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Protein kinase activity associated with simian virus 40 T antigen

(immunoprecipitation assay/casein phosphorylation/tsA mutant protein)

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ABSTRACT **Incubation of simian virus 40 (SV40) tumor (T) antigen-containing immunoprecipitates with [γ-^{32}P]ATP results in the incorporation of radioactive phosphate into large T antigen. Highly purified preparations of large T antigen from a SV40-transformed cell line, SV80, are able to catalyze the phosphorylation of a known phosphate acceptor, casein. The kinase activity migrates with large T antigen through multiple purification steps. Sedimentation analysis under non-T-antigen-aggregating conditions reveals that kinase activity and the immunoreactive protein comigrate as a 6S structure. The kinase activity of purified preparations of large T antigen can be specifically adsorbed to solid-phase anti-T IgG, and partially purified T antigen from a SV40 *tsA* transformant is thermolabile in its ability to phosphorylate casein when compared to comparably purified wild-type T antigen. These observations indicate that the SV40 large T antigen is closely associated with protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) activity.**

The simian virus 40 (SV40) tumor (T) antigens play a role in initiating rounds of viral DNA replication, regulating viral gene expression, and promoting a state of virus-induced neoplastic transformation (1–13). Two distinct SV40 T proteins have been characterized to data, large T antigen (T, M_r ≈80,000) and small T antigen (M_r 17,000). They are encoded by partially overlapping cistrons and are present in both SV40-infected and SV40-transformed cells (6, 7, 13–22). T is a nuclear phosphoprotein (23, 24) that has specific DNA binding properties (25–28), and it is active in each of the biochemical processes noted above. Small T antigen is believed to be important for transformation (18, 21, 22).

Recently, another virus-coded transforming protein, the *src* protein of Rous sarcoma virus, has been shown to be closely associated with protein kinase activity (29, 30). Subsequently, protein kinase activity has been observed in immunoprecipitates formed between anti-adenovirus 5 T antigen antiserum and T antigen containing extracts of adenovirus 5-infected and -transformed cells (31). In this report, we present the results of experiments designed to assess whether any of the SV40 T antigens have a similar biochemical activity. The data strongly suggest that large T antigen is closely associated with protein kinase activity (ATP:protein phosphotransferase, EC 2.7.1.37). Recently, comparable results have been obtained by others for an adenovirus–SV40 hybrid T antigen (D2 protein) that is an analog of large T antigen (32).

MATERIALS AND METHODS

Cells and Viruses. The propagation of virus, SV80 cells (33), and CV1-P green monkey kidney cells (a gift from Thomas Shenk) were as described (12, 14, 27). SVCHL and *CHLtsA30* are isogeneic Chinese hamster lung cell lines transformed by wild-type SV40 and SV40-*tsA30*, respectively (2, 34). They were grown at 33°C in glass roller bottles in standard medium (14).

Immunoprecipitation of SV40 T Antigens. Immunoprecipitation was performed as described (14, 35). Where indicated, hamster anti-T or control IgG was purified and coupled to cyanogen bromide-activated Sepharose 4B beads (36). The protein content of each immunoadsorbant was approximately 10 mg/g.

Purification of Large T Antigen from SV80 Cells. Large T antigen was purified from 80- to 200-g lots of SV80 cells (grown at the Massachusetts Institute of Technology Cell Culture Center, Cambridge, MA) by a modification of the method of Tenen *et al.* (37) as described (12). The standard sequence of purification steps was: nuclear soaking, 25–35% ammonium sulfate fractionation, DEAE-cellulose chromatography (0.10–0.40 M NaCl gradient elution), QAE-Sephadex chromatography (0.18–0.40 M NaCl gradient elution), heparin-Sepharose chromatography (0.15–0.50 M NaCl gradient elution), and 5–40% glycerol gradient centrifugation. Large T antigen from 15- to 20-g lots of SVCHL and *CHLtsA30* grown at 33°C was partially purified by the same method through the QAE-Sephadex step. Large T antigen was assayed by complement fixation (27).

