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Protein kinase and its regulatory effect on reverse transcriptase activity of Rous sarcoma virus

(phosphorylation/fibroblasts/phosphatase/RNA-dependent DNA polymerase)

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ABSTRACT **We have studied the effect of protein phosphokinase (EC 2.7.1.37; ATP:protein phosphotransferase) and phosphoprotein phosphatase (EC 3.1.3.16; phosphoprotein phosphohydrolase) on reverse transcriptase (RNA-dependent DNA nucleotidyltransferase) activity of Rous sarcoma virus. Protein kinase from Rous sarcoma virus-transformed chick embryo fibroblasts was purified by DEAE-cellulose chromatography, Sephadex gel filtration, and isoelectric focusing. Purified reverse transcriptase from Rous sarcoma virus was preincubated with protein kinase and ATP under conditions allowing incorporation of phosphate into substrate protein. After the preincubation, reverse transcriptase activity was assayed in the presence of poly(rA)·oligo(dT) as template. A 2- to 5-fold increase of reverse transcriptase activity was found after the preincubation of reverse transcriptase with protein kinase and ATP. Incubation of reverse transcriptase with heat-treated, inactive protein kinase and ATP had no effect on transcriptase activity. When the transcriptase preparation was incubated with protein kinase and [γ-^{32}P]ATP and subsequently purified by chromatography on phosphocellulose and Sephadex gel filtration, significant amounts of ^{32}P-labeled proteins were found in the fractions exhibiting reverse transcriptase activity, suggesting ^{32}P incorporation into transcriptase or transcriptase-associated proteins. A 20–60% decrease of reverse transcriptase activity was observed after incubation of reverse transcriptase with phosphatase. The results suggest that phosphorylative modification of reverse transcriptase may be critical in the regulation of reverse transcriptase-catalyzed DNA synthesis.**

It is well recognized that regulation of enzyme activity may be achieved by several distinct mechanisms. In recent years evidence has accumulated implicating an enzymatic interconversion of active and inactive forms of enzymes as a major regulatory control mechanism. The activity of an increasing number of enzymes may be regulated and modulated through phosphorylation or dephosphorylation involving the coordinated action of protein phosphokinases and phosphoprotein phosphatases on their enzyme substrates (1–7). The opportunities that such systems provide for coordinated control of enzymic activity are amply illustrated by the now well-established phosphorylative and functional modifications of glycogen synthetase and glycogen phosphorylase (2, 3).

There is evidence that infection and transformation of susceptible cells by oncogenic RNA viruses, such as Rous sarcoma virus (RSV), is critically dependent upon the activity of the reverse transcriptase (RNA-dependent DNA nucleotidyltransferase) (8, 9). In view of this finding, it is of importance to determine whether or not the enzymatic activity of the reverse transcriptase may be altered and modified and whether the modification occurs as a consequence of protein phosphokinase (EC 2.7.1.37; ATP:protein phosphotransferase) and phosphoprotein phosphatase (EC 3.1.3.16; phosphoprotein phosphohydrolase) action. In the present communication we wish to report that protein phosphokinase isolated from RSV-transformed chick embryo fibroblasts can enhance reverse transcriptase activity of Rous sarcoma virus and that the increase in activity can be reversed through the action of phosphatase.

Abbreviation: RSV, Rous sarcoma virus.

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MATERIALS AND METHODS

Chemicals. The biochemical reagents were purchased from Sigma Chemical Co., St. Louis, Mo. [γ-^{32}P]Adenosine 5′-triphosphate, ammonium salt (30 Ci/mmol), was obtained from ICN, Nuclear and Isotope Division, Irvine, Calif. and [^{3}H]thymidine 5′-triphosphate, ammonium salt (54 Ci/mmol), from New England Nuclear, Boston, Mass. ^{32}P-Labeled protamine sulfate was prepared with [γ-^{32}P]ATP and beef heart cyclic AMP-dependent protein kinase by the method of Kato and Bishop (10).

Cells and Viruses. Schmidt-Ruppin RSV, subgroup D, (SR-RSV-D) was grown in type c/o chick embryo fibroblasts and purified as described (11). Two weeks after infection by SR-RSV-D, the transformed cells were removed with scraping, washed with a solution of 0.14 M NaCl/0.1 M sodium phosphate, pH 7.2, and pelleted by centrifugation at 1000 × *g*. The recovered cells were used for the isolation of protein kinase.

