol/vol] containing 1 M KCl, 10 mM Tris-hydrochloride [pH 8.0], 2 mM MgCl$_2$, and 0.1% NP-40), and centrifuged at 50,000 rpm for 21.5 h in an SW65 rotor at 4 C. Fifteen-drop fractions were collected from the bottom of the tube. Protein kinase activity of each fraction was determined as described in Materials and Methods both with and without 25 µg of histone fraction 3 added as phosphate acceptor protein. The γ-^{32}P-ATP contained 190 counts per min per pmol. Sedimentation is from right to left.*

ated histone fraction 3 at greater rate than it phosphorylated other calf thymus histone fractions. In contrast, the muscle enzyme and several other cAMP-dependent protein kinases, such as histone (23) or protamine (13) kinase, most readily phosphorylated histone fraction 2b.

The frog virus enzyme could be further differentiated from other protein kinases by its lack of dependence on added cyclic nucleotides. Neither cAMP nor cGMP significantly effected the rate of incorporation by this enzyme under conditions where the activity of the muscle enzyme was markedly stimulated by the addition of cAMP (Table 4). Concentrations of cyclic nucleotides 100-fold higher were only slightly inhibitory or without significant effect on the incorporation by the viral enzyme. Control experiments in which beef heart and FV3 kinases were assayed together showed that these was nothing present in the frog virus enzyme preparation which modified the stimulation of a cAMP-dependent enzyme by cAMP (Table 4). No stimulation of phosphorylation was observed by addition of cyclic nucleotides to reactions catalyzed by native or detergent-disrupted frog virus or where other proteins (e.g.,

heated FV3 or R-MLV) were tested as substrate for the virion enzyme.

Since the FV3 enzyme would phosphorylate other viruses, experiments were performed to determine which viral polypeptides were active as substrate. Previous experiments had indicated that several of the structural proteins of R-MLV could be phosphorylated by a R-MLV protein kinase (42). It appeared that they were also active as substrates when the phosphorylation was catalyzed by the frog virus enzyme (Fig. 8A-C). The frog virus kinase also phosphorylated the structural polypeptides of reovirus (Fig. 8D, E). When heated FV3 was substrate for the partially purified viral enzyme, the pattern of phosphorylated proteins on SDS-polyacrylamide gels was very similar to that obtained with native virions (as in Fig. 4), suggesting that solubilization of the enzyme or heating of virions does not change the overall specificity of phosphorylation. In control experiments, heated frog virus, R-MLV, or reovirus failed to incorporate significant radioactivity in the absence of added protein kinase, demonstrating that the incorporation observed above was specifically catalyzed by exogenous enzyme.

TABLE 3. *Substrates of virion and beef heart kinases*

Substrate[a]	Picomoles incorporated per 10 min	
	Frog virus[b]	Beef heart[b]
None	4	3
Frog virus (35 µg)	38	3
R-MLV (35 µg)	29	5
Reovirus (34 µg)	24	3
Herpes simplex (30 µg)	27	4
Histone fraction 1 (100 µg)	29	490
Histone fraction 2a (100 µg)	28	175
Histone fraction 2b (100 µg)	26	830
Histone fraction 3 (100 µg)	61	90
Protamine sulfate (100 µg)	5	340

[a] The reaction mixtures, as described in Materials and Methods, contained substrate as shown, 100 pmol of cAMP, and enzyme as described below. Before use, R-MLV, reovirus, and herpes simplex virus were heated at 60 C for 5 min, and frog virus at 75 C for 5 min as concentrated virus suspensions (0.5–3.4 mg of protein per ml) to inactivate the protein kinase associated with virions. The γ-^{32}P-ATP contained 230 counts per min per pmol.