DNA-Cellulose Chromatography. Calf thymus DNA (Sigma) was adsorbed to Whatmann CF-11 cellulose powder (approximately 0.58 mg of DNA per g of cellulose) by the method of Alberts and Herrick (38). Columns were equilibrated in application buffer [50 mM Hepes, pH 6.6/10% (vol/vol) glycerol/1 mM dithiothreitol] at 4°C. After sample application, the column was washed successively with 4 column volumes of application buffer and 4 volumes of 50 mM Tris, pH 8/10% glycerol/1 mM dithiothreitol (pH 8.0 wash) and then was eluted with a linear NaCl gradient in 50 mM Tris, pH 8.0/10% glycerol/1 mM dithiothreitol (20 column volumes).

Protein Kinase Assays. Protein kinase activity in immunoprecipitates was detected by incubating the Sepharose-protein A bound immune complexes (25 μl of of packed beads exhaustively washed with extraction buffer) in 100 μl of 50 mM Tris·HCl, pH 9.0/10 mM $MgCl_2$/0.1% Triton (New England Nuclear) containing 0.1 μM [γ-^{32}P]ATP (250 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) (New England Nuclear). The mixture was incubated at 37°C for 10 min, and the reaction was terminated by adding 1.0 ml of Tris·HCl, pH 9.0/0.5M LiCl/150 mM 2-mercaptoethanol (sEtOH). The beads were washed twice in this buffer prior to elution with 30 μl of 20 mM Tris·HCl, pH 9.0/5% (wt/vol) sodium dodecyl sulfate ($NaDodSO_4$)/700 mM sEtOH (elution buffer) at 100°C for 3 min.

Protein kinase activity of column or gradient fractions was determined in a 100-μl reaction mixture containing 50 mM Tris·HCl (pH 9.0), 10 mM $MgCl_2$, 0.1% Triton, 0.1 μM [γ-^{32}P]ATP (2500 Ci/mmol), and, where indicated, casein at approximately 3 μM as a phosphate acceptor. After 10 min at

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Abbreviations: SV40, simian virus 40; T antigen, tumor antigen of SV40; sEtOH, 2-mercaptoethanol; $NaDodSO_4$, sodium dodecyl sulfate; ts-T, *tsA* mutant encoded large T antigen; wt-T, wild-type large T antigen.

37°C, reactions were terminated by the addition of 1.0 ml of cold 95% ethanol followed by 2 μl of RNase A (10 mg/ml) (Sigma) as carrier. After 1 hr at 0°C, the precipitated proteins were pelleted at 12,000 × *g* for 15 min; the pellet was washed once with cold 20% (wt/vol) trichloroacetic acid/30 mM sodium pyrophosphate and once with acetone. After vacuum drying, 30 μl of elution buffer was added, and the samples were solubilized and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (39). Discontinuous $NaDodSO_4$/polyacrylamide gels (13 × 15 × 0.1 cm; 4% stacking; 12% running) were prepared and electrophoresed as described by Laemmli (39). Staining and autoradiography (with intensifying screens) were performed as described (14). The incorporation of radioactive phosphate into casein was measured semiquantitatively by densitometric tracing of the casein region of each lane in the gel autoradiogram and, for each assay point, is expressed as the weight of a cutout of the tracing.

Phosphothreonine and Phosphoserine Analyses. Radiolabeled protein product bands were located in $NaDodSO_4$/polyacrylamide gels, excised, and eluted overnight into 0.1% NH_4HCO_3/0.1% $NaDodSO_4$/2% sEtOH and the polypeptides were hydrolyzed in 6M HCl prior to high-voltage (3.5 kV) paper electrophoresis in 2.5% formic acid/7.8% acetic acid, pH 1.9, followed by ninhydrin staining of marker phosphoamino acids (Sigma) and autoradiography (40, 41).

RESULTS

Detection of Protein Kinase Activity in Immunoprecipitates of SV40-Transformed Cell Line. Immunoprecipitates of extracts of the SV40-transformed human cell line SV80 contain five major large T antigen polypeptides [apparent M_r, 94,000, 57,000, 55,000, 17,000, and 8000 (Fig. 1*A*)] (14). To determine if such immunoprecipitates also contained protein kinase activity, protein A-adsorbed immune complexes were incubated with [γ-^{32}P]ATP in an assay mixture similar to that of Collett and Erikson (29). The antibody-bound proteins were eluted and electrophoresed in $NaDodSO_4$ polyacrylamide gels.

