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Biochemistry

Protein kinase activity associated with the D2 hybrid protein related to simian virus 40 T antigen: Some characteristics of the reaction products

(adenovirus–simian virus 40 hybrid/Ad2$^+$D2/nuclear proteins/[γ-^{32}P]ATP/sodium dodecyl sulfate–polyacrylamide gel electrophoresis)

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ABSTRACT **Protein kinase activity (ATP:protein phosphotransferase, EC 2.7.1.37) has been found associated with the D2 hybrid protein, a highly purified protein of 107,000 daltons specified by the adenovirus–simian virus 40 (SV40) hybrid Ad2$^+$D2, which has many properties associated with authentic SV40 T antigen [Tjian, R. & Robbins, A. (1979) *Proc. Natl. Acad. Sci. USA* 76, 610–614]. We have now examined some of the biochemical characteristics of the reaction products. Acceptors for the terminal phosphoryl group of [γ-^{32}P]ATP are the purified protein itself and at least four proteins extracted from nuclei of uninfected cells. Purified histones do not serve as substrate for the enzyme. Phosphorylation is markedly reduced by heating the D2 hybrid protein to 50°C for 30 min. The products of phosphorylation are stable to treatment with ethanol/ether, DNase, and RNase, but completely degraded by digestion with Pronase, demonstrating their protein nature. The phosphate bonds are labile to hot alkali and sensitive to digestion with alkaline phosphatase but stable to treatment with hot acid or hydroxylamine. These results provide evidence that ^{32}P is incorporated into *O*-phosphoserine or *O*-phosphothreonine residues of acceptor proteins, indicating that the enzymatic activity is characteristic for protein kinase, and that cell-specified nuclear proteins other than histones may serve as substrates for the enzyme.**

Simian virus 40 (SV40) T antigen, the product of the virus *A* gene (1–5), plays an important role in a number of viral and cellular processes. (*i*) It is required for the initiation of viral replication (6) and binds to specific regions near or at the origin of viral DNA replication (7, 8). (*ii*) It is associated with the stimulation of cellular DNA synthesis (3). (*iii*) It is involved in the induction and maintenance of cell transformation (9–13). (*iv*) It provides helper function for the growth of adenovirus in primate cells (14–16). (*v*) It regulates the transcription of its own gene (2, 17). (*vi*) It stimulates RNA synthesis in isolated nuclei (18) and nucleoli (19). With the recent discovery of protein kinase activity (ATP:protein phosphotransferase, EC 2.7.1.37) associated with the D2 hybrid protein, a protein closely related to authentic SV40 T antigen (20), a potential explanation for the multiple effects of T antigen has become available. The defective adenovirus–SV40 hybrid Ad2$^+$D2 specifies the synthesis of this protein of 107,000 daltons, which can be purified in large quantities from HeLa cells infected with the hybrid virus (21). The protein is specifically immunoprecipitated by antisera from hamsters bearing SV40-induced tumors (22), and it shares extensive peptide homology with, and has many properties of, authentic T antigen (23). The association of a kinase activity with a tumor antigen immediately suggests that such activity may be involved in transformation.

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Other studies have also suggested that protein kinases play a role as mediators of cellular transformation. The appearance of a tumor-specific protein kinase activity not found in normal tissues has been demonstrated in Ehrlich ascites carcinoma, Novikoff ascites hepatoma, and Walker tumor cell nuclei (24). More specifically, the *src* gene product of avian sarcoma virus, a protein required for transformation *in vitro* and sarcoma induction *in vivo* (25), either possesses or is associated with a protein kinase activity (26, 27). Furthermore, in extracts from adenovirus 5-infected cells, antiserum directed against viral tumor antigens immunoprecipitates a protein kinase activity (28). Both these enzyme activities result in phosphorylation of the heavy chain of the IgG used to precipitate the antigen. On the other hand, data indicating that the enzymes are true protein kinases, that is, that the phosphorylated products have the characteristics usually associated with the products of this class of enzyme reactions, have been presented only for the *src*-associated enzyme (27). Also, the substrates in these studies have been the heavy chain of IgG, the egg protein phosvitin, or other proteins that are unlikely candidates for natural substrates for the enzymes.