Enzyme Preparations. Protein kinase from the cytosol (105,000 × *g* supernatant fraction) of RSV-transformed cells was isolated and purified as described (6). After DEAE-cellulose chromatography, the pooled fractions containing protein kinase activity were additionally purified by Sephadex G-200 gel filtration (elution buffer: 0.05 M Tris buffer, pH 7.2, containing 2 mM $MgCl_2$, and 1 mM 2-mercaptoethanol) followed by preparative isoelectric focusing (12). The protein kinase preparation used in these studies had a specific activity of 116 pmol of ^{32}P incorporated/mg of protein per min using protamine sulfate as substrate. Addition of cyclic AMP (1 μM) to this kinase preparation resulted in a 1.54-fold stimulation of the kinase activity.

Reverse transcriptase from RS-RSV-D was purified by the method of Grandgenett *et al.* (13). The major peak of reverse transcriptase activity eluting at 0.22 M phosphate buffer from phosphocellulose chromatography was used in this study. Protein concentration was determined by the method of Lowry *et al.* (14), using crystalline serum albumin as standard.

Reverse Transcriptase Assay. The reaction mixture contained, in a total volume of 0.125 ml, 0.65 μg of poly(rA)·oligo(dT)$_{12-18}$ (Collaborative Research, Waltham, Mass.), 0.125–0.5 μg of reverse transcriptase, 50 mM Tris buffer (pH 8.3), 12 mM $MgCl_2$, 40 mM KCl, 10 mM dithiothreitol,

and 40 μM [^{3}H]dTTP (2.5 μCi). When alkaline phosphatase was present in the reverse transcriptase assay, potassium phosphate buffer (50 mM, pH 8.0) and $MnCl_2$ (0.4 mM) were used in the place of Tris buffer and $MgCl_2$. Incubation was carried out at 35° for the times indicated in the *text*. The reaction was stopped by the addition of 2 ml of ice-cold 10% trichloroacetic acid containing 50 mM sodium pyrophosphate. Fifty microliters of bovine serum albumin (1 mg/ml) were added as carrier protein to the samples prior to the addition of trichloroacetic acid. The samples were filtered on Millipore filters and washed five times each with 2 ml of ice-cold 10% trichloroacetic acid. The filters were air-dried, and the retained radioactivity was determined in Beckman liquid scintillation spectrometer using toluene-based scintillant.

Protein Kinase Assay. Phosphorylation of substrate protein was carried out as described (6) in a total incubation volume of 0.2 ml containing various amounts of protein kinase and substrate proteins as indicated in the *text*, 50 mM sodium glycerol phosphate buffer (pH 7.0), 5 mM NaF, 10 mM $MgCl_2$, 5 mM dithiothreitol, 2 mM theophylline, and 20 μM (0.5 μCi) of [γ-^{32}P]ATP, without or with 1 μM cyclic AMP. Incubation was carried out for 15 min at 35°. The reaction was terminated by the addition 2 ml of 10% trichloroacetic acid. The samples were filtered on Millipore filters and the radioactivity was determined as described above.

Phosphoprotein Phosphatase Assay. Phosphoprotein phosphatase activity was measured with alkaline phosphatase (*Escherichia coli*, electrophoretically purified, Worthington Biochemical Co.) using 10–50 μg of [^{32}P]protamine sulfate (400 cpm of ^{32}P/μg) as substrate in a solution of 50 mM Tris buffer (pH 8.0), containing 5 mM $MgCl_2$ (10). Incubation was carried out at 35° for 10 min. The reaction was stopped by the addition of 2 ml of 10% trichloroacetic acid, and the samples were filtered on Millipore filters. The loss of ^{32}P label from [^{32}P]protamine sulfate after incubation was used as index of phosphatase activity. When reverse transcriptase from phosphocellulose fractions was used as a substrate for alkaline phosphatase dephosphorylation, the transcriptase was dialyzed extensively against 10 mM Tris buffer (pH 8.0); 2 mM 2-mercaptoethanol; 0.1 mM EDTA to remove orthophosphate before the reaction.

Heat inactivation of protein kinase activity was carried out by incubation of the preparation for 15 min at 75°. Alkaline phosphatase was heated at 95° for 15 min. After the heat treatment, the enzymatic activities were totally destroyed.