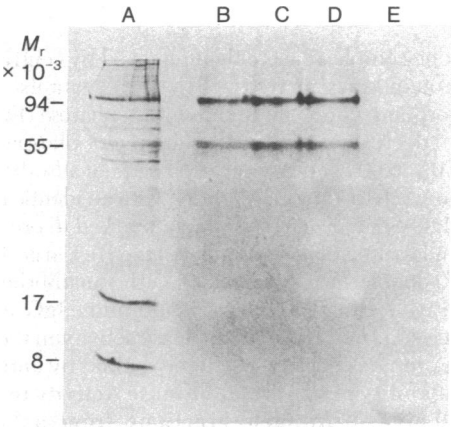

FIG. 1. Detection of protein kinase activity in SV40 large T antigen immunoprecipitates from SV80 cells. Extracts of SV80 cells were incubated with either anti-T (lanes A–D) antiserum or nonimmune hamster serum (lane E), and immune complexes were isolated and assayed for protein kinase activity. Molecular weight markers include phosphorylase *a* (97,000), bovine serum albumin (68,000), hamster IgG heavy chain (55,000), ovalbumin (43,000), IgG light chain (25,000), and RNase A (13,000). Lanes: A, precipitate of [^{35}S]methionine-labeled SV80 cells (approximately 2 × 10^6 cells) with anti-T antiserum; B, anti-T antiserum precipitate of SV80 cells incubated in the standard [γ-^{32}P]ATP-containing reaction mixture; C, anti-T antiserum precipitate plus 1 mM cyclic AMP and 1 mM cyclic GMP incubated as in B; D, anti-T antiserum precipitate plus 0.5 μg of SV40 DNA (form I) similarly incubated; E, nonimmune serum precipitate similarly incubated.

Newly phosphorylated proteins were visualized by autoradiography (Fig. 1, lane A). Nonimmune serum precipitate gave virtually no radiolabeled bands. In contrast, anti-T precipitates contained labeled proteins of M_r 94,000 (large T antigen) and a doublet of $M_r \approx$ 55,000–57,000 (Fig. 1, lane B). No labeling of the small T antigen (17,000) or the 8000 polypeptide was detected. The 57,000 and 55,000 bands likely represent phosphorylated forms of two "middle-sized" T antigens observed in SV80 extracts (14). These polypeptides are also phosphorylated *in vivo* under standard cell growth conditions (unpublished observations). The heavy chain of IgG was not phosphorylated in these experiments. Cyclic AMP and cyclic GMP (1 mM each) failed to stimulate labeling of these three bands (Fig. 1, lane C). In subsequent experiments, addition of these compounds separately also had no apparent effect. Likewise, 0.5 μg of SV40 DNA (Fig. 1, lane D) was without apparent effect. The phosphorylation reaction in these immune complexes was time-dependent, with maximal labeling of the large T antigens occurring after 30 min at 37°C. It was also temperature-dependent, with greater labeling of the three bands after 10 min at 37°C than at 4°C (data not shown). Attempts to detect large T antigen-dependent phosphorylation of a heterologous substrate were repeatedly unsatisfactory with this system. Thus, protein kinase activity was sought in purified large T antigen fractions.

Detection of Protein Kinase Activity Associated with Purified SV80 Large T Antigen. Column fractions from each of the three chromatographic steps in the standard large T antigen purification procedure from SV80 cells were assayed for protein kinase activity, as measured by the labeling of casein. At each step, large T antigen-containing fractions (identified by complement fixation) proved to contain casein phosphorylating activity. An aliquot of a pool of large T antigen-containing fractions eluting from the heparin-Sepharose column between 0.40 and 0.48 M NaCl was dialyzed and sedimented through a 5–40% glycerol gradient containing 1 M NaCl (Fig. 2*A*). A single, symmetrical peak of large T antigen was present (migrating at 6 S, with *Escherichia coli* alkaline phosphatase as marker) and comigrating with a peak of protein kinase activity. Additional protein kinase activity was present at the top of the gradient. When the same experiment was performed with another aliquot of heparin-Sepharose-purified material without NaCl in the transport medium, major large T antigen peaks of 6 S and 10–15 S were observed, both of which comigrated with protein kinase activity (data not shown). The 6S material has been found to be composed of a species of ≈95% pure, full-size large T antigen (12, 37) and is the monomeric species of the protein (14). The presence of the non-T-antigen-associated ≤4S kinase species in the glycerol gradient profiles was an inconstant finding from antigen preparation to antigen preparation. Preliminary results suggest that these activities arise from kinase activity eluting at somewhat higher salt concentration than the peak large T antigen fractions during both the QAE-Sephadex and heparin-Sepharose steps.

