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Phosphorylation In Vitro of *Escherichia coli*-Produced 235R and 266R Tumor Antigens Encoded by Human Adenovirus Type 12 Early Transformation Region 1A

LYNNE A. LUCHER, PAUL M. LOEWENSTEIN, AND MAURICE GREEN*

Institute for Molecular Virology, St. Louis University Medical Center, St. Louis, Missouri 63110

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The tumor (T) antigens encoded by the human adenovirus early transforming region 1A (E1A) are gene regulatory proteins whose functions can immortalize cells. We have recently described the synthesis in *Escherichia coli* and the purification of the complete T antigens encoded by the adenovirus type 12 (Ad12) E1A 12S mRNA (235-residue [235R] T antigen) and 13S mRNA (266R T antigen). In this study, we show that the Ad12 E1A T antigens are extensively phosphorylated in Ad12-infected mammalian cells but are not phosphorylated in *E. coli*. Inasmuch as posttranslational phosphorylation at specific amino acid sites may be important for biological activity, we have studied the phosphorylation of the *E. coli*-produced T antigens in vitro by using a kinase activity isolated from cultured human KB cells. The kinase was purified about 300-fold and appears to be a cyclic AMP-independent, Ca^{2+}-independent protein kinase requiring only ATP and Mg^{2+} for activity. To determine which amino acids are phosphorylated and whether phosphorylation in vitro occurs at the same amino acid sites that are phosphorylated in vivo, the Ad12 E1A T-antigen species synthesized by infected cells were metabolically labeled with $^{32}P_i$ and compared with the *E. coli*-produced E1A T antigens labeled in vitro with [γ-^{32}P]ATP by using the partially purified kinase. Partial V8 proteolysis analysis gave similar patterns for in vivo- and in vitro-phosphorylated T antigen. Two-dimensional maps of tryptic phosphopeptides and of chymotryptic phosphopeptides suggested that mainly the same amino acid sites are phosphorylated in vitro and in vivo and that phosphorylation occurred at multiple sites distributed throughout the T-antigen molecule. Serine was the only amino acid that was phosphorylated both in vivo and in vitro, and, surprisingly, most serines appeared to be phosphorylated. The feasibility of faithfully phosphorylating T antigens in vitro suggests that the *E. coli*-produced Ad12 E1A 235R and 266R T antigens may prove useful for molecular studies on T-antigen function.

We have described recently the synthesis in *Escherichia coli* and the purification to near homogeneity of the complete T-antigen molecules encoded by the adenovirus type 12 (Ad12) early 1A (E1A) 12S mRNA (235-residue [235R] T antigen) and the Ad12 E1A 13S mRNA (266R T antigen) (21). Ferguson et al. (12) have reported the expression in *E. coli* of an Ad2-Ad5 hybrid E1A T antigen and its purification. The adenovirus E1A T antigens function as regulatory proteins that can activate the expression of viral early genes and some cellular genes (1, 18, 20, 23) and can immortalize cells (for a review, see reference 15). The Ad12 E1A T antigens are of particular interest because of the high oncogenicity of Ad12 (14) and because the expression of the Ad12 E1A 266R T antigen is associated with the suppression of class I major histocompatibility genes in cultured primary rodent cells (2, 28). The availability of highly purified E1A T antigens will permit in vitro studies to elucidate molecular properties that may be relevant to their functions in gene regulation and cell growth.

A potential drawback of producing biologically interesting proteins in *E. coli* is that posttranslational modifications unique to eucaryotic cells may be required for activity in vitro. The adenovirus E1A T antigens are posttranslationally phosphorylated (13, 22, 34), as are other tumor virus transformation proteins. Phosphorylation can play an important role in the activity of regulatory proteins (for a review, see reference 19). The biological activities of several tumor virus transforming proteins appear to be regulated by the phosphorylated state of the molecule. For example, the simian virus 40 (SV40) large T antigen is phosphorylated at eight or more sites, and correlations have been reported among the state of phosphorylation, oligomeric forms of large T, and binding to viral DNA (see reference 27 and references therein). The Rous sarcoma virus pp60$^{v\text{-}src}$ protein contains two major phosphorylated sites, phosphoserine near the N terminus at residue 17 and phosphotyrosine at residue 416. Studies with Rous sarcoma virus mutants have suggested that phosphorylation at these sites is not essential for protein kinase activity or for morphological transformation (10, 31). However, modified forms that possess enhanced protein kinase activity and are phosphorylated at new tyrosine residues in the N-terminal region have been identified recently in Rous sarcoma virus-transformed cells, suggesting that secondary phosphorylation may regulate phosphotransferase activity (7). The polyomavirus middle T antigen is phosphorylated mainly at tyrosine residue 315, presumably by the activity of the complexed pp60$^{c\text{-}src}$ (9), and also at serine and threonine residues, probably by other cellular protein kinases. A polyomavirus mutant with a phenylalanine substituted for a tyrosine at residue 315 is transformation defective (4).

As described in this study, the Ad12 E1A 235R and 266R T antigens synthesized in *E. coli* are not phosphorylated. We have partially purified a protein kinase activity from uninfected KB cells and have used the enzyme to phosphorylate the *E. coli*-produced T antigens in vitro. Phosphorylation appears to occur in the T antigens mainly at the same amino acid sites in vitro as in Ad12-infected cells.

* Corresponding author.