[b] The amounts of protein kinase in each assay were as follows: beef heart, 1.0 µg; frog virus, 0.01 ml of partially purified virion protein kinase derived from 200 µg of FV3(BHK) as described in Fig. 7. The peak glycerol gradient fractions (no. 10–16) were pooled and were found to contain less than 20 µg of protein per ml.
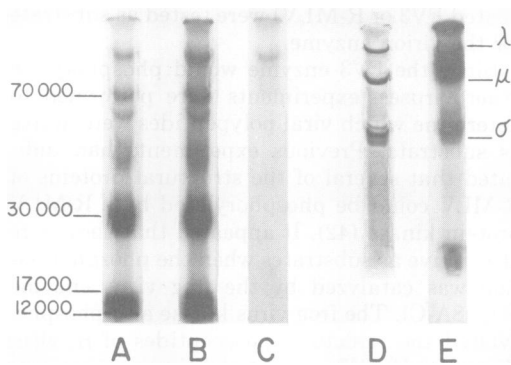
FIG. 8. *Phosphorylation of R-MLV and reovirus proteins by protein kinase. Heated R-MLV and reovirus proteins were phosphorylated by the partially purified frog virus protein kinase in 1.5-fold reaction mixtures of 0.3 ml as described in Table 3 except that cAMP was omitted and 110 μg of heated R-MLV or 85 μg of heated reovirus was added. As a control the incorporation of the FV3 enzyme without added acceptor protein was also included. The γ-³²P-ATP contained 625 counts per min per pmol. R-MLV proteins, 110 μg, were phosphorylated by the endogenous R-MLV protein kinase by using unheated virus in the same volume of reaction mixture as above without added protein kinase. The γ-³²P-ATP contained 1875 counts per min per pmol. After incubation at 30 C for 30 min, the reactions were terminated by the addition of 1 M EDTA (pH 7.0) to a final concentration of 20 mM, and the samples were chilled on ice. The samples were then dialyzed at 4 C overnight in 80 mM Tris-hydrochloride (pH 7.0), and 20% glycerol, and were analyzed by high-resolution SDS-polyacrylamide gel electrophoresis and autoradiography performed as described in Materials and Methods. A, autoradiogram of R-MLV proteins phosphorylated by the endogenous protein kinase; B, autoradiogram of R-MLV proteins phosphorylated by the frog virus protein kinase; C, autoradiogram of proteins phosphorylated by the frog virus protein kinase without added substrate; D, gel of reovirus stained for protein; E, autoradiogram of D showing the proteins of reovirus phosphorylated by the frog virus protein kinase.*

DISCUSSION

Evidence presented in this study indicates that purified virions of frog virus 3, an enveloped DNA virus which replicates and matures within the cellular cytoplasm, contain a relatively high activity of protein kinase in comparison with the activity demonstrated with several other viruses. The protein kinase appeared to be an integral component of the virion, although with proper dissociating conditions it was possible to solubilize most of the virion protein kinase activity. Partial purifica-

tion, achieved by velocity sedimentation on a glycerol gradient, suggested a molecular weight of 65,000 for the virion enzyme.

Studies of several properties of the virion protein kinase indicated that it was different from other well characterized protein kinases. Particularly noteworthy was the ability of the virion protein kinase to phosphorylate a wide variety of viral polypeptides, including proteins of frog virus, R-MLV, herpes simplex, and reovirus, while a cyclic nucleotide-dependent protein kinase was inactive with these viral substrates. Although additional experiments are necessary to determine whether the virion enzyme is different from host cell protein kinases, it seems possible that this enzyme, be it viral or host specified, represents a new class of protein kinase which is active in the phosphorylation of viral proteins.

Several recent studies, which have focused on the analysis of virions labeled in vivo with ³²Pi, have indicated that a limited number of the polypeptides of rhabdoviruses (38, S. Moyer, personal communication), and none of the proteins of oncornaviruses (F. Sokol, personal communication) were phosphorylated. In contrast, many of the proteins of these viruses were phosphorylated by an endogenous protein kinase on incubation of virions in vitro with ATP (42). In lieu of the finding that virions of frog virus, as well as many other enveloped viruses (including rhabdo and oncornaviruses), also contained a phosphoprotein phosphatase activity (H. Silberstein and J. T. August, Abstr.

TABLE 4. *Effect of cyclic nucleotides*

Enzyme[a]	Picomoles incorporated per minute		
	None[b]	cAMP[b]	cGMP[b]
Beef heart	2	18	3
FV3(BHK)	18	17	18
FV3(FHM)	8	7	7
Beef heart plus FV3(BHK)	10	16	11

[a] Reaction mixtures, as described in Materials and Methods, contained 50 μg of histone fraction 3, 100 pmol of cyclic nucleotide (as indicated), and protein kinase as follows: beef heart and FV3(BHK), as described in Table 3; FV3(FHM), 0.01 ml (less than 0.2 μg) of an enzyme purified as in Fig. 7 by using 200 μg of FV3(FHM) as starting material. For the mixing experiment 0.5 μg of beef heart kinase was combined with 0.005 ml of FV3(BHK) protein kinase. The γ-³²P-ATP contained 48 counts per min per pmol. Reactions were incubated for 2 min at 30 C.

[b] Cyclic nucleotide added.

Annu. Meet. Amer. Soc. Microbiol., p. 258, 1973), it appears that these viruses carry the enzymatic machinery to catalyze the turnover of phosphoproteins. Thus the possibility exists that the phosphorylation and dephosphorylation of certain viral polypeptides may be regulated during the course of virus replication.

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