

Protein Kinase from Avian Myeloblastosis Virus

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A protein kinase associated with purified virions of avian myeloblastosis BAI strain A was partially purified by ion-exchange chromatography and gel filtration. The transfer of phosphate catalyzed by this enzyme required a divalent metal ion and ATP as phosphate donor. GTP could not be substituted for ATP, and the reaction was unaffected by either cyclic AMP or beef-heart protein-kinase inhibitor. Of the virus and nonvirus proteins tested as phosphate acceptors, only acidic proteins were phosphorylated. In particular, purified preparations of reverse transcriptase from avian myeloblastosis virus did not accept phosphate. The enzyme is a basic protein ($pI = 9.3$), and, on the basis of molecular sieving through Sephadex G-200 and velocity sedimentation on glycerol gradient, the protein kinase has a molecular weight of 45,000.

Protein kinase occurs in a variety of enveloped animal viruses (4, 5, 7, 9-11, 13-21, 23-25, 27) and has been isolated in purified preparations from vaccinia (9, 10) and frog virus (FV3) (19), of which the enzyme is a virion-specific component (20). The presence of protein kinase has been noted, also, in avian myeloblastosis virus (AMV) (18), and a recent publication (11) reported studies on phosphorylation of preparations of reverse transcriptase (RDDP) from Rous sarcoma virus by protein kinase extracted from Rous sarcoma virus-transformed chicken embryo fibroblasts. These results, together with possible applicability of protein kinase to developmental work in this laboratory, prompted continued exploration of procedures for isolation of the enzyme from AMV. Preliminary investigations showed that the protein kinase was present in fractions derived in the process of isolation of RDDP from AMV. This report describes studies on purification and characterization of protein kinase from AMV.

MATERIALS AND METHODS

Chemicals. Nucleotides were obtained from either Miles Laboratories, Inc., Elkhart, Ind., or P-L Biochemicals, Milwaukee, Wis. New England Nuclear Corp., Boston, Mass. was the source of ATP and GTP labeled with ³²P in the gamma position (10 to 30 Ci/mmol). Whatman DEAE-cellulose (DE-52), phosphocellulose (P1), and GF/C paper for filter disks were from Reeve Angel, Clifton, N.J.; Sephadex G-200, G-100, and blue dextran were obtained from Sigma Chemical Co., St. Louis, Mo. Reagents for disc gel electrophoresis were from E-C Apparatus Corp., St. Petersburg, Fla., carrier ampholytes were purchased from Bio-Rad Laboratories, Richmond, Calif.,

and molecular-weight markers were from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. A Bio-Rad Gel Pro-pHiler was used to measure the pH gradient in acrylamide gels.

Virus. AMV, BAI strain A, was obtained from plasma of chicks diseased with the agent in this laboratory. Blood drawn into heparin by heart puncture was spun twice at $3,000 \times g$ for 10 min to remove cellular material, and virus was then sedimented at $59,000 \times g$ for 40 min. The pellets were suspended at concentrations of 20 to 50 mg of virus per ml in TNE (10 mM Tris-hydrochloride [pH 8.0]-0.15 M NaCl-1 mM EDTA) and homogenized with 8 to 10 strokes of a loose-fitting pestle in a tissue grinder. The virus-containing suspension was centrifuged at $3,000 \times g$ for 10 min, and the resulting pellet was rehomogenized to wash trapped virus from the fibrin pellet and spun again at $3,000 \times g$ for 10 min. The supernatant fractions were pooled and centrifuged again at $59,000 \times g$ for 40 min to resediment the virus. This cycle of low and high speed centrifugation was carried out three times, and virus of the final pellet was suspended in TNE. Virus concentrations are cited as wet weight estimated by adenosine 5'-triphosphatase activity (1). Gradient centrifugation was not used in any aspect of the process.