MATERIALS AND METHODS

Preparation of $^{32}P_i$-labeled total cell and nuclear and cytoplasmic extracts of Ad12 early-infected KB cells. Cell monolayers (75 cm^2) containing 3.5×10^6 KB cells were grown in Eagle minimal essential medium containing 10% calf serum and infected with 3 PFU of Ad12 (strain Huie) per cell. Cells were then incubated in Eagle minimal essential medium containing 2% agamma calf serum and 20 μg of 1-β-D-arabinofuranosylcytosine (araC) per ml to enhance the accumulation of adenovirus early gene products (13). At 24 h postinfection, cell monolayers were incubated for 1 h in 2 ml of phosphate-free Eagle minimal essential medium containing 2% dialyzed calf serum and then labeled for 6 to 8 h with 1 to 4 mCi of $^{32}P_i$ (ICN Pharmaceuticals Inc., Irvine, Calif.). Monolayers were washed twice with phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1% Trasylol (Aprotinin; 10,000 Kallikrein Inactivation units per ml; Mobay Chemical Corp., New York, N.Y.) and either were sonically disrupted in 3 ml of whole-cell sonication buffer (20 mM Tris hydrochloride [pH 7.4], 50 mM NaCl, 5 mM EDTA, 1 mM β-mercaptoethanol, 0.5 M urea, 10% glycerol, 1% deoxycholate, 0.5% Nonidet P-40, 1 mM PMSF, 0.1% Trasylol) and clarified at 100,000 × *g* (22) or were fractionated into cytoplasm and nucleoplasm as follows (16). Cells were suspended in 10 times the pellet volume of buffer containing the following: 10 mM Tris (pH 8.5), 250 mM sucrose, 3.7 mM $CaCl_2$, 12 mM $MgCl_2$, 1% Nonidet P-40, 1 mM PMSF. The suspension was incubated on ice for 20 min with occasional mixing. Completeness of cell lysis was checked by phase microscopy. Nuclei were centrifuged at 2,400 rpm for 15 min in a centrifuge (model J-6B; Beckman Instruments, Inc., Fullerton, Calif.), washed with 10 volumes of the above buffer, and recentrifuged. The combined supernatant was defined as the cytoplasmic fraction. Nuclei were suspended in 10 times the pellet volume of whole-cell sonication buffer and sonicated for 2 min at a setting of 4 to 5 with the standard microtip probe of the sonifier (model W-350; Branson Sonic Power Co., Danbury, Conn.). Cytoplasm and nucleoplasm were clarified at 100,000 × *g* for 1 h and stored at −20°C until used for immunoprecipitation analysis.

Preparation of extracts of [^{35}S]methionine-labeled and $^{32}P_i$-labeled *E. coli* PR13^q cells transformed by plasmids pAd416 and pAd418. Plasmids pAd416 and pAd418, which contain cDNA copies of the Ad12 E1A 12S and 13S mRNAs, respectively, under the control of the *tac* promoter, were used to transform *E. coli* PR13^q (21). Clones of transformed cells were grown for 2 h at 37°C in M9CAT medium (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 0.2% Casamino Acids [Difco Laboratories, Detroit, Mich.], 2 mM $MgSO_4$, 0.2% glucose, 0.1 mM $CaCl_2$, 0.012% thiamine [pH 7.4]) containing 50 mg of ampicillin per liter. Isopropyl-β-D-thiogalactopyranoside (Bethesda Research Laboratories, Gaithersburg, Md.) was added to 2 mM to derepress the *tac* promoter and to induce the synthesis of T antigen. Cells were incubated for an additional 60 min at 37°C, centrifuged at 8,000 × *g* for 10 min, and washed with and suspended in phosphate-free medium (M9CAT with phosphate replaced by 50 mM Tris [pH 7.4] and Casamino Acids replaced by 50 mg each of methionine, threonine, leucine, proline, arginine, histidine, isoleucine, and valine per liter). Cells (1-ml portions) were labeled for 30 min at 37°C with either 20 μCi of [^{35}S]methionine (1,100 Ci/mmol; New England Nuclear Corp., Boston, Mass.) or 500 μCi of $^{32}P_i$. Cells were centrifuged, washed twice with phosphate-free medium, suspended in sodium dodecyl sulfate (SDS)-urea buffer (1% SDS, 5 M urea, 1% 2-mercaptoethanol, 30 mM NaCl, 50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.0]), boiled for 3 min, and stored at −20° until used for immunoprecipitation analysis.

Purification of Ad12 E1A T antigens from pAd416- and pAd418-transformed *E. coli*. Purification of E1A T antigens was performed as described (21; B. Ferguson and M. Rosenberg, personal communication). Preparations used as substrates for in vitro phosphorylation in these studies were from 40 to 90% pure.

Preparation of KB cell extracts containing kinase activity that phosphorylates Ad12 E1A T antigens in vitro. The activity that phosphorylates the Ad12 E1A T antigens is hereafter referred to as kinase. All procedures were performed at 4°C. Crude cell extracts were prepared by sonicating KB cells harvested from suspension cultures in 10 ml of 10 mM HEPES (pH 7.7)–10 mM NaCl–5% glycerol–1 mM dithiothreitol–1 mM PMSF–0.1% Trasylol per ml of cell pellet by sonication (model W-350; Branson) and clarifying by centrifugation at 100,000 × *g* for 5 min in an airfuge (Beckman). Cytoplasmic and nuclear extracts were prepared by disrupting KB cells in 10 ml of buffer A (10 mM HEPES, [pH 7.7], 10 mM NaCl, 1 mM PMSF, 0.1% Trasylol) per ml of cell pellet with 10 strokes of a Dounce homogenizer. Nuclei were centrifuged at 2,400 rpm for 15 min in a Beckman J-6B centrifuge, washed with 2 to 5 ml of buffer A, and recentrifuged. The combined supernatant was defined as the cytoplasmic extract. Nuclei were suspended in 10 ml of buffer A per ml of nuclear pellet, sonicated as described above, and clarified by centrifugation at 2,400 rpm for 15 min. Crude kinase extracts were stored at −20°C.

Purification of the kinase from KB cell cytoplasm. All steps were performed at 4°C. The cytoplasmic fraction from KB cells was dialyzed against buffer B (10 mM potassium phosphate, [pH 7.0], 20 mM KCl, 10% glycerol); a flocculent precipitate was removed by centrifugation at 12,000 rpm for 30 min in a Beckman J-21B centrifuge (JA-20 rotor). A DEAE-cellulose column (1.1 by 5.0 cm; Whatman DE-52; Whatman, Inc., Clifton, N.J.) was equilibrated in buffer B, loaded with dialyzed cytoplasm (26 mg of protein) at a flow rate of 0.2 ml/min, and washed with 15 ml of buffer B. The column was eluted with an 80-ml linear gradient of 20 to 400 mM KCl in buffer B at a flow rate of 0.3 ml/min, followed with 10 ml of buffer B containing 800 mM KCl. Fractions (2 ml) were collected and assayed for kinase activity with T antigen as substrate (see below). Active fractions were pooled, dialyzed against buffer B, and loaded on a phosphocellulose column (1.1 by 2 cm; Whatman P11; Whatman) equilibrated in buffer B at a flow rate of 0.2 ml/min. The column was washed with 5 ml of buffer B and eluted with a 40-ml linear gradient of 20 to 500 mM KCl in buffer B, followed with 4 ml of column buffer B containing 1 M KCl. Fractions (1.3 ml) were collected and assayed for kinase activity. Active fractions were pooled, concentrated by dialysis against 20% polyethylene glycol (molecular weight, 20,000; Sigma Chemical Co., St. Louis, Mo.) and 30% glycerol in buffer B, dialyzed against buffer B containing 30% glycerol, and stored at −20°

Assay of the kinase activity towards purified T antigens. The kinase assay mixture contained from 0.1 to 5 μg of purified T antigen, 0.072 to 225 μg of kinase protein, 8 to 18 μM ATP, 10 to 33.3 mM $MgCl_2$, and 8 to 50 μCi of [γ-^{32}P]ATP (2,500 Ci/mmol; New England Nuclear Corp.) in 20 to 220 μl of buffer B. The mixture was incubated for 5 min to 2 h at 37°C and placed at 4°C, and 1 volume of SDS-urea buffer was

added. Water was added to 0.9 ml, and immunoprecipitation was immediately performed (see below). Radioactive bands of phosphorylated T antigens detected by autoradiography were excised from dried gels, and counts were measured by Cerenkov counting.

Immunoprecipitation of phosphorylated Ad12 T antigens. Phosphorylated T antigens were immunoprecipitated from kinase reaction mixtures or from extracts of $^{32}P_i$-labeled infected cells by using 10 μl of antipeptide 204 antibody (22). Immunoprecipitates were processed and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (17).

Partial proteolysis of phosphorylated Ad12 T antigens with V8 protease. Partial digestion of phosphorylated T antigens was performed as described by Cleveland et al. (5). Immunoprecipitated bands of phosphorylated T antigens were excised from a dried 10% SDS–polyacrylamide gel and placed in the sample wells of a 12% gel. A 20-μl volume of buffer C (10 mM Tris hydrochloride [pH 6.8], 10% glycerol, 5% 2-mercaptoethanol, 0.1% SDS, 0.05% bromphenol blue) was added to each well, and the gel slices were allowed to swell for 15 min. V8 protease (type XVII, *S. aureus* V8; Sigma) in 10 mM Tris hydrochloride (pH 6.8)–10% glycerol was added, and the wells were filled with buffer C. Electrophoresis was performed at 15 mA per gel until the dye front ran 9.5 cm. The gel was dried, and autoradiography was performed.

Phosphopeptide maps of phosphorylated Ad12 T antigens. Immunoprecipitated bands of phosphorylated T antigens were excised from dried SDS-polyacrylamide gels and digested with trypsin or chymotrypsin (17). Peptides were separated by two-dimensional electrophoresis-chromatography (17); butyric acid-pyridine-acetic acid-butanol-water (65:5:3:2:29) was used for the second dimension (26).

Phosphoamino acid analysis of phosphorylated Ad12 T antigens. Phosphoamino acid analysis was performed by the procedure of Collett et al. (8; Hans-Peter Biemann and R. Erikson, personal communication). Immunoprecipitated bands of phosphorylated T antigens were excised from dried SDS-polyacrylamide gels and washed for 1 h with 5% methanol–7.5% acetic acid and for 1 h with water. Gel bands were crushed in Reacti-Vials (Pierce Chemical Co., Rockford, Ill.) with a glass rod, immersed in 1 ml of 6 N HCl, flushed with nitrogen, and incubated at 110°C for 2.5 h. Vials were centrifuged at 1,100 rpm for 3 min in a Beckman J-6B centrifuge, and supernatants were transferred to siliconized glass test tubes. HCl was removed by lyophilization through a NaOH trap, and the residues were dissolved in 0.5 ml of water, lyophilized again, and dissolved in 10 to 30 μl of water. Samples were spotted on Whatman no. 1 paper together with 25 μg each of marker phosphoserine, phosphothreonine, and phosphotyrosine (Sigma) and electrophoresed at pH 3.5 (pyridine-acetic acid-water; 1:10:189) at 2,000 V for 2.5 h; the progress of the run was monitored with a tracking dye (1% xylene cyanol FF, 2% orange G, 1% acid fuchsin). The paper was air dried and sprayed with 0.8 g of ninhydrin in 80 ml of acetone plus 10 ml of a cadmium chloride solution (6.4 g of $CdCl_2$, 320 ml of water, 168 ml of acetone). ^{32}P-labeled phosphoamino acids were detected by autoradiography.

RESULTS

Phosphorylated state of Ad12 E1A T antigens synthesized by pAd416- and pAd418-transformed *E. coli* cells. *E. coli* PR13^q cells transformed by pAd416 and pAd418, which express the Ad12 E1A 235R and 266R T antigens, respectively, were induced by treatment with isopropyl β-D-thiogalactopyranoside and metabolically labeled with $^{32}P_i$ or [^{35}S]methionine. For comparison, Ad12-infected human KB cells were incubated with $^{32}P_i$ to label the E1A T antigens. The Ad12 E1A T antigens were immunoprecipitated from the labeled cell extracts with antipeptide 204 antibody, which is targeted to the common C terminus of the Ad12 E1A T antigens (22). Two phosphorylated species of apparent M_rs of 45,000 and 47,000 were detected in $^{32}P_i$-labeled Ad12-infected KB cells (Fig. 1B). These represent the two major forms of the Ad12 E1A T antigens found in productively infected cells after long labeling periods (22). The Ad12 E1A 235R (Fig. 1A, lane 2) and 266R (Fig. 1A, lane 3) T antigens produced in *E. coli* incorporate [^{35}S]methionine and were detected by immunoprecipitation (21, 22). However, neither T antigen was phosphorylated in *E. coli*, as shown by the absence of immunoprecipitable ^{32}P-labeled proteins (Fig. 1A, lanes 4, 5, and 6). *E. coli* proteins in pAd416- and pAd418-transformed cells were extensively phosphorylated under our conditions (data not shown). These results indicate that posttranslational phosphorylation of Ad12 E1A T antigens occurs in mammalian cells but not in *E. coli* cells.

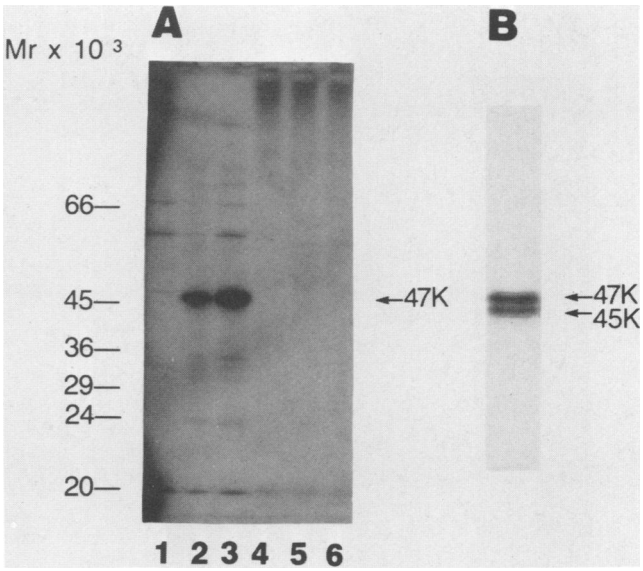

FIG. 1. In vivo phosphorylation of Ad12 E1A T antigens. Extracts of [^{35}S]methionine- or $^{32}P_i$-labeled pAd416 or pAd418 transformed *E. coli* and Ad12 early araC-infected KB cells were immunoprecipitated with antipeptide 204 antibody, electrophoresed on 10% SDS–polyacrylamide gels, and autoradiographed as described under Materials and Methods. (A) Cultures of *E. coli* PR13^q transformed with pAd416 or pAd418 were labeled with [^{35}S]methionine or $^{32}P_i$ as described in the text. Lane 1, [^{35}S]methionine-labeled *E. coli* (nontransformed); lane 2, [^{35}S]methionine-labeled *E. coli* transformed by pAd416; lane 3, [^{35}S]methionine-labeled *E. coli* transformed by pAd418; lane 4, $^{32}P_i$-labeled *E. coli* (nontransformed); lane 5, $^{32}P_i$-labeled *E. coli* transformed by pAd416; lane 6, $^{32}P_i$-labeled *E. coli* transformed by pAd418. (B) Ad12 early infected KB cells labeled with $^{32}P_i$ as described in the text.

In vitro phosphorylation of Ad12 E1A T antigens produced in *E. coli*. Our purpose in overproducing Ad12 E1A T antigens in *E. coli* is to purify sufficient quantities for in vitro studies. Since phosphorylation of proteins can play a role in biological function, we attempted to phosphorylate the *E. coli*-produced T antigens with a mammalian source of kinase

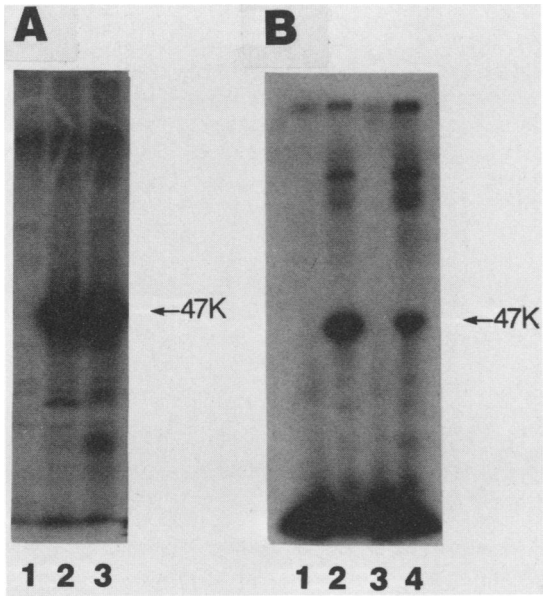

FIG. 2. In vitro phosphorylation of *E. coli*-produced Ad12 E1A T antigens. Crude kinase reaction mixtures containing [γ-^{32}P]ATP-labeled, *E. coli*-produced T antigen were immunoprecipitated and analyzed by SDS-gel electrophoresis. (A) Purified T antigen from *E. coli* (2.8 μg of Ad12 E1A 235R T antigen or 5 μg of Ad12 E1A 266R T antigen) was incubated with 50 μl of a sonicated KB cell extract (225 μg of protein) in HEPES buffer (pH 7.7) containing 18 μM ATP, 18 mM $MgCl_2$, and 50 μCi of [γ-^{32}P]ATP for 30 min at 37°C. Lane 1, No T antigen; lane 2, 235R T antigen; lane 3, 266R T antigen. (B) Purified T antigen from *E. coli* was incubated as described in panel A with 25 μCi of [γ-^{32}P]ATP and 25 μl of a dialyzed, sonicated KB cell extract plus 29 μl of a deproteinized KB cell extract. The deproteinized extract was prepared by adding 5 μl of cold 30% perchloric acid to 200 μl of a KB cell extract, neutralizing with KOH, and removing the $KClO_4$ precipitate by centrifugation. The extract was adjusted to 10 mM HEPES (pH 7.7), 10 mM NaCl, and 10% glycerol. Incubation was for 5 min at 37°C. Lane 1, 235R T antigen without dialyzed KB cell extract; lane 2, 235R T antigen plus dialyzed KB extract; lane 3, 266R T antigen without dialyzed KB extract; lane 4, 266R T antigen plus dialyzed KB extract.

activity. Purified *E. coli*-produced 235R and 266R T antigens were incubated with a crude KB cell extract in the presence of [γ-^{32}P]ATP and $MgCl_2$, and the reaction mixture was immunoprecipitated with antipeptide 204 antibody. Each T antigen was extensively phosphorylated in vitro, as shown by the immunoprecipitation of a ^{32}P-labeled 47,000-molecular-weight species (47K species) (Fig. 2A, lanes 2 and 3). No phosphorylated species were detected in the absence of added T antigen (Fig. 2A, lane 1). Immunoprecipitation of the ^{32}P-labeled 47K species was blocked when antipeptide 204 antibody was preincubated with peptide 204, providing evidence that the phosphorylated species was indeed the Ad12 E1A T antigen (data not shown).

In vitro phosphorylation could be due to kinase activity present in the cell extract or possibly to autophosphorylating activity of the E1A T-antigen molecule. To distinguish between these alternatives, purified T antigen was incubated with [γ-^{32}P]ATP and $MgCl_2$ in the absence and presence of a dialyzed KB cell extract; a deproteinized KB cell extract was added to provide a source of possible factors other than ATP and Mg^{2+} that may be required for autophosphorylating activity. There was no detectable phosphorylation of T antigen in the absence of the cell extract (Fig. 2B, lanes 1 and 3). Additon of the cell extract resulted in the phosphorylation of both the 235R and 266R T antigens (Fig. 2B, lanes 2 and 4, respectively). Therefore, neither T antigen, as purified from *E. coli*, possesses autophosphorylating protein kinase activity.

Purification of the kinase activity from KB cells that phosphorylates purified *E. coli*-produced Ad12 E1A T antigens. To study further the kinase activity that phosphorylates the E1A T antigens, to characterize the products of in vitro phosphorylation, and to develop procedures for the preparative phosphorylation of T antigen, we partially purified the kinase from KB cells. Kinase activity was assayed by measuring the incorporation of ^{32}P (in counts per minute) from [γ-^{32}P]ATP into purified 235R or 266R T antigen followed by immunoprecipitation of radioactive T antigens with antipeptide 204 antibody. When KB cells were fractionated into nucleus and cytoplasm, about 70% of the kinase activity was found in the cytoplasmic fraction. The dialyzed KB cytoplasmic fraction was subjected to column chromatography on DEAE-cellulose. T-antigen kinase activity eluted between 150 and 250 mM KCl buffer (Fig. 3A). Fractions containing kinase activity were pooled and purified further by column chromatography on phosphocellulose. T-antigen kinase activity eluted at a KCl concentration greater than 500 mM (Fig. 3B). For large-scale purification, the kinase-containing fractions from DEAE-cellulose were loaded onto phosphocellulose and washed stepwise with 20 mM KCl and 500 mM KCl buffer. Kinase was eluted with 1.0 M KCl buffer, dialyzed, concentrated, and used for further study. Results of a typical purification are shown in Table 1. About a 300-fold purification of kinase was obtained.

Reaction conditions for the in vitro phosphorylation of Ad12 E1A T antigens by the purified KB cell kinase. The kinase reaction was linear for at least 2 h at 37°C (Fig. 4A). Reaction velocity was directly proportional to T-antigen concentration up to at least 200 ng of kinase protein per 60 μl of reaction mixture (Fig. 4B). Velocity was linear with protein concentration up to about 500 ng of protein per 60 μl of reaction mixture and reached a plateau at about 1 μg (Fig. 4C). The optimum pH was 8.5 (Fig. 4D); the reaction velocity was similar between pH 8.5 and 10 (data not shown). An absolute requirement was found for divalent cation and ATP. At 10 to 20 mM Mg^{2+}, maximum velocity was observed with 20 to 40 mM ATP (Fig. 4E). The optimal Mg^{2+} concentration was about 20 mM (Fig. 4F). Mn^{2+} gave 1/10th the reaction velocity of Mg^{2+} at the optimal Mn^{2+} level of 1 mM. The reaction velocity remained constant at KCl levels from 0 to 100 mM. The addition of Ca^{2+} (10 mM) and cyclic AMP (10 μM) did not stimulate the reaction.

Phosphoamino acid analysis of the 235R and 266R T antigens phosphorylated in vivo and in vitro. To determine which amino acids were phosphorylated, immunoprecipitated bands of ^{32}P-labeled T antigen were eluted with 6 N HCl and hydrolyzed to free amino acids. Phosphoserine, phosphothreonine, and phosphotyrosine were resolved by high-voltage paper electrophoresis at pH 3.5, and the presence of ^{32}P was detected by autoradiography. Only serine was phosphorylated in the in vivo-^{32}P-labeled 45K and 47K T antigens (Fig. 5, lanes A and B, respectively) and in the in vitro-^{32}P-labeled 235R and 266R T antigens (Fig. 5, lanes C and D, respectively). Identical results were obtained when T antigen was phosphorylated by preparations of purified or crude kinase.

Analysis of in vitro and in vivo ^{32}P-labeled E1A T antigens

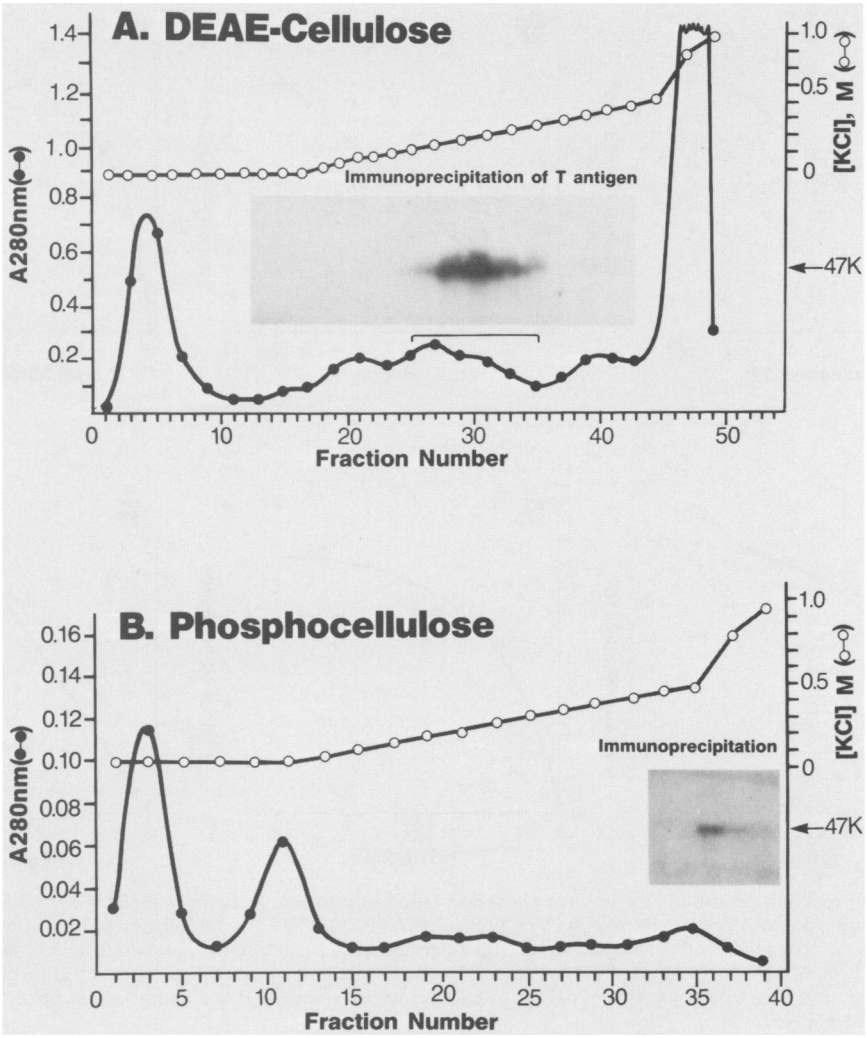

FIG. 3. Partial purification of a kinase from KB cytoplasm which phosphorylates purified *E. coli*-produced Ad12 E1A T antigens. (A) Dialyzed cytoplasm from KB cells was loaded on a DEAE-cellulose column and eluted as described in Materials and Methods. The column fractions were assayed for kinase activity by using purified 266R T antigen as substrate. The figure insert shows the kinase assay for fractions 13 to 43; fractions 25 to 35 contain kinase activity, as revealed by immunoprecipitation of ^{32}P-labeled T antigen. (B) Pooled fractions (brackets) from the DEAE-cellulose column in panel A containing kinase activity were purified by chromatography on phosphocellulose, and fractions were assayed for kinase activity. The insert shows kinase activity mainly in fractions 35 to 38.

by partial V8 proteolysis. To determine whether the same serine sites in T antigen are phosphorylated in vitro as in vivo, we performed partial proteolysis experiments. Immunoprecipitates were prepared by using antipeptide 204 antibody with in vitro-phosphorylated 235R and 266R T antigens and with lysates of $^{32}P_i$-labeled Ad12 early araC-infected cells. Bands of phosphorylated T antigen obtained by electrophoresis on 10% polyacrylamide gels were identified by autoradiography, excised, and digested with V8 protease (5). V8 protease digestion patterns were relatively simple and consisted of a major polypeptide at 22,000, material at 14,000 near the bottom of the gel, and minor species between undigested 45K-to-47K T antigen and the 22K polypeptide (Fig. 6). The material running at 14,000 was

TABLE 1. Purification of the kinase from KB cell cytoplasm[a]

Step	Total protein (mg)	Sp act (pmol of P per μg of protein per min)	Purification (fold)	Total activity (pmol of P per min)	% Yield
1. Dialyzed cytoplasm	26.3	8.07×10^{-5}	0	2.12×10^{-3}	100
2. DEAE-cellulose	3.1	8.28×10^{-4}	10	2.57×10^{-3}	100
3. Phosphocellulose	0.03	2.57×10^{-2}	318	7.71×10^{-4}	36

[a] Assays were performed as described in Materials and Methods, using the 235R T antigen purified from *E. coli* as substrate.

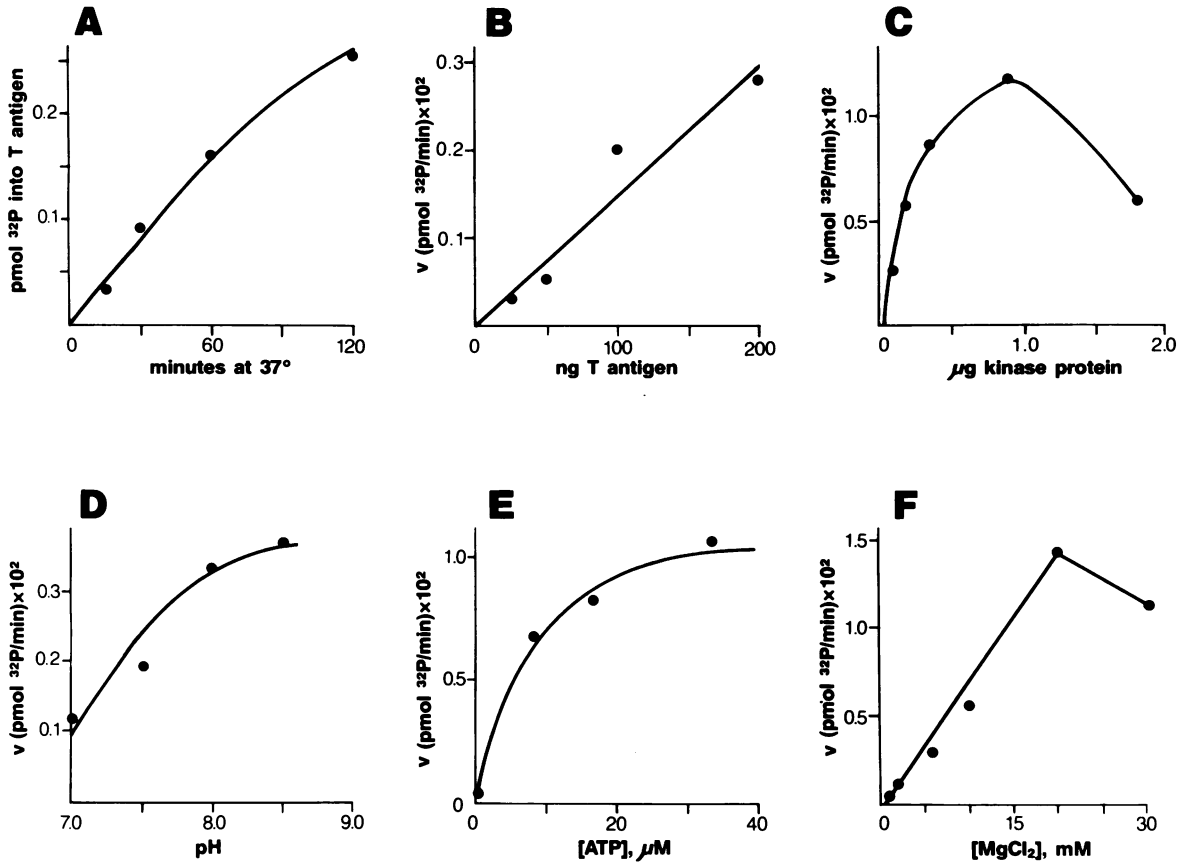

FIG. 4. Optimization of reaction conditions for in vitro phosphorylation of purified, *E. coli*-produced Ad12 E1A T antigens. The kinase reaction mixture, containing 8.3 μM ATP, 10 mM $MgCl_2$, 8 μCi of [γ-^{32}P]ATP, 20 mM KCl, 10% glycerol, 10 mM potassium phosphate (pH 7.0), 200 ng of purified *E. coli*-produced 266R T antigen, and 72 ng of purified kinase protein was incubated at 37°C for 30 min except as noted in the figure. (A) Incorporation of ^{32}P into T antigen and time of incubation. (B) Reaction velocity and concentration of T antigen. (C) Reaction velocity and level of kinase. (D) Reaction velocity and pH. (E) Reaction velocity and ATP concentration (at pH 8.5). (F) Reaction velocity and Mg^{2+} concentration (at pH 8.5).

resolved into several small peptides when the proteolysis digest was electrophoresed on a 20% polyacrylamide gel (data not shown). The major 22K polypeptide was resistant to further digestion by V8 protease (data not shown). No appreciable differences in proteolysis pattern were observed between in vivo-phosphorylated 45K and 47K and in vitro-labeled 235R and 266R T antigens (Fig. 6). Partial proteolysis experiments of T antigens phosphorylated by purified and by crude kinase preparations gave identical results.

Two-dimensional tryptic and chymotryptic phosphopeptide maps of Ad12 E1A T antigens phosphorylated in vivo and in vitro. As shown above, within the limits of the partial proteolysis data which compared only several polypeptide

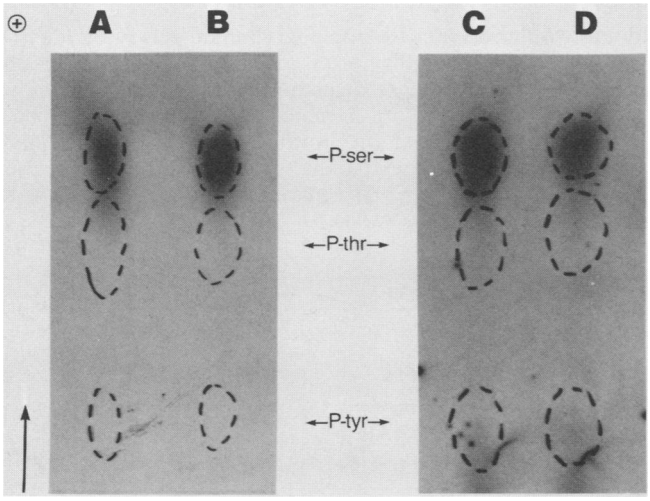

FIG. 5. Phosphoamino acid analysis of phosphorylated Ad12 E1A T antigens. ^{32}P-labeled T-antigen preparations, phosphorylated in vivo or in vitro, were immunoprecipitated with antipeptide 204 antibody and electrophoresed on an SDS-polyacrylamide gel as described in Materials and Methods. Bands containing T antigen were hydrolyzed to free amino acids, and marker phosphoserine (P-ser), phosphothreonine (P-thr), and phosphotyrosine (P-tyr) were added. Phosphorylated amino acids were resolved by paper electrophoresis and visualized by ninhydrin staining (dotted circles). ^{32}P-labeled phosphoserine was detected by autoradiography. Panels A and B contain Ad12 E1A 45K and 47K T antigen, respectively, immunoprecipitated from a $^{32}P_i$-labeled Ad12 early infected whole-cell extract. Panels C and D contain purified *E. coli*-produced 235R and 266R T antigen, respectively, phosphorylated in vitro by a KB cytoplasmic extract.

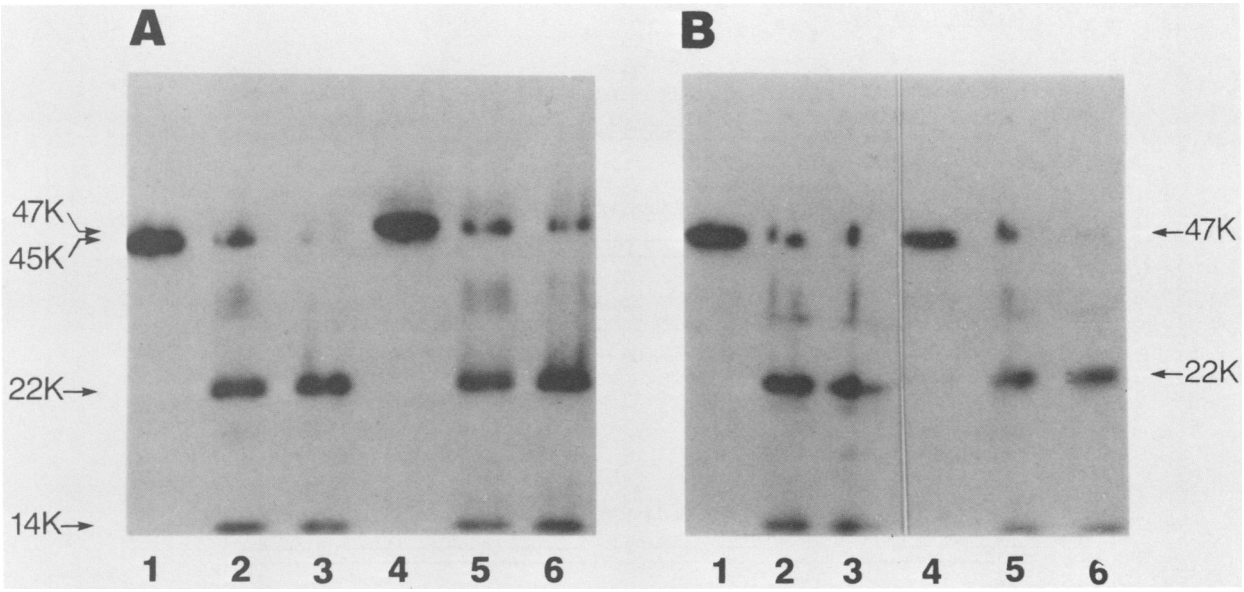

FIG. 6. Partial proteolysis of phosphorylated Ad12 E1A T antigens by V8 protease. (A) Phosphorylated Ad12 E1A T antigens immunoprecipitated from a $^{32}P_i$-labeled Ad12 early infected whole-cell extract were electrophoresed on a 12% SDS–polyacrylamide gel in the presence of V8 protease as described in Materials and Methods. Lanes 1, 2, and 3 contain the 45K T antigen plus 0, 10, and 40 ng of protease, respectively; lanes 4, 5, and 6 contain the 47K T antigen plus 0, 10, and 40 ng of protease, respectively. (B) Purified Ad12 E1A T antigens produced in *E. coli* were phosphorylated with [γ-^{32}P]ATP by a KB cytoplasmic extract, immunoprecipitated, and electrophoresed on a 12% SDS–polyacrylamide gel. Lanes 1, 2, and 3 contain the 235R T antigen with 0, 10, and 40 ng of protease, respectively; lanes 4, 5, and 6 contain the 266R T antigen with 0, 10, and 40 ng of protease, respectively.

products, the sites of T antigens phosphorylated in vitro and in vivo appeared very similar. To further study the structural relationship between in vitro- and in vivo-phosphorylated T antigens by a procedure that provided greater resolution of individual peptides, we analyzed two-dimensional phosphopeptide maps of tryptic digests. Trypsin cleaves at the C-terminal side of the basic amino acids lysine and arginine. Based on the amino acid sequence deduced from the cDNA sequence (24), all 3 lysine residues and 10 of the 11 arginine residues are located in the C-terminal half of the Ad12 235R T-antigen molecule. The 235R T antigen contains six tryptic peptides with serine residues which are potential sites of phosphorylation (Fig. 7A). These are located between amino acid residues 3 and 142, 145 and 177, 178 and 182, 183 and 193, 205 and 219, and 220 and 230. Comparison of the tryptic phosphopeptide map of the in vivo-phosphorylated 45K species (Fig. 8A, map A) with that of the in vitro-phosphorylated *E. coli*-produced 235R T antigen (Fig. 8A, map B) reveals a similar pattern of about six phosphopeptides. The tryptic phosphopeptide map of the 47K species (Fig. 8A, map C) is virtually identical to that of the 45K species (Fig. 8A, map A). This can be explained as follows. With the 6- to 8-h $^{32}P_i$ labeling time used, the 47K species represents mainly the intact 12S mRNA gene product (235R T antigen), whereas the 45K species is the 235R T antigen modified at the N terminus; the 266R T antigen has a high turnover and is a minor species of lower mobility under these labeling conditions (L. A. Lucher, J. S. Symington, K. H. Brackmann, and M. Green, manuscript in preparation).

The 266R T antigen contains eight tryptic peptides with serine residues which are potential sites of phosphorylation (Fig. 7B). These are located between amino acid residues 3 and 142, 161 and 166, 167 and 182, 183 and 208, 209 and 213, 214 and 224, 236 and 250, and 251 and 261. Five tryptic phosphopeptides were detected in the in vitro-phosphorylated 266R T antigen (Fig. 8A, map D). Four of these (spots 1 through 4) appear very similar to phosphopeptide spots 1, 2, 5, and 6 found in the 235R T antigen phosphorylated in vitro and in vivo (Fig. 8A, maps A, B, and C). Spot 5 appears to be different (Fig. 8A, map D). These observations are consistent with the fact that five tryptic peptides containing serine residues are shared between the Ad12 E1A 235R and 266R T-antigen molecules (Fig. 7A and B).

Because of the unique distribution of basic amino acid residues susceptible to cleavage with trypsin, as discussed above, all but one of the tryptic peptides in the Ad12 E1A T antigens are located in the C-terminal half of the molecule (Fig. 7A and B). To characterize the sites of phosphorylation in the N-terminal half of the T-antigen molecule, we performed chymotryptic phosphopeptide mapping. Chymotrypsin cleaves at the C-terminal end of the amino acids tyrosine, phenylalanine, and tryptophan. Tyrosine and phenylalanine are distributed throughout the T-antigen molecule with about a twofold-higher frequency in the N-terminal half (Fig. 7A and B). The 235R T antigen contains eight chymotryptic peptides with potential phosphoserine residues, all located within the N-terminal 147 amino acids, between amino acid residues 1 and 12, 28 and 37, 38 and 42, 46 and 64, 66 and 78, 79 and 110, 115 and 147, and 148 and 235 (Fig. 7A). The two-dimensional chymotryptic phosphopeptide maps of the in vitro- and in vivo-phosphorylated Ad12 235R E1A T antigens are shown in Fig. 8B, maps A, B, and C. They reveal similar patterns of about eight major phosphopeptides.

Several spots in these phosphopeptide maps are of different intensity. This could be caused by a variety of factors, including: (i) difference in the number of serine residues in certain chymotryptic peptides (Fig. 7A and B) (for example, the number of serine residues varies from one to seven in different chymotryptic peptides); (ii) incomplete digestion