RESULTS

Partial Characterization of Protein Kinase. Protein kinase isolated from the cytosol of RSV-transformed chick embryo fibroblasts and partially purified by DEAE-cellulose chromatography, Sephadex G-200 gel filtration, and preparative isoelectric focusing exhibited an average isoelectric point of pH 5.7 (Fig. 1). The preparation obtained after isoelectric focusing appears to consist of multiple forms of kinase. One form of protein kinase, which is cyclic AMP-dependent, was eluted in fractions 24–28 and a form of protein kinase that was insensitive to cyclic AMP stimulation (1 μM cyclic AMP) was eluted in fractions 30–34. For the purpose of this study fractions 24 to 34 were pooled and used as the source of protein kinase.

Effect of Protein Kinase on Reverse Transcriptase Activity. Previously reported evidence that the activity of a number of enzymes can be modified through the action of protein kinase (2–7), considered with the fact that both protein kinase and reverse transcriptase activity are present in the virion of oncogenic RNA viruses (13, 15), prompted us to investigate whether or not reverse transcriptase activity may be subject to phosphorylative and functional modification through the action of protein kinase. To test this possibility, the rate of [^{3}H]dTMP incorporation into RNA·DNA hybrid catalyzed by reverse transcriptase was measured as a function of time in the presence and absence of protein kinase and in the presence of heat-treated protein kinase. The results of such studies are shown in Figs. 2 and 3. Incubation of reverse transcriptase in the presence of protein kinase led to a significant increase of the activity of reverse transcriptase. The incorporation of [^{3}H]dTMP into RNA·DNA hybrid was stimulated about 250% after 30 min of incubation in the presence of protein kinase. The addition of heat-treated protein kinase, which was inactive in phosphorylating histone F1 (not shown here), had no effect on the activity of reverse transcriptase. The addition of 1 μM cyclic AMP in the presence of protein kinase did not further enhance reverse transcriptase activity observed in the presence of protein kinase. Furthermore, addition of cyclic AMP alone to the reverse transcriptase assay had no effect on reverse transcriptase activity. The degree of stimulation of reverse transcriptase activity achieved in the presence of protein kinase varied from one preparation of reverse transcriptase to another and varied between 1.5- and 4-fold. Varying degrees of reverse transcriptase activation were also observed when the reverse transcriptase preparations were obtained at different stages of purification.

Fig. 3 shows the increase of reverse transcriptase activity as a function of increasing amounts of protein kinase present in the incubation samples. The stimulation of reverse transcriptase activity was essentially proportional to the amount of protein kinase added, and a 5-fold stimulation of reverse

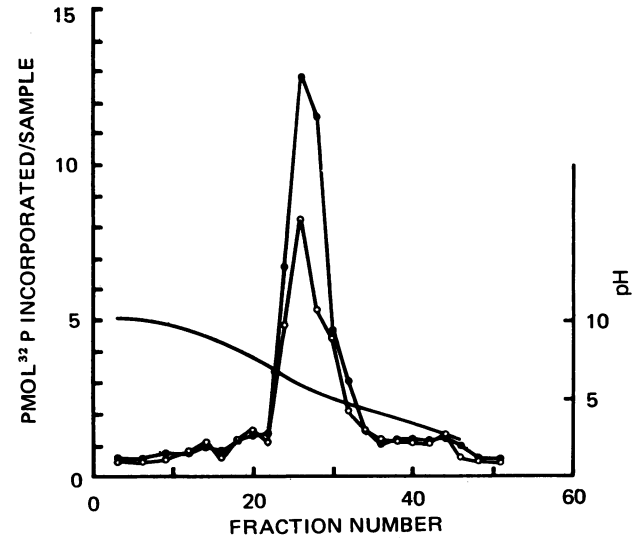

FIG. 1. Pattern of protein kinase activity after isoelectric focusing. Protein kinase obtained after DEAE-cellulose chromatography and Sephadex G-200 gel filtration (6) was dialyzed against 10% glycerol/0.1 mM EDTA/1 mM 2-mercaptoethanol before isoelectric focusing. Preparative isoelectric focusing was carried out in a 110-ml glass column (model 8100-10, LKB Instrument, Inc.) as described by Hung *et al.* (12). The dialyzed protein kinase preparation (3–5 mg of protein) was mixed in 30% sucrose containing 1 mM 2-mercaptoethanol. A linear gradient of pH 3.5 to 10 was formed by applying a constant voltage (600 V) for 20 hr at 2°. A 50-μl aliquot was taken from various fractions for assay of protein kinase activity with histone F1 as substrate. ●, Protein kinase activity assayed in the presence of cAMP (1 μM); O, protein kinase activity assayed in the absence of cAMP; —, pH. For experimental details see *Materials and Methods*.