Polyacrylamide gel electrophoresis. Isoelectric focusing was carried out in 9% polyacrylamide gels (6 by 70 mm) containing 1.7% carrier ampholytes. To achieve reproducible, linear gradients in the upper pH range, each 25 ml of gel solution contained 0.9 ml of 3-10 carrier ampholyte (40%), 0.35 ml of 8-10 carrier ampholyte (20%), and 0.2 mg of riboflavin 5'-phosphate. The gels were photopolymerized overnight before use. The upper reservoir contained 0.06 N H₂SO₄ and the lower, 0.06 N NaOH. Each gel was exposed to 1.5 mA until a maximum of 200 V was reached. The gradient was formed within 1 h, and migration of the polypeptides reached equilibrium within 5 h. After determination of pH at 0.5-cm intervals, the gel

was frozen in dry ice and sliced into 1-mm sections. Polypeptides were extracted from the sections overnight at 4°C with 100 mM potassium phosphate (pH 7.3) and 10% glycerol. Protein kinase activity in gel sections was determined as indicated. Dissociating gel electrophoresis was done in Tris-acetate-buffered sodium dodecyl sulfate (pH 6.4) as recommended by Bio-Rad Laboratories.

Purification of protein kinase. Although a small amount of protein kinase co-purified with RDDP, the bulk of the kinase activity was present in the DEAE flow-through fraction derived during the large-scale process of RDDP purification (G. E. Houts, M. Miyagi, D. Beard, C. Ellis, and J. W. Beard, manuscript in preparation; 8). Protein kinase was purified further by adsorption to phosphocellulose (P1, 2.6 by 20 cm) and stepwise elution with 0.05, 0.2, and 0.4 M potassium phosphate (pH 7.3; Fig. 1). Enzyme activity in the 0.4 M eluate was pooled, precipitated with 45% $(\text{NH}_4)_2\text{SO}_4$, and dialyzed against 0.1 M Tris-hydrochloride (pH 9.0) and 0.5 M KCl. The material then was filtered through Sephadex G-100 (2.6 by 90 cm) equilibrated with dialysis buffer (Fig. 2). The activity peak was pooled, dialyzed against 50 mM Tris-hydrochloride (pH 8.0) containing 50% glycerol, and stored at -20°C. Protein kinase activity was measured by transfer of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to acid-insoluble product at 35°C in a mixture containing 50 mM potassium phosphate (pH 7.3), 20 mM MgCl_2 , 0.5 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, and phosphate acceptor protein as indicated. The acid-insoluble product of this reaction was collected on GF/C filter disks and processed for detection of radioactivity by liquid scintillation spectrometry as previously described (2). One unit of protein kinase was that amount of activity converting 1 nmol of ^{32}P to acid-insoluble product in 30 min. The reaction was not linear within the time interval of 30 min, but 30 min was chosen as the period usually yielding the more reproducible results.

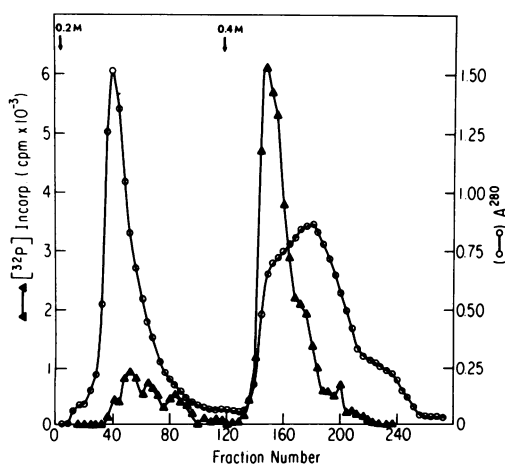


FIG. 1. Stepwise elution of protein kinase from phosphocellulose. Effluent 7.0-ml fractions were monitored for protein by UV absorbance at 280 nm and for protein kinase activity (see text for details of assay). Fractions 138 to 168 were pooled for additional purification of protein kinase (see Fig. 2).