An aliquot of the same pool of heparin-Sepharose-purified large T antigen used in the experiment described in Fig. 2*A* was also applied to a column of calf thymus DNA-cellulose equilibrated at pH 6.6. The column was washed at the same pH, then washed with pH 8 buffer, and finally exposed to a linear salt elution gradient (1.0–2.5 M NaCl). Three peaks of large T antigen immunoreactivity were observed (Fig. 2*B*). None was observed in the column application and wash fractions; the first peak eluted in the pH 8.0 fraction. Two more were observed in the NaCl elution gradient, one at approximately 1.8 M NaCl and the other at approximately 2.2 M NaCl. Four peaks of protein kinase activity were observed. The first did not bind to the column; it appeared in the application/pH 6.6 wash seg-

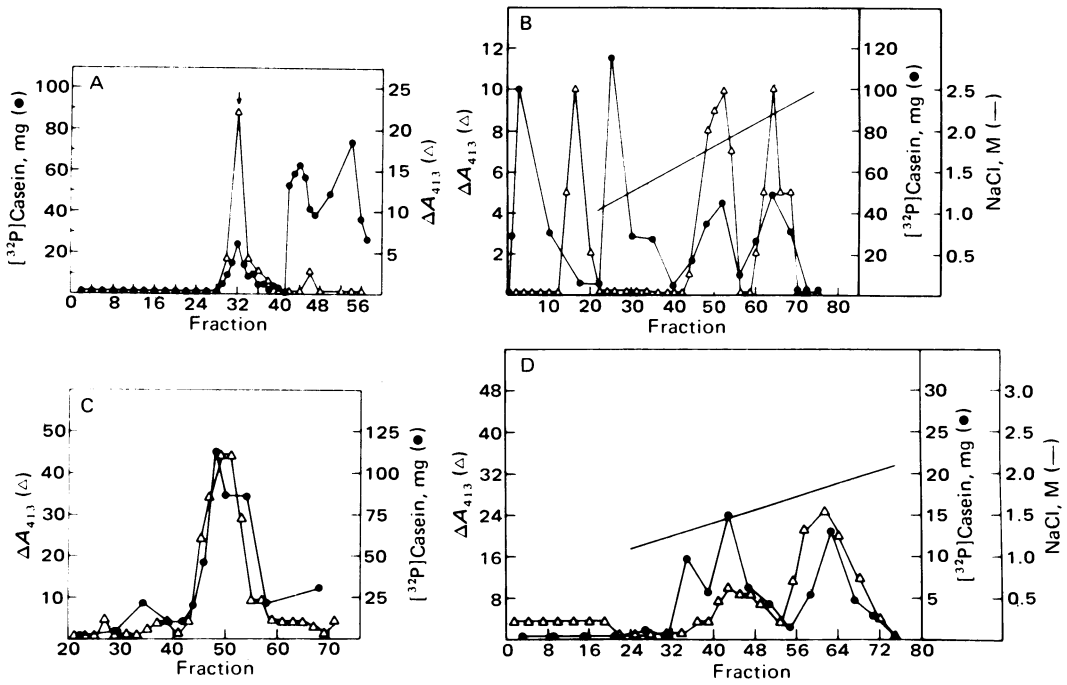

FIG. 2. Comigration of SV80 large T antigen immunoreactivity with protein kinase activity. (*A*) Large T antigen-containing fractions (eluting between 0.40 and 0.48 M NaCl) from the heparin-Sepharose step were pooled and dialyzed against buffer A (37) containing 4% glycerol (buffer B) for 2 hr, and an aliquot [≈6 μg of protein (42)] was applied to a 5–40% glycerol gradient containing 1 M NaCl. This was centrifuged for 24 hr at 42,000 rpm at 2°C in a Beckman SW 50.1 rotor. After collection, fractions were assayed for complement fixation (13 μl) activity (ΔA_{413}) and casein phosphorylating activity (9 μl). The position of *E. coli* alkaline phosphatase (6 S) run in a parallel gradient is indicated by an arrow. (*B*) Another aliquot (≈10 μg of protein) of the pooled heparin-Sepharose large T antigen was applied to a column (1 ml) of calf thymus DNA-cellulose; the column was washed successively with pH 6.6 buffer, pH 8.0 buffer, and a linear 1–2.5 M NaCl gradient. Samples were assayed by complement fixation (19 μl) and for protein kinase activity (15 μl). (*C*) Large T antigen from the peak of the heparin-Sepharose fractions was further purified by sedimentation in a 5–40% gradient containing 1 M NaCl as described above. Large T antigen peak fractions from several such gradients run in parallel were pooled and dialyzed, and 3 μg was applied to a 2-ml DEAE-cellulose (DE52) column equilibrated in 50 mM Tris, pH 7.8/25% glycerol/3 mM dithiothreitol/0.02% Triton/0.1% Nonidet P40. The column was washed and then eluted with a 0.1–0.4 M NaCl gradient (12 column volumes) in the same buffer and fractions were assayed for complement fixing activity (30 μl) and protein kinase activity (50 μl). (*D*) Peak large T antigen-containing heparin-Sepharose fractions from another preparation were pooled and purified through the glycerol gradient step, and an aliquot (≈5 μg) of the 6S peak was dialyzed and then applied to a calf thymus DNA-cellulose column (1 ml) that was washed and eluted as above, except that a 1.2–2.2 M NaCl gradient was applied. Aliquots were assayed for complement fixation activity (20 μl) and protein kinase activity (25 μl).