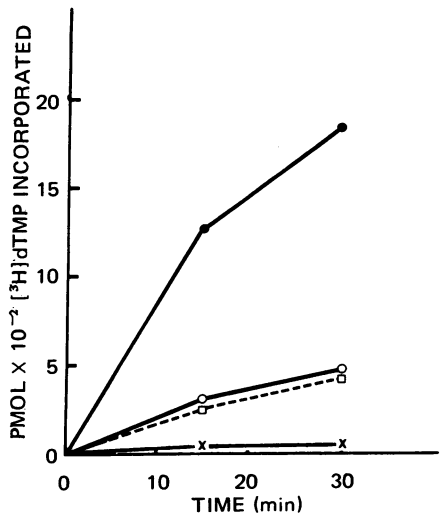

FIG. 2. Effect of protein kinase on reverse transcriptase activity. Reverse transcriptase (0.3 μg) was phosphorylated with 20 μg of protein kinase obtained after isoelectric focusing in the presence of nonradioactive ATP under the conditions described under *Materials and Methods*. Phosphorylation was carried out for 10 min at 35°. At the end of the preincubation period, the samples were cooled in ice and then incubated again at 35° to measure [³H]dTMP incorporation after the ingredients (0.05 ml) for reverse transcriptase assay were added. For experimental details, see *Materials and Methods*. O——O, reverse transcriptase only; □ - - - □, reverse transcriptase and heat-treated protein kinase; ●——●, reverse transcriptase and protein kinase: X——X, protein kinase only.

transcriptase activity was observed with the highest concentration of protein kinase (20 μg). The protein kinase preparation used in this study did not exhibit reverse transcriptase activity as shown in Fig. 2.

The effect of protein kinase reaction on the specific activity of reverse transcriptase was studied. Having been incubated with or without protein kinase in the presence of ATP, the transcriptase was reisolated by phosphocellulose chroma-

Table 1. Effect of protein kinase on the specific activity of reverse transcriptase reisolated from phosphocellulose

Preparations	Protein recovered (μg)	Specific activity (pmol of dTMP incorporated per μg)
Reverse transcriptase incubated with ATP and reisolated from phosphocellulose*	6.5	6.3
Reverse transcriptase incubated with protein kinase and ATP then reisolated from phosphocellulose	11	14
Protein kinase incubated with ATP then chromatographed on phosphocellulose	0	0

* Reverse transcriptase (15 μg) was incubated with ATP (0.2 mM) or ATP (0.2 mM) plus protein kinase (40 μg) in a solution containing α-glycerol-phosphate buffer (50 mM, pH 7.0), dithiothreitol (5 mM), and $MgCl_2$ (10 mM). After 30 min at 35°, the reaction mixtures were chilled and diluted and the transcriptase was reisolated from phosphocellulose columns (1 × 5 cm) as described in *Materials and Methods*. In a control experiment, protein kinase was incubated and chromatographed in the identical manner.

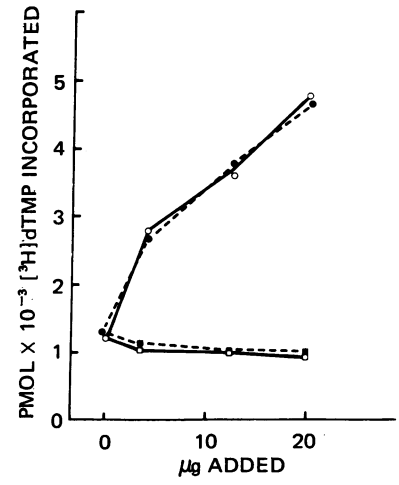

FIG. 3. Effect of increasing amounts of protein kinase on reverse transcriptase activity. Reverse transcriptase (0.5 μg) was phosphorylated with the indicated amounts of protein kinase in the presence of nonradioactive ATP as described in legend of Fig. 2. The total incubation time for the reverse transcriptase assay was 30 min. For experimental details see *Materials and Methods*. O——O, reverse transcriptase and protein kinase; ● - - - ●, reverse transcriptase, protein kinase, and cyclic AMP (1 μM); □——□, reverse transcriptase and heat-treated protein kinase; ■——■, reverse transcriptase, heated protein kinase, and cyclic AMP (1 μM).