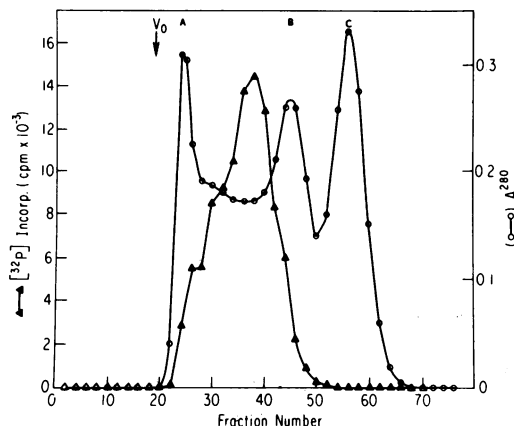


FIG. 2. Fractionation of protein kinase (fractions 138 to 168, Fig. 1) on Sephadex G-100. Effluent 5.5-ml fractions were monitored for protein by UV absorbance at 280 nm and for protein kinase activity. Fractions 31 to 41 were pooled, dialyzed against 50 mM Tris-hydrochloride containing 50% glycerol, and used for further characterization of protein kinase.

RESULTS

Purity of protein kinase. Only 5 to 10% of the protein in the crude AMV lysate adsorbs to DEAE (Houts et al., in preparation), while the bulk of the remaining protein fraction containing the kinase activity adsorbs to P1. Unambiguous assays for protein kinase activity were not obtainable until the enzyme was eluted from P1 (Fig. 1). The enzyme was recovered from P1 by stepwise increase of buffer to 0.4 M potassium phosphate (pH 7.3) rather than by gradient elution. Stepwise elution afforded complete separation of contaminating RDDP, which eluted in 0.2 M buffer, while gradient elution of the column resulted in a diffuse profile of protein kinase, poor recovery of the enzyme, and difficulty in the elimination of RDDP activity. The elution position of protein kinase after filtration through G-100 was between two major peaks (Fig. 2), and the kinase was thus contaminated by proteins from both of these adjacent positions. Purification to homogeneity was significantly impeded by the facts that protein kinase appears to be a minor component of the virus and that the enzyme co-purified with the bulk of AMV protein.

Stability. The enzyme was routinely stored at either -20°C or 4°C. Under these conditions, no loss of activity was observed after 6 months. Furthermore, there was no loss of activity after 48 h at room temperature or after 18 h at 37°C, but, after 2.5 min at 55°C, the activity was diminished by 50% (data not shown). No change in activity was observed when the enzyme was stored at various pH values ranging from 4.0 to

11.0 for 48 h. Although enzyme activity was inhibited by molar concentrations of salt (KCl, guanidine · HCl), inhibition was reversible, and no loss of activity was detected (data not shown).

Isoelectric point. The pI of AMV protein kinase was 9.3, as determined by migration of a 20- μ l sample into the focusing gel as described above. The focused gels were sliced, polypeptides were extracted, and protein kinase activity of each fraction was determined. The peak of activity focused between pH 9.2 and 9.4 (Fig. 3); thus, AMV protein kinase is a basic protein.

Molecular weight. Previous efforts to determine molecular weight of the protein kinase by filtration through Sephadex G-200 in neutral buffers resulted in poorly defined, heterogeneous peaks of protein kinase activity. Filtration of protein kinase through a column of G-200 (1.5 by 90 cm) equilibrated with 0.1 M Tris-hydrochloride (pH 9.0) containing 0.5 M KCl resulted in a homogeneous elution profile of activity (Fig. 4). By comparison with the elution positions of other proteins, the molecular weight of this kinase was estimated to be 45,000. Velocity sedimentation of the enzyme on glycerol gradient indicated a molecular weight of 42,000 to 47,000 relative to bovine serum albumin (Fig. 5). This agreed well with the value determined by filtration through Sephadex G-200 as just noted.