ment. The second eluted at the beginning of the salt gradient, and the remaining peaks eluted in association with the two peaks of large T antigen. Thus, three peaks of T antigen with increasing affinities for DNA were observed, two with and one without associated kinase activity. Because this profile was obtained with the same heparin-Sepharose-purified material utilized in the sedimentation velocity experiment shown in Fig. 2*A*, we assume that the two species of non-T-antigen-associated kinase eluting in this experiment correspond to the non-T-antigen-associated low molecular weight kinase species in the glycerol gradient.

In a subsequent experiment, the 6S T antigen fraction present in the 1 M NaCl/glycerol gradient was dialyzed and then applied to a column of DEAE cellulose (Fig. 2*C*). When a linear salt gradient was applied, a T antigen and a protein kinase peak coeluted at approximately 0.2 M NaCl. When an aliquot of 6S glycerol gradient-purified material from another preparation was applied to a calf thymus DNA cellulose column (Fig. 2*D*), two major peaks of T antigen and protein kinase activity were observed, eluting together at approximately 1.6 and 2.0 M NaCl. A less discrete immunoreactive species with associated protein kinase activity eluted at approximately 1.3 M NaCl

Selective Anti-T Immunoadsorption of Casein Phosphorylating Activity from a Fraction Containing Highly Purified SV80 Large T Antigen. Aliquots of highly purified T antigen were incubated (4°C) with identical amounts of either nonimmune IgG or anti-T IgG covalently coupled to Sepharose (36). Immune complexes were then removed by centrifugation, and the protein kinase activity of the supernatants was measured. Absorption with nonimmune IgG-Sepharose (Fig. 3, lane A) failed to decrease significantly subsequent phosphorylation of casein (M_r 30,000 bands) compared to a nonadsorbed control (Fig. 3, lane C). In contrast, absorption with an identical amount of anti-T IgG-Sepharose resulted in a marked decrease in the subsequent labeling of casein (Fig. 3, lane B). Large T antigen (M_r 94,000 doublet) was also labeled in the unadsorbed control reaction (Fig. 3, lane B) and after nonimmune IgG adsorption (Fig. 3, lane A). Thus, the protein kinase activity in the purified T antigen preparation was selectively bound by anti-T IgG.

Thermolability of the Protein Kinase Activity in Partially Purified Large T Antigen Fractions from a Cell Line Transformed by SV40 tsA30. Large T antigen was partially purified (through the QAE step in the standard preparatory procedure) from isogeneic Chinese hamster lung cell strains transformed at a permissive temperature by *tsA30* and wild-type SV40 and cultivated at 33°C. The *tsA* mutant-encoded large T antigen (ts-T) has been shown to be relatively thermolabile, both as a DNA binding protein and as an antigen, compared to comparably purified wild-type T antigen (wt-T) (6, 7, 34, 43).

When identical aliquots of these ts-T or wt-T antigen preparations were assayed for protein kinase activity after preincubation at a permissive temperature (33°C), an equivalent amount of radioactive phosphate was incorporated into casein

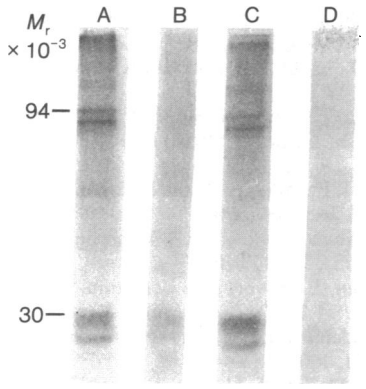

FIG. 3. Immunoabsorption of protein kinase activity in purified preparations of large T antigen: SV80 large T antigen was purified through all five steps of the standard procedure. Aliquots of T antigen [20 μl; protein concentration, 15 μg/ml (42)] were incubated with either nonimmune IgG-Sepharose [25 μl of a suspension of Sepharose beads in water, 50:50 (vol/vol), 30 min at 4°C], or an identical amount of anti-T IgG coupled to Sepharose followed by removal of the Sepharose beads by centrifugation. This cycle was repeated twice, and protein kinase activity was measured in the final supernatants with casein (30,000 M_r bands) as a phosphate acceptor; 10 μl of each fraction was assayed. Lanes: A, assay of nonimmune IgG-Sepharose-adsorbed T antigen; B, assay of anti-T IgG-Sepharose-adsorbed T antigen; C, T antigen (10 μl) assayed without prior absorption; D, no T antigen was added to the reaction mixture. Both the 94,000 and 84,000 bands were specifically immunoprecipitated by anti-T antiserum (data not shown). It is likely that the lower molecular weight band represents a proteolytic cleavage product of large T antigen.

(Fig. 4, curves A and C). In contrast, when similar aliquots were preincubated at a nonpermissive temperature (40°C) for 30 min prior to assay, there was decrease (to 29%) in casein phosphorylation in the ts-T antigen-containing reaction mixture and a 1.5-fold stimulation in the wt-T antigen reaction. Heated (90°C) wt-T was inactive. These results indicate that a significant fraction of the protein kinase activity of partially purified large T antigen from *CHLtsA30* is temperature sensitive, compared to similarly purified wild-type T antigen.

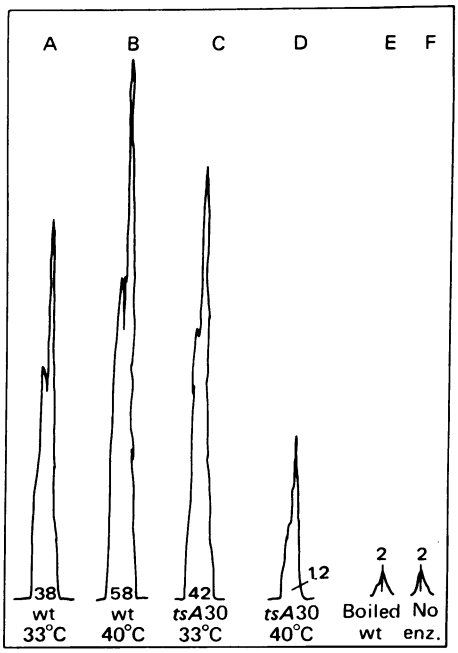

FIG. 4. Thermolability of the protein kinase activity in partially purified large T antigen fractions from a temperature-sensitive SV40-transformed cell line. Large T antigen was partially purified from SVCHL and *CHLtsA30*. Aliquots [20 μl; protein concentration, 20 μg/ml (42)] of wt-T (curves A, B, and E) or ts-T (curves C and D) were assayed for protein kinase activity with casein as a phosphate acceptor at either 33 or 40°C after the T antigen had been preincubated for 30 min at either 33 or 40°C, respectively. The casein regions of each lane in the gel autoradiograph were traced densitometrically and are shown here. For curve E the T antigen fraction was heated at 90°C for 10 min before being added to the reaction mixture. The numbers on each tracing indicate the weight of the cutout (in mg).

Characterization of the *In Vitro* Phosphorylation Products and of Divalent Cation Requirements. Phosphoserine and phosphothreonine analyses of two *in vitro* labeled products, large T antigen and casein, were undertaken in an effort to characterize the nature of the enzymatic reaction (40, 41). *In vitro* ^{32}P-labeled large T antigen contained both radioactive phosphoserine and phosphothreonine, although there was significantly more of the former (Fig. 5*A*). *In vitro* labeled casein contained both phosphoserine and phosphothreonine with a preponderance of phosphothreonine (Fig. 5*B*).

With either casein or large T antigen as substrate, an absolute requirement for certain divalent cations was apparent. Magnesium (optimal concentration, 10 mM) and manganese (optimal concentration 0.1 mM) were equivalent in supporting the phosphorylation of these proteins. In addition, no stimulation of the casein phosphorylation reaction catalyzed by highly purified T antigen preparations was observed in the presence of cyclic AMP or cyclic GMP.

DISCUSSION

The results presented here strongly suggest that SV40 large T antigen from two strains of SV40-transformed cells is associated with protein phosphorylating activity. The extensive coincidence of elution of T antigen immunoreactivity and kinase activity in multiple chromatographic systems and their comigration in a zonal sedimentation system makes it unlikely that the kinase activity present in highly purified antigen fractions is an adventitious contaminant. This conclusion is further strengthened by the observations that the kinase activity can be specifically adsorbed by anti-T IgG and, more importantly, that it is thermolabile when isolated from cells transformed by a temperature-sensitive gene *A* mutant. Because large T antigen is the product of gene *A* (2, 6, 7, 13, 16, 17, 34, 43–45), this result suggests that the stability of the protein phosphorylating activity depends, to some extent, upon the structural integrity of large T protein.

Although these results are consistent with large T antigen alone being the source of the observed kinase activity, other reasonable possibilities remain. The enzymatic activity could be a property of another polypeptide which is tightly bound to T antigen. One example of a tight complex with protein kinase activity is rat brain tubulin. In this case, tubulin is noncovalently complexed with a higher molecular weight, non-colchicine-binding protein kinase (46). Another plausible expla-

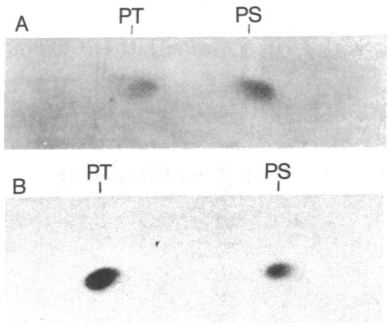

FIG. 5. Analysis of phosphoserine and phosphothreonine contents of *in vitro* phosphorylated large T antigen (*A*) and casein (*B*). PT, phosphothreonine; PS, phosphoserine.

nation is that a specific complex of T antigen with another protein exists and that the appearance of enzymatic activity depends on both subunits being properly liganded to one another.

The protein kinase activity associated with large T antigen is apparently not stimulated by addition of cyclic AMP or cyclic GMP. Nevertheless, we cannot rule out the possibility that the enzyme is isolated as a stable protein–cyclic nucleotide complex.

Multiple species of large T antigen were observed during chromatography of heparin-Sepharose-purified T antigen on DNA-cellulose. Indications of such heterogeneity have been evident before with preparations of SV40 T antigen both during DNA-cellulose chromatography (25) and phosphocellulose chromatography (47). Of interest here is the absence of protein kinase activity in a T antigen fraction that bound least tightly to the column (pH 8 eluted fraction). How these molecules differ from one another structurally is unclear at present. The possibility exists that these species differ by virtue of some relatively simple structural feature such as the presence or absence of an external covalent modification or a complexed polypeptide (see above). The antigen-associated activity is able to phosphorylate both large T antigen itself and phosphate acceptor proteins such as casein and phosvitin. Large T antigen is a nuclear phosphoprotein, and it will be of interest to determine whether the T antigen-associated protein kinase plays a role in the *in vivo* phosphorylation of T antigen. In this regard, the effects of phosphorylation of large T antigen on its DNA binding properties *in vitro* and on its role in the initiation of rounds of viral DNA replication or in control of early transcription are obvious subjects for investigation.

Finally, the association of protein kinase activity with transforming proteins of three quite different viruses (29–31) suggests a role for protein phosphorylation in the mechanism of transformation specific to each virus. This raises the possibility of common elements among these reactions, and in this regard, it will be of particular importance to define the natural *in vivo* targets of the T antigen-related kinase activity.

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