In the present report, we demonstrate that at least four nuclear proteins from uninfected cells can serve as substrates for the kinase activity associated with the D2 hybrid protein and that the reaction products have the biochemical characteristics of phosphoproteins.

MATERIALS AND METHODS

Cells and Viruses. HeLa cells (human) and CV-1 cells, a line of African green monkey kidney cells (29), were grown in plastic dishes in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10% vol/vol), gentamicin sulfate (50 μg/ml), and amphotericin B (0.25 μg/ml). The defective hybrid virus Ad2$^+$D2 was a gift from J. Hassell (Université de Sherbrooke, Quebec, Canada). Stocks were grown in CV-1 cells as described (22).

Purification of D2 Hybrid Protein. D2 hybrid protein was purified essentially as described by Tjian (21). In brief, confluent cultures of HeLa cells grown in roller bottles were infected with Ad2$^+$D2 at a multiplicity of 5–10 plaque-forming units per cell. At 50 hr after infection, the cells were scraped from the surface with a rubber policeman and washed three times with cold phosphate-buffered saline. Nuclei were released from the cells by Dounce homogenization and extracted with 0.4 M LiCl. The extract was brought to 55% saturation with ammonium sulfate. The precipitate formed was dissolved in a buffer containing 1 M NaCl, cleared by centrifugation, and

Abbreviations: SV40, simian virus 40; CF, complement-fixing; NaDodSO$_4$, sodium dodecyl sulfate.

loaded onto a column (2.5 × 75 cm) of Ultrogel AcA 34 (LKB). Gel filtration was at a flow rate of 30 ml/hr. Fractions (6 ml) were collected and assayed for complement fixation (see below) and absorbance at 280 nm. The fractions containing the peak of complement-fixing (CF) activity were pooled, dialyzed, and passed through a column (0.9 × 15 cm) of DEAE-Sephadex A-50 at a flow rate of 7 ml/hr. The proteins retained on the column were eluted with a linear gradient (100 ml) of 0.1–0.5 M NaCl. Fractions (2 ml) were collected and analyzed for CF activity and absorbance. The fractions containing D2 hybrid protein eluted between 0.3 and 0.4 M NaCl and were again pooled, dialyzed, and passed through a column (0.5 × 6 cm) of phosphocellulose P[11] (Whatman) at a flow rate of 10 ml/hr. The proteins were eluted with a 34-ml gradient of 0.1–1 M NaCl. The fractions from the main peak with CF activity were pooled and concentrated on a DEAE-cellulose DE52 (Whatman) column. The final preparation contained approximately 60 μg of D2 hybrid protein in a volume of 0.6 ml.

Complement Fixation Assay. Microtiter plates containing 25 μl each of Veronal-buffered diluent (3.25 mM diethylbarbituric acid/1.8 mM sodium barbital/0.1 mM calcium lactate, 0.5 mM $MgSO_4$/0.15 M NaCl, pH 7.4), antigen, anti-T serum (inactivated by heating for 30 min at 56°C and diluted 1:100) and 50 μl of guinea pig complement (Flow Laboratories, McLean, VA, diluted 1:90) were incubated for 16 hr at 4°C. Residual complement was assayed by incubating at 37°C for 30 min in the presence of 8×10^6 sensitized sheep erythrocytes (Institut Armand Frappier, Montreal) followed by centrifugation of the microtiter plates at 1000 rpm for 2 min in a PR-6 centrifuge (International). The highest dilution of antigen giving complete lysis was chosen as the end point. Hamster anti-SV40 T serum was provided by R. E. Wilsnack (National Cancer Institute, Bethesda, MD). All other materials for the complement fixation assay were generously supplied by A. F. Doss of McGill University.