Characterization of the kinase reaction. The reaction at the pH optimum, 6.5 to 7.5 (Fig. 6), required divalent metal, ATP, and phosphate-acceptor protein (Table 1), but was unaf-

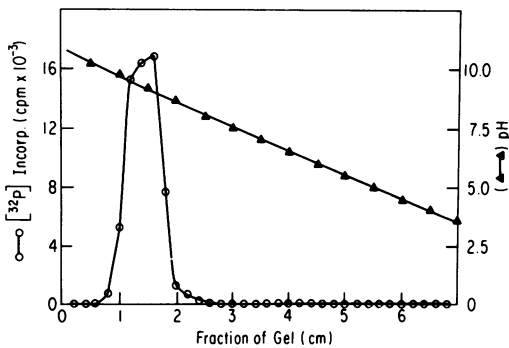


FIG. 3. Isoelectric focusing of protein kinase (fractions 31 to 41, Fig. 2). The material was focused in 6% polyacrylamide gels containing 1.7% carrier ampholytes (1.4% of 3-10 and 0.3% of 8-10 carrier). After determination of the pH gradient with miniature electrodes (Bio-Rad Pro-pHler), the gel was frozen and sliced into 1-mm sections. Polypeptides were extracted from the sections overnight at 4°C with 0.1 M potassium phosphate (pH 7.3) containing 10% glycerol. A 10- μ l sample of sliced-gel extract was added to 50 μ l of the protein kinase reaction mixture, incubated for 15 min at 37°C, and assayed for acid-insoluble 32 P.

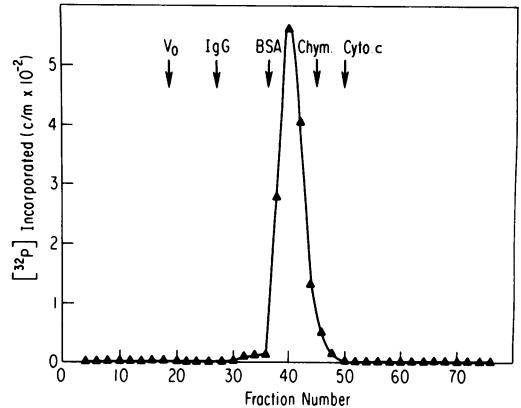


FIG. 4. Molecular sizing of AMV protein kinase on Sephadex G-200. A 1.0-ml sample of enzyme containing 2 mg of blue dextran was eluted from a column (1.5 by 90 cm) of G-200 with 0.1 M Tris-hydrochloride (pH 9.0) containing 0.5 M KCl. A 5- μ l sample of alternate fractions (volume, 2.9 ml) was added to 50 μ l of the protein kinase reaction mixture, incubated for 15 min at 37°C, and assayed for acid-insoluble 32 P. The elution positions of proteins of known molecular weights (immunoglobulin G [IgG], 150,000; bovine serum albumin [BSA], 68,000; chymotrypsinogen A [Chym], 25,000; cytochrome c [Cyto-c], 12,200) were determined in separate experiments.

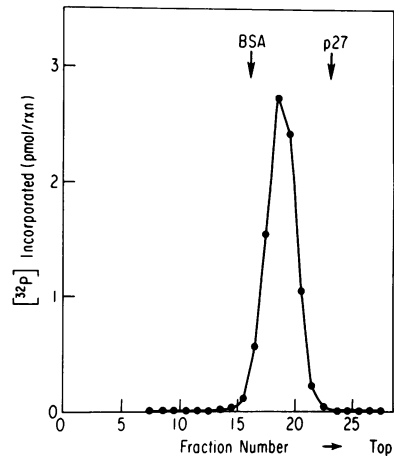


FIG. 5. Velocity sedimentation of protein kinase. A 0.5-ml sample of protein kinase layered on top of a 10 to 30% glycerol gradient containing 50 mM Tris-hydrochloride (pH 7.5), 0.2 M KCl, 3 mM dithiothreitol, and 0.2 mM EDTA was centrifuged for 45 h at 40,000 rpm in an SW41 rotor at 2°C. The gradients were fractionated by collecting 25-drop samples from the bottom of the tube. A 10- μ l sample of each fraction was added to 50 μ l of protein kinase reaction mixture, and, after incubation for 5 min at 37°C, the preparation was assayed for acid-insoluble 32 P.