tography to determine its specific activity from the reverse transcriptase activity and protein concentration. As shown in Table 1, the specific activity of the transcriptase increased more than 2-fold under the experimental conditions. In a parallel study, the protein kinase preparation did not produce any measurable protein concentration in the eluates of phosphocellulose column.

Phosphorylation of Fractions Containing Reverse Transcriptase Activity. To gain information as to whether or not the stimulation of reverse transcriptase activity may involve phosphorylation of reverse transcriptase or reverse transcriptase-associated proteins, a phosphorylation experiment was carried out (Fig. 4). Protein kinase and reverse transcriptase preparations were incubated in the presence of [γ-^{32}P]ATP under conditions of optimal protein kinase activity. After the incubation the reaction mixture was dialyzed and subjected to chromatography on phosphocellulose to isolate reverse transcriptase. Trichloroacetic acid-insoluble ^{32}P radioactivity, reverse transcriptase, and protein kinase activities were assayed in the eluted fractions. Protein kinase activity and some trichloroacetic acid-precipitable radioactivity were found in the fractions that did not adsorb onto the phosphocellulose column. As shown in Fig. 4A, reverse transcriptase activity was eluted with 0.22–0.25 M potassium phosphate. Significant amounts of ^{32}P radioactivity were eluted in the fractions containing reverse transcriptase activity but not in the other fractions. Sephadex gel filtration on the combined transcriptase fractions from phosphocellulose column was carried out further according to the method of Faras *et al.* (16). Again, the reverse transcriptase activity coincided with the radioactivity (Fig. 4B). Considering the partially purified state of the eluted reverse transcriptase, the data allow the tentative conclusion that reverse transcriptase or reverse transcriptase-associated proteins is selectively phosphorylated.

Effect of Phosphatase on Reverse Transcriptase Activity. The observations of a protein kinase-mediated activation of reverse transcriptase and phosphorylation of reverse transcriptase or reverse transcriptase-associated proteins suggest-

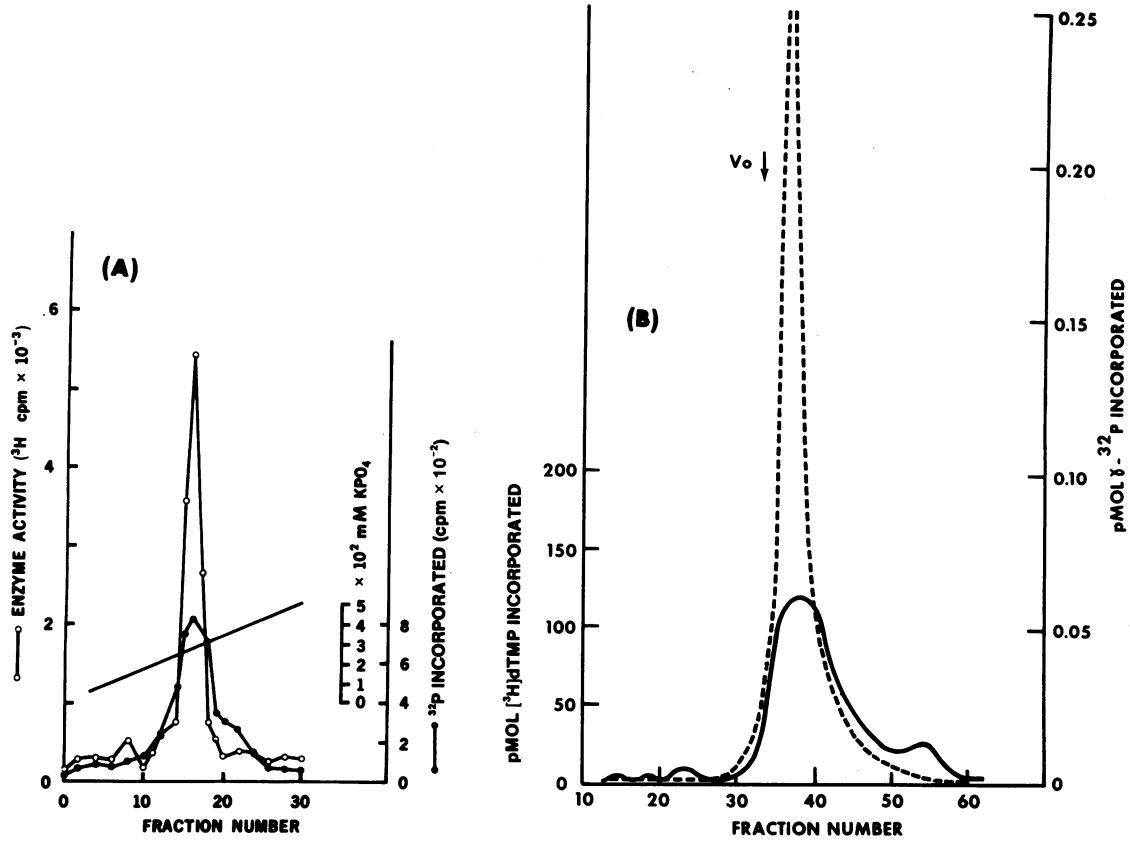

FIG. 4. Chromatographic profiles of radioactive label and reverse transcriptase activity after incubation with protein kinase. (A) *Phosphocellulose column:* Reverse transcriptase (4.5 μg) was phosphorylated with 42 μg of protein kinase obtained after isoelectric focusing in the presence of 4 nmol of [γ-^{32}P]ATP (9 μCi) in a final volume of 5 ml under the conditions described in *Materials and Methods.* After 30 min of incubation at 35° the reaction mixture was cooled in ice and dialyzed against potassium phosphate buffer (pH 8.0), containing 0.1 mM EDTA, 1 mM mercaptoethanol, and 10% glycerol. Dialysis was carried out for 20 hr with three changes of dialysis buffer. The dialyzed ^{32}P-labeled protein fraction was subjected to chromatography on phosphocellulose (13). Elution was carried out with a linear gradient of 10–500 mM potassium phosphate. Aliquots (0.1 ml) of the eluted fractions were taken for the assay of reverse transcriptase activity (○) and for assay of ^{32}P radioactivity (●). (B) *Sephadex G-100 column:* The fractions containing enzyme activity from panel A were pooled and applied to Sephadex G-100 column (1.5 × 88 cm) as described by Faras *et al.* (16). Fractions of 1.6 ml were collected and analyzed for ^{32}P counts and enzyme activity. Broken line, transcriptase activity; solid line, ^{32}P counts.

ed to us an apparent correlation between the phosphorylative and functional modification of reverse transcriptase. Such a correlation may under certain conditions assume considerable physiological importance, particularly since phosphoprotein phosphatase activity has been observed in viruses (17) and chick embryo fibroblasts (S. G. Lee, R. A. Jungmann, and P. P. Hung, unpublished observation). The coordinated action of protein kinase and phosphoprotein phosphatase on reverse transcriptase may conceivably constitute a regulatory system permitting control of reverse transcriptase activity. In this regulatory system phosphoprotein phosphatase would act to dephosphorylate and simultaneously deactivate reverse transcriptase. To gain preliminary data to test this possibility, ^{32}P-labeled reverse transcriptase of known enzymatic activity was subjected to treatment with alkaline phosphatase under conditions allowing hydrolysis of esterified ^{32}P-labeled phosphate groups from protein. Since we have not purified chick embryo fibroblast phosphatase to a high degree of purity at this time we have chosen alkaline phosphatase from *E. coli,* which was capable of removing ^{32}P-labeled phosphate from [^{32}P]protamine sulfate. The data of Table 2 demonstrate that addition of increasing amounts of alkaline phosphatase to a ^{32}P-labeled reverse transcriptase preparation that had been dialyzed against Tris buffer to remove orthophosphate resulted in an increasing loss of ^{32}P label from the reverse transcriptase preparation. The loss of phosphatase-mediated ^{32}P label was accompanied by a corresponding decrease of the reverse transcriptase activity, which was carried out in the presence of 50 mM orthophosphate in a separate experiment. The alkaline phosphatase used in the experiments did not hydrolyze [^{3}H]dTTP (40 μM) in the presence of its potent inhibitor, orthophosphate (18) during the reverse transcriptase assay, as revealed by thin-layer chromatography (19) when control experiments were carried out.

DISCUSSION

The results reported in this communication suggest that the reverse transcriptase activity of an oncogenic RNA virus may be determined by the degree of phosphorylation of the enzyme. Stimulation of the rate of DNA synthesis appears to be associated with increased phosphorylation of reverse transcriptase mediated by protein kinase, and conversely, dephosphorylation of the enzyme by the action of phosphatase decreases reverse transcriptase activity. This type of regulation of enzyme activity, consisting of an enzymatic interconversion of the active and inactive or less active forms of reverse transcriptase, has not been reported heretofore for nucleic acid polymerases of viruses.

It is unknown at present whether reverse transcriptase is subject to phosphorylative modification in the virion of RSV.

Table 2. Effect of varying amounts of alkaline phosphatase on reverse transcriptase activity and on dephosphorylation of ^{32}P-labeled reverse transcriptase

Alkaline phosphatase (μg)	Reverse transcriptase activity (%)	pmol of ^{32}P hydrolyzed
Exp. 1: 0	100	—
3.5	85	—
7.0	62	—
13	48	—
20	37	—
Exp. 2: 0	—	0
5	—	0.24
10	—	0.33

Reverse transcriptase preparation (0.5 μg) that had been dialyzed against Tris buffer to remove orthophosphate was dephosphorylated with the indicated amounts of alkaline phosphatase in Tris buffer (50 mM, pH 8.0) and $MgCl_2$ (5 mM). Dephosphorylation was carried out for 10 min at 35°. At the end of the incubation period, the samples were cooled in ice, and incubated again at 35° for 30 min to measure [^{3}H]dTMP incorporation after the ingredients (0.05 ml) for transcriptase assay were added. Reverse transcriptase activity in the presence of heat-inactivated alkaline phosphatase was used as 100% for calculating reverse transcriptase activity as affected by the same amount of alkaline phosphatase. ^{32}P-Labeled reverse transcriptase (0.2 μg, total radioactivity 650 cpm, 1300 cpm/pmol) was dephosphorylated with the indicated amounts of alkaline phosphatase under the conditions described above. The samples were incubated for 15 min at 35°, and subsequently assayed for acid-precipitable ^{32}P as described under *Materials and Methods.*

We have found that the degree of activation *in vitro* of different reverse transcriptase preparations by the same protein kinase preparation varied widely (1.5- to 4-fold), suggesting that the isolated reverse transcriptase preparations may have been phosphorylated to varying degrees prior to their isolation. Additionally, we have obtained evidence that preincubation of reverse transcriptase with alkaline phosphatase decreased the activity of reverse transcriptase preparations that had not been subjected to protein kinase treatment *in vitro*, which also indirectly suggests some degree of phosphorylation of the reverse transcriptase preparation prior to its isolation from the virions.

Protein kinase activities are associated with many enveloped viruses (15, 20). The origin of protein kinase as a host or virus coded component and its physiological role remain obscure. In RSV, we have observed a cAMP-independent protein kinase activity associated with the viral core (unpublished observation). Immunological and enzyme kinetics studies may clarify its relationship to the cellular protein kinase. The mechanism of reverse transcriptase activation by phosphorylation is yet to be studied. It is not known whether the activated transcriptase would transcribe the viral template into products different from those by transcriptase without activation, or whether the activated enzyme has a better affinity to the primer (21) and the template of reverse transcription. Neither is known whether lack of the activation is related to the low DNA polymerase activity of certain viruses, such as the reticuloendotheliosis virus group, or to the temperature sensitivity of reverse transcriptase from certain oncornavirus mutants (22).

The observation that reverse transcriptase can be subjected to phosphorylative and functional modification may be of biological significance. Upon infection, activation of reverse transcriptase by the protein kinase of the virion or of the host cell may precede the actual replication of viral RNA in RSV. On the other hand, reverse transcriptase may be dephosphorylated by phosphatase of viral or cellular origin into an inactive or less active state for packaging in the virions where viral replication may not be desired.

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