Protein Kinase Assay. This assay was a modification of the procedure of Sen *et al.* (30). The reaction mixture (usually 50 μl) contained buffer A (20 mM Hepes, pH 7.5/120 mM KCl/5 mM Mg acetate/2 mM dithiothreitol), 0.2 mM [γ-^{32}P]ATP (1–2 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels), and, where indicated, D2 hybrid protein at approximately 20 μg/ml dialyzed against buffer A just before use, and nuclear extract (see below) at approximately 1.2 A_{280} units/ml. Incubation was at 30°C for 1 hr. Prior to addition, the nuclear extract was heated to 56°C for 30 min to destroy endogenous protein kinase activity. When the heat treatment was omitted, the endogenous kinase activity obscured that of the D2 hybrid protein.

Filter Assay for Kinase Activity. At the end of the incubation period for the protein kinase reaction, 200 μg of bovine serum albumin was added to each sample and proteins were precipitated with 1 ml of cold 10% trichloroacetic acid containing 2% sodium pyrophosphate. The precipitate was collected by centrifugation and redissolved in 0.1 ml of cold 1 M NaOH. The material was reprecipitated, collected, and washed on Whatman GFC filters. Radioactivity retained on the filters was measured after addition of 5 ml of toluene containing diphenyloxazole at 4 g/liter and 1,4-bis[2(5-phenyloxazolyl)]benzene at 50 mg/liter.

Sodium Dodecyl Sulfate ($NaDodSO_4$)/Polyacrylamide Gel Electrophoresis and Autoradiography. Electrophoresis was carried out in either 7% or 10% $NaDodSO_4$/polyacrylamide slab gels, with a 3% stacking gel according to the procedure of Weber and Osborn (31). Samples for electrophoresis were precipitated with 10% trichloroacetic acid/2% sodium pyrophosphate. The precipitated proteins were dissolved in 20 μl of sample buffer and heated to 100°C for 2 min before being applied to the gels. After electrophoresis, gels were stained with 0.1% Coomassie brilliant blue in 50% methanol and 7.5% (vol/vol) acetic acid and destained in 50% methanol and 7.5% acetic acid. For autoradiography gels were dried and exposed to Kodak X-Omat x-ray film.

Preparation of Nuclear Extracts. CV-1 cells, grown to subconfluence in roller bottles, were washed three times with phosphate-buffered saline, scraped with a rubber policeman, and centrifuged. The pellet was resuspended in 2 vol of hypotonic buffer (10 mM Hepes, pH 7.5/15 mM KCl/1.5 mM Mg acetate/1 mM dithiothreitol) and the cells allowed to swell for 10 min at 0°C before they were disrupted by 20 strokes in a Dounce homogenizer with a tight-fitting pestle. Concentrated buffer was added to give the final concentrations of buffer A used in the protein kinase assay.

Nuclei were pelleted at 1000 × *g* for 10 min, resuspended in buffer A, and disrupted by sonication (Branson sonifier W350, Microtip, position 3, 1 min). The suspension was centrifuged at 10,000 × *g* for 20 min and the supernatant was stored at −20°C until used.

Protein Determination. Protein determinations were performed according to the method described by McKnight (32) with bovine serum albumin as a standard.

Chemicals. [γ-^{32}P]ATP was purchased from New England Nuclear; Pronase, calf thymus histone (type II AS), and alkaline phosphatase were obtained from Sigma; deoxyribonuclease I (DNase), from Worthington; and ribonuclease (RNase), from Boehringer Mannheim.

RESULTS

Purification of D2 Hybrid Protein. D2 hybrid protein was purified from HeLa cells infected with the defective adenovirus-SV40 hybrid Ad2+D2 by using the procedure of Tjian (21). The precipitated nuclear extract was subjected to chromatography on columns of Ultrogel, DEAE-Sephadex, and phosphocellulose. In contrast with the results of Tjian and Robbins (20), but in agreement with the earlier work of Tjian (21), we found a single peak of CF activity eluting at 0.35 M NaCl from DEAE-Sephadex columns. The final chromato-

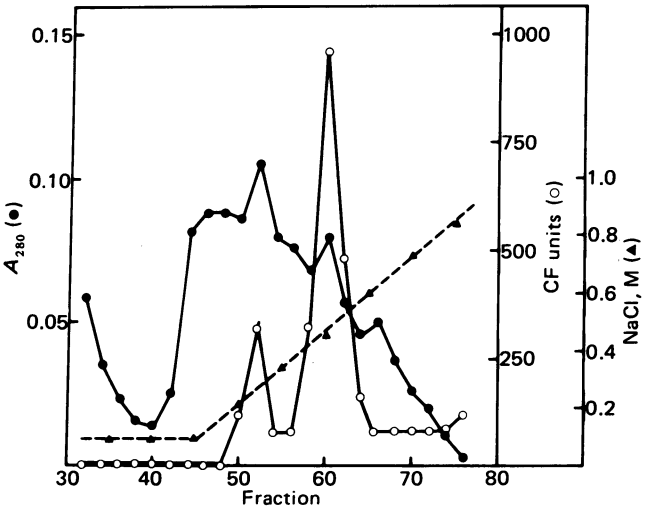

FIG. 1. Phosphocellulose chromatography of D2 hybrid protein. After DEAE-Sephadex chromatography, fractions containing CF activity were pooled, applied to a phosphocellulose column, and washed with several column volumes of buffer. Proteins retained on the column were eluted with a linear gradient of NaCl (▲). Fractions (1 ml) were analyzed for CF activity (○) and absorbance at 280 nm (●). A CF unit corresponds to the reciprocal of the highest dilution of antigen showing complete fixation of complement.

graphic separation on columns of phosphocellulose revealed two peaks with CF activity when tested with hamster anti-SV40 T serum, in agreement with the findings of Tjian and Robbins (20) (Fig. 1). The smaller peak, containing less than 20% of the total CF activity, was not characterized further. The fractions from the major peak were pooled, concentrated, and used for subsequent experiments. The extent of purification after each step, as analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis, is shown in Fig. 2. After phosphocellulose chromatography, only one band could be detected by electrophoresis when 3 μg of protein was applied to the gel. The recovery in the final preparation of D2 hybrid protein was 10% and the increase in specific activity was 220-fold as judged by complement fixation.

Phosphorylation of D2 Hybrid Protein. D2 hybrid protein was incubated in a standard protein kinase assay in the presence of [γ-^{32}P]ATP and in the absence of any other protein substrate. The reaction was terminated by precipitation with 10% trichloroacetic acid, and the precipitated protein was dissolved in sample buffer and subjected to electrophoresis on a 10% polyacrylamide gel. Autoradiography of the gel showed one labeled band at the position of D2 hybrid protein (Fig. 3, lane a). If the protein was first heated to 50°C for 30 min, a procedure that destroys most of the CF activity of authentic T antigen (33), the incorporation of ^{32}P was markedly reduced (Fig. 3, lane b). These results indicate that D2 hybrid protein undergoes phosphorylation in the presence of a phosphate donor.

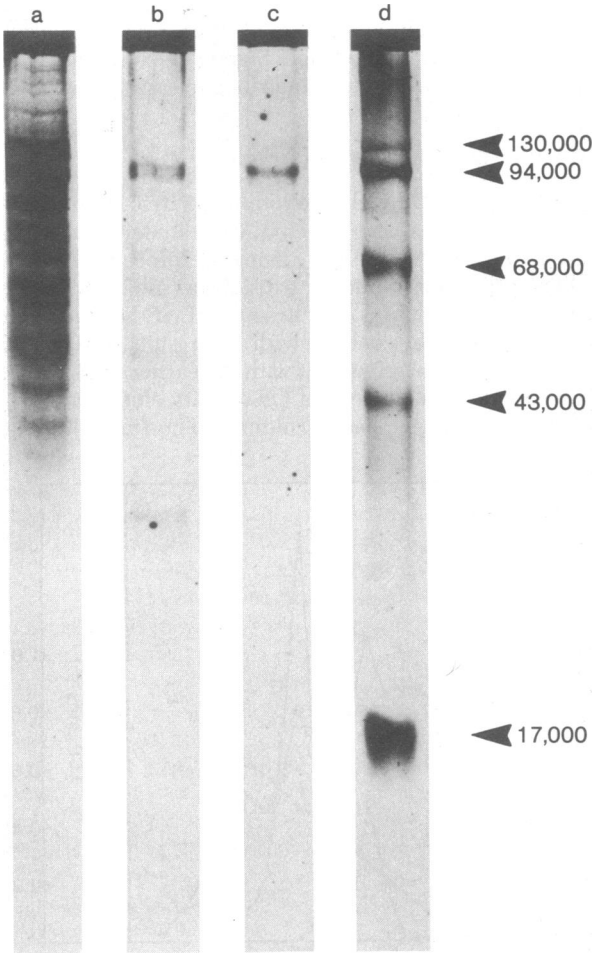

FIG. 2. Purification of D2 hybrid protein. After Ultrogel (lane a), DEAE-Sephadex (lane b) and phosphocellulose (lane c) chromatography, samples of the pooled fractions containing CF activity were precipitated with 10% trichloroacetic acid, collected by centrifugation, resuspended in sample buffer, and subjected to electrophoresis on $NaDodSO_4$/polyacrylamide (7%) slab gels. In lane c, 3 μg of protein was applied to the gel. Molecular weight markers (lane d) were run at the same time. The gels were stained with Coomassie brilliant blue.

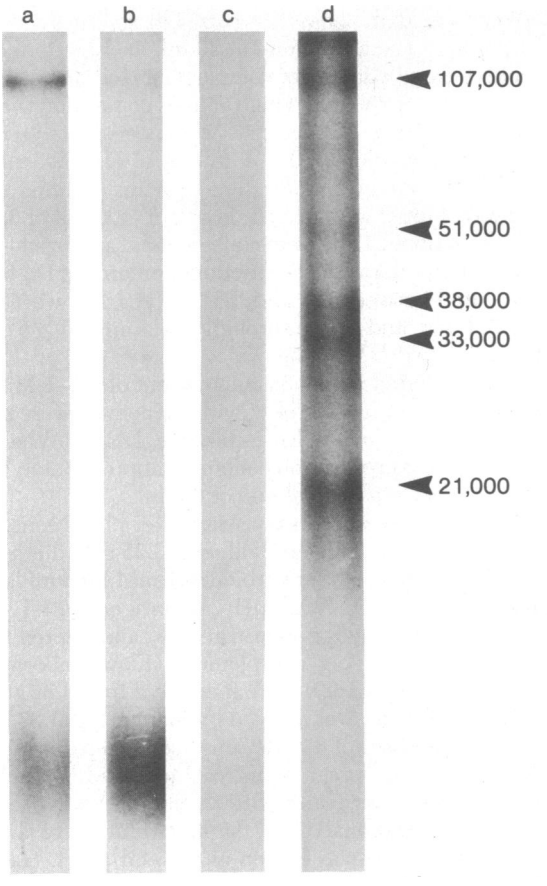

FIG. 3. Protein phosphorylation by D2 hybrid protein. D2 hybrid protein was incubated in a standard protein kinase assay with [γ-^{32}P]ATP either alone (lanes a and b) or in the presence of a nuclear extract (lane d). As a control, nuclear extract was also incubated without added D2 hybrid protein (lane c). In one sample (b), D2 hybrid protein was heated to 50°C for 30 min prior to incubation in the kinase assay. The reaction products were subjected to electrophoresis on $NaDodSO_4$/polyacrylamide (10%) slab gels and analyzed by autoradiography. The samples in lanes a and b and those in lanes c and d were run in two different gels. The molecular weights of the polypeptide bands in lane d were estimated in comparison to stained marker proteins run on the same gel.

Phosphorylation of Nuclear Protein Substrates. Nuclear extracts were prepared from CV-1 cells. In order to destroy endogenous protein kinase activity, extracts were heated to 56°C for 30 min. If these extracts were incubated in the protein kinase reaction mixture with [γ-^{32}P]ATP in the presence of D2 hybrid protein, no incorporation of ^{32}P could be detected after gel electrophoresis and autoradiography (Fig. 3, lane c). In fact, in the filter assay (see below), it was found that incorporation under these conditions in the absence of D2 hybrid protein amounted to about 2% of that obtained in the presence of the protein. If the extracts were incubated in its presence, at least four labeled bands in addition to the labeled D2 hybrid protein appeared on the autoradiogram (Fig. 3, lane d). By comparison to marker proteins of known molecular weights, these four proteins were estimated to have molecular weights of 51,000, 38,000, 33,000, and 21,000. Heating the nuclear extract to 100°C for 2 min prior to addition of D2 hybrid protein destroyed the ability of these nuclear proteins to serve as substrates;

Table 1. Characteristics of the protein kinase reaction products

	Treatment of reaction product	^{32}P incorporated cpm	% of control
a	None	78,432	100
	None (no D2 added)	1,458	2
	1 M KOH	531	1
	Hydroxylamine	63,162	81
b	None	108,035	100
	None (no D2 added)	2,806	3
	10% trichloroacetic acid, 90°C	86,881	80
c	None	44,917	100
	None (no D2 added)	839	2
	DNase	54,856	122
	RNase	41,054	93
	Pronase	514	1
	Alkaline phosphatase	23,493	52

In experiments a and b, 0.04-ml samples were removed from the reaction mixture and precipitated two times with trichloroacetic acid in the presence of 200 μg of carrier bovine serum albumin. They were then incubated with ethanol/ether (3:1 vol/vol) at 37°C for 30 min and centrifuged, and the pellets were air dried. Duplicate samples received no treatment or were treated in experiment a with 0.25 ml of 1 M KOH at 100°C for 15 min; or 0.25 ml of 0.8 M hydroxylamine (pH 5.4), freshly prepared according to Blat and Harel (34), at 37°C for 30 min; and in experiment b with 1 ml of 10% trichloroacetic acid at 90°C for 20 min. After these treatments, proteins were precipitated with 10% trichloroacetic acid containing 2% sodium pyrophosphate and collected on GFC filters. In experiment c, 0.04-ml samples were taken from a reaction mixture and heated at 100°C for 3 min to terminate the reaction. Duplicate samples were then incubated at 37°C for 1 hr (the Pronase-treated sample for 2 hr) in 0.5 ml of 0.2 M Tris·HCl, pH 7.0. The control contained no other additives, while experimental reaction mixtures contained 50 μg of DNase I and 1.5 mM $MgCl_2$, 50 μg of RNase, 50 μg of Pronase (preincubated for 15 min at 37°C) plus 1.5 mM $CaCl_2$ at pH 7.8, or 50 μg of alkaline phosphatase at pH 8.0. After these treatments, samples were precipitated two times with trichloroacetic acid in the presence of 200 μg of carrier bovine serum albumin and collected on GFC filters. Controls without D2 were incubated under identical conditions except that the D2 hybrid protein was omitted.

^{32}P was incorporated only into the D2 protein itself under these conditions (data not shown). Purified histones did not serve as phosphate acceptors in the presence of the protein.

Chemical Characterization of Labeled Products. The nature of the protein–phosphate linkage was investigated by using a filter assay for radioactivity incorporated into protein as shown in Table 1. The phosphorylated product is protein because ^{32}P-labeled material was stable to extraction with ethanol/ether and to digestion with RNase and DNase but was rendered completely acid soluble by digestion with Pronase. The reaction product was partially sensitive to treatment with alkaline phosphatase, very labile to hot alkali, but stable in hot acid, as would be expected for phosphate linked to serine and threonine residues (35). There was little or no acylphosphate present, because the product was insensitive to treatment with hydroxylamine at pH 5.4 (34), nor was there evidence for the presence of phosphohistidine, which would have been hydrolyzed in acid and stable in alkali (36). These results provide strong evidence that the enzymatic activity causes the incorporation of ^{32}P into *O*-phosphoserine or *O*-phosphothreonine, as would be expected for a protein kinase.

DISCUSSION

The purified D2 hybrid protein, a protein very similar to SV40 T antigen, is closely associated with protein kinase activity (20). That the enzyme activity may also be associated with the functions of T antigen is suggested by the findings presented in this report: (*i*) At least four proteins in a nuclear extract prepared from uninfected cells are phosphorylated in its presence, whereas no such phosphorylation is observed in the absence of D2 hybrid protein under our experimental conditions. These proteins might serve as natural substrates for the enzyme under physiological conditions. (*ii*) There is incorporation of ^{32}P from [γ-^{32}P]ATP into proteins and, more specifically, into phosphoserine or phosphothreonine residues in the presence but not in the absence of D2 hybrid protein, showing that the products have the attributes of those from well-characterized protein kinase reactions (35, 37). (*iii*) The enzymatic activity is reduced by heating the protein to 50°C for 30 min, a procedure that reduces the CF activity of authentic SV40 T antigen (33), and the ability of the nuclear extract to serve as substrate is destroyed by denaturing the acceptor proteins by heating to 100°C for 2 min.

There is the possibility that the enzyme activity does not reside in D2 hybrid protein itself but rather in an impurity in the preparation. Against this is the finding that anti-T gamma globulin inhibits the kinase activity (20). In addition, neither in the Coomassie blue-stained gels nor in the autoradiograms of gels on which the purifed protein was subjected to electrophoresis was any band other than the D2 hybrid protein detected. The purification procedure increased the specific activity for complement fixation 220-fold, and we estimate the maximum level of contamination to be less than 3% because a single band was detected by staining with Coomassie blue when 3 μg of protein was applied to the gel. Nevertheless, the only criterion of purity used in this and other studies (20, 21) on the protein has been a single band on $NaDodSO_4$/polyacrylamide gel electrophoresis. Certainly additional studies with more rigorous criteria for purity are indicated before the enzyme activity can be attributed to the protein.

Whether authentic T antigen also exhibits protein kinase activity is also not yet known. But Tjian and Robbins (20) have presented arguments against the prospect that the enzyme activity resides in the adenovirus-specific part of the D2 hybrid protein, and we agree with them that the most likely possibilities are that it is a property of the protein itself or results from the specific binding of a cellular or viral product to the protein.

The D2 hybrid protein becomes phosphorylated in the presence of a phosphate donor and both it and T antigen have been shown to be phosphorylated *in vivo* (22, 38). In the case of T antigen, the phosphate has been shown to be bound to serine residues contained in one tryptic peptide (38). Because these phosphate groups have been shown to turn over several times within the lifetime of T antigen in cells transformed by wild-type SV40, but not in cells infected with *A* mutants, phosphorylation and dephosphorylation may be important to the function of T antigen (37). Furthermore, if T antigen is associated functionally with the phosphorylation of other, especially nuclear, proteins, this is a very appealing explanation for the diverse functions attributed to this protein in productive and transforming infections. Protein phosphorylation has been implicated in the regulation of nuclear processes such as transcription and replication and may also be required for regulation of processes involved in maintenance of cell architecture (39, 40). Protein kinase activity has been found in association with the avian sarcoma virus *src* gene product by Collett and Erikson (26) and by Levinson *et al.* (27), and with human adenovirus type 5 T antigen(s) by Lassam *et al.* (28). Because these proteins are required for the transformation of cells by these viruses, the findings imply that phosphorylation is a critical step in transformation.

Cell transformation by the D2 hybrid protein has not been directly demonstrated, but the SV40-specific sequences of the

$Ad2^{+}D2$ viral genome are reported to complement gene A mutants of SV40 (21). Because this protein, so closely related to SV40 T antigen, also is associated with protein kinase activity, and nuclear proteins serve as substrates, it may be that alterations in phosphorylation play a central role in the molecular events leading to cell transformation by SV40 and perhaps by all tumor viruses.

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