ected by the presence of either cyclic AMP or beef-heart protein-kinase inhibitor, which is known to inhibit cyclic nucleotide-dependent

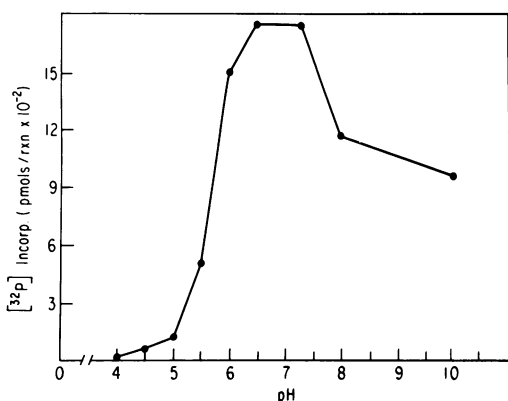


FIG. 6. Influence of pH on protein kinase activity. Protein kinase reaction mixtures were buffered at the indicated pH values and incubated at 37°C for 60 min. At intervals, 20- μ l samples from the respective mixtures were assayed for acid-insoluble ³²P. The values presented were obtained after 30 min of incubation at 37°C.

TABLE 1. Requirements for AMV protein kinase reaction

Reaction components	pmol of ³² P incorporated per 30 min
Complete ^a	1,115
- Acceptor protein	6
- MgCl ₂	54
- Enzyme	9
+ Cyclic AMP (0.04 to 1.6 mM)	1,076
+ Protein kinase inhibitor (50 mg/ml)	1,091
+ KCl (0.1 M)	789

^a Complete reaction mixture contained: 50 mM potassium phosphate (pH 7.3); 0.5 mM [γ -³²P]ATP; 2.5 mg of phosvitin per ml, 10 mM MgCl₂, and about 1 U of enzyme.

protein kinase. KCl was progressively inhibitory at concentrations of 20 mM to 1.0 M (10 to 80%), but exerted no effects at concentrations of 10 mM or less. The reaction occurred in the presence of several divalent metals (Table 2) other than Mg²⁺ (as shown in tests at the optimum concentrations of the respective metals) and at much lower concentrations, but this requirement was satisfied by Mg²⁺ two- to threefold better than the other metals. Of ATP and GTP tested as phosphate donors with both virus and nonvirus proteins as phosphate acceptors, only ATP functioned as phosphate donor (Table 3). Moreover, more than 78% of the radioactivity in the phosphorylated product (virus or nonvirus) became trichloroacetic acid-soluble after treatment with *Escherichia coli* alkaline phosphatase, a well-characterized enzyme with a specificity for phosphomonoester bonds.

Various proteins were tested as potential

phosphate acceptors (Table 3), and the effect of protein concentration on kinetics of the reactions was determined for three of these (Fig. 7). Of the nonvirus proteins tested, only the two acidic proteins, α -casein and phosvitin, accepted phosphate. In view of recent reports (11, 25, 26) describing phosphorylation and associated increase in the activity of RDDP purified from AMV or Rous sarcoma virus, we assayed a series of 12 different preparations each of 90% or more pure RDDP (Houts et al., in preparation) for the ability to accept phosphate. With all except one preparation, RDDP was present in the reaction mixtures in concentrations of 27 to 134 μ g/ml; however, one of the preparations (G-677) was further concentrated and used in the reaction at 810 μ g/ml. None of the preparations of RDDP, including G-677 (Fig. 7), accepted detectable amounts of phosphate (Table 3). Fur-

TABLE 2. Effect of various metal ions on AMV protein kinase activity

Metal (optimum concn [mM])	pmol of ³² P incorporated per 30 min
Mg ²⁺ (10)	909
Mn ²⁺ (0.005)	394
Co ²⁺ (0.05)	426
Zn ²⁺ (0.0125)	415

TABLE 3. Comparison of proteins as phosphate acceptors with ATP or GTP as phosphate donor

Protein ^a	³² P incorporated per 30 min (pmol)	
	ATP	GTP
α -Casein	726	7
Phosvitin	1,008	15
Arginine-rich histone	17	8
Lysine-rich histone	3	3
Protamine sulfate	7	5
Purified RDDP ^b (27 to 134 μ g/ml)	3	
Purified RDDP ^c (810 μ g/ml)	0	
Pools A, B, and C ^d (296, 464, 360 μ g/ml)	0	
0.2 M pool ^e (4 mg/ml)	426	

^a All non-virus proteins were 1 mg/ml in the reaction mixture. Virus proteins were used at the concentration indicated in parentheses.

^b This value is representative of reactions with 12 different preparations of purified RDDP coded as: G-1476, G-1976, G-177, G-277, G-477, G-577, G-677, G-777, G-1277, G-1377, G-977, and G-1577.

^c G-677 (footnote b) was further concentrated for use in this reaction.

^d These three fractions were derived during G-100 fractionation of protein kinase (see Fig. 2).

^e This fraction was derived during phosphocellulose chromatography of protein kinase (see Fig. 1), and contained RDDP in a concentration of 0.300 mg/ml which accepted no phosphate (see Fig. 8).

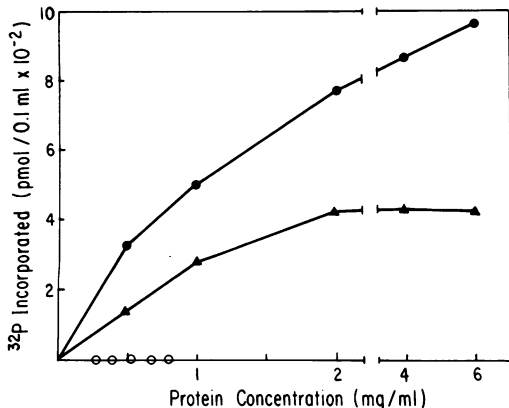


FIG. 7. Effect of phosphate-acceptor protein concentration on phosphorylation by protein kinase from AMV. Various concentrations of phosphovitin (\bullet), 0.2 M eluate (\blacktriangle), or RDDP purified from AMV (\circ) were incubated with approximately 1 U of protein kinase for 30 min under reaction conditions as described in the text. A 25- μ l sample from each reaction specimen was plated on a GF/C disk and processed for detection of acid-insoluble 32 P.

thermore, all of the preparations were tested for RDDP activity before and after attempted phosphorylation, and no change in RDDP activity was observed in any (data not shown).

The evaluation of other virus proteins as phosphate acceptors included fractions derived during purification of protein kinase (Table 3). Proteins that co-eluted from phosphocellulose with protein kinase (0.4 M buffer, Fig. 1), separated on Sephadex G-100 (Fig. 2) and designated as pools A, B, and C, failed to accept phosphate in the protein kinase reaction. However, protein eluted from the phosphocellulose column at 0.2 M buffer (Fig. 1) could be phosphorylated (Table 3). It is important to note that a small amount of RDDP activity always passed through DE-52, adsorbed to phosphocellulose, and was subsequently eluted by the 0.2 M buffer. To determine the molecular weight and pI of these phosphorylated proteins, the following experiments were made. A sample of the 0.2 M eluate was phosphorylated for 1 h at 37°C, precipitated with 70% $(\text{NH}_4)_2\text{SO}_4$, and then dialyzed overnight against 1,000 volumes of 50 mM Tris-hydrochloride (pH 8.0) containing 0.1 M KCl. Final dialysis was against the same buffer containing 50% glycerol without KCl. The dialyzed, 32 P-labeled proteins were then analyzed by both sodium dodecyl sulfate-gel electrophoresis and isoelectric focusing. In each system, gels were frozen and sliced into 1-mm sections which were then assayed for the presence of 32 P. More than 80% of the 32 P in the sodium dodecyl sulfate gel was associated with proteins ranging in molecu-

lar weight from approximately 10,000 to 25,000 (Fig. 8). The remaining radioactivity was associated with proteins of up to an approximate molecular weight of 50,000. There was no detectable 32 P associated with either α - or β -polypeptides of reverse transcriptase (Fig. 8; by densitometric tracing of a separate gel stained with Coomassie blue, we estimated that RDDP constituted 7.5% of the total protein in the 0.2 M eluate indicated in Fig. 1 and, therefore, was present in the above reaction at a concentration of approximately 0.300 mg per ml, see Table 3). All of these phosphorylated proteins focused between pH 5.1 and 6.8 (Fig. 9), and most of the radioactivity was associated with two sharply defined peaks, one positioned at pH 5.6 and the other at pH 6.5. Thus, virus phosphate-acceptor proteins are acidic, a property consistent with that of the nonvirus proteins α -casein and phosphovitin.

DISCUSSION

Protein kinases are currently classified in two distinct categories: those that are cyclic nucleotide dependent, and those that are cyclic nucleotide independent. None of the protein kinase activities associated with animal viruses is stimulated by addition of cyclic nucleotides to in vitro assays (for review, see 19). AMV protein kinase activity also was not increased by various concentrations of cyclic AMP and was unaffected by the presence of cyclic nucleotide-dependent protein kinase inhibitor protein. It is

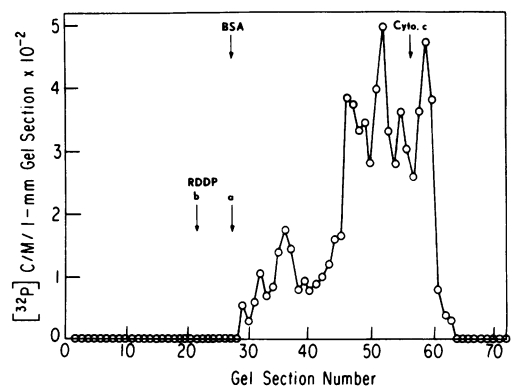


FIG. 8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of in vitro-phosphorylated AMV proteins. A sample of AMV proteins (0.2 M fraction, Fig. 1) was phosphorylated for 1 h at 37°C with $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ by AMV protein kinase, precipitated from the reaction mixture with 70% $(\text{NH}_4)_2\text{SO}_4$, dialyzed against two changes of 50 mM Tris-hydrochloride (pH 8.0), and fractionated in 8% sodium dodecyl sulfate-polyacrylamide gels buffered at pH 6.4 with Tris-acetate. The gels were frozen, sliced into 1-mm sections, and assayed for 32 P.

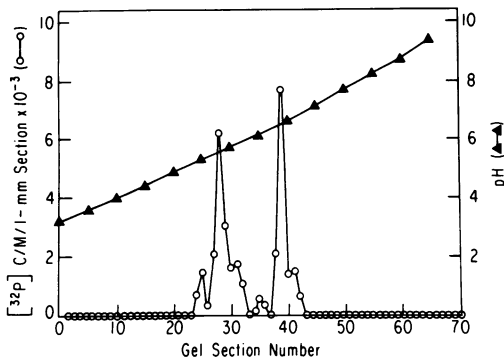


FIG. 9. Isoelectric focusing of AMV proteins phosphorylated *in vitro*. A sample of AMV proteins (0.2 M fraction, Fig. 1) was phosphorylated for 1 h at 37°C with ^{32}P ATP by AMV protein kinase, precipitated from the reaction mixture with 70% $(\text{NH}_4)_2\text{SO}_4$, dialyzed against two changes of 50 mM Tris-hydrochloride (pH 8.0), and focused in 6% polyacrylamide gels containing 2% 3-10 carrier ampholytes. After determination of the pH gradient, the gels were frozen and sliced into 1-mm sections. Measurement of ^{32}P in each section was done by scintillation spectrometry.

significant to note that the AMV enzyme could not use GTP as a phosphate donor. The utilization of GTP in the phosphotransferase reaction has been observed with several cyclic nucleotide-independent activities; however, cyclic nucleotide-dependent kinases are active only with ATP as the phosphate donor. Thus, the AMV enzyme shares properties with both groups, in that it is cyclic nucleotide independent but will not utilize GTP as a phosphate donor.

The molecular weight of the AMV enzyme (45,000) is in the same general size range as vaccinia virus protein kinase (62,000 [9]) and frog virus protein kinase (50,000 to 55,000 [19]). In contrast, a protein kinase of mouse mammary tumor virus has a molecular weight of 105,000 (5). AMV protein kinase displays a rather strict specificity relative to proteins that can serve as phosphate acceptors. Only acidic phosphoproteins, both virus and nonvirus, function in the reaction, and this specificity may be related to the strong basicity of the enzyme ($\text{pI} = 9.3$). On the other hand, frog virus protein kinase appears to prefer acidic phosphoproteins, but will utilize basic proteins to some extent.

The biological role of protein phosphorylation by cyclic nucleotide-independent protein kinase in systems other than animal viruses appears to be regulatory in a variety of biosynthetic pathways (22). However, the function of such phosphorylation in animal virus systems is currently unknown. Uncertainty of the results of studies, thus far, is of particular significance relative to

the performance of reverse transcriptase. Access to the enzyme in large quantities has afforded extension of its use in a broadening variety of investigations (R. Devos, J. V. Emmelo, P. Celen, E. Gillis, and W. Fiers, *Eur. J. Biochem.*, in press; E. Y. Friedman and M. Rosbash, submitted for publication; 3, 12, 14). In consequence, limitations on the ultimate usefulness of the enzyme are dependent in large part on the provision and maintenance of the full integrity of its capabilities. In this respect, it has been reported that a protein kinase from Rous sarcoma virus-transformed chicken embryo fibroblasts in tests *in vitro* effected an increase in Rous sarcoma virus transcriptase activity (11). More recently, it was shown that the DNA polymerase of the B77 strain of Rous sarcoma virus was phosphorylated, and that the phosphorus was associated with the β component of the transcriptase (6). In this instance, phosphorylation was effected by *in vivo* labeling with ^{32}P in tissue culture.

Earlier publications (25, 26) also reported the enhancement of purified RDDP from AMV by AMV protein kinase. Moreover, there were questions of the size of DNA copies and of the fidelity of transcription by the purified enzyme from AMV. That some of these results and assumptions are of questionable tenability has been indicated by the results of the present work and those of other investigators (Devos et al., in press; Friedman et al., submitted for publication; 3, 12, 14). As the findings showed, tests *in vitro* on 12 different preparations of purified RDDP from AMV with AMV protein kinase gave no evidence of the acceptance of detectable phosphate nor of any change or increase in activity after exposure to the protein kinase. Furthermore, consistent production of lengthy copies of high fidelity (Devos et al., in press; Friedman et al., submitted for publication; 3, 12, 14) gives no indication of incomplete or faulty activity of the purified RDDP of AMV. Although modification of RDDP activity by phosphorylation is an interesting possibility, current evidence obtained in studies *in vitro* is insufficient to warrant assumption of this role for protein kinase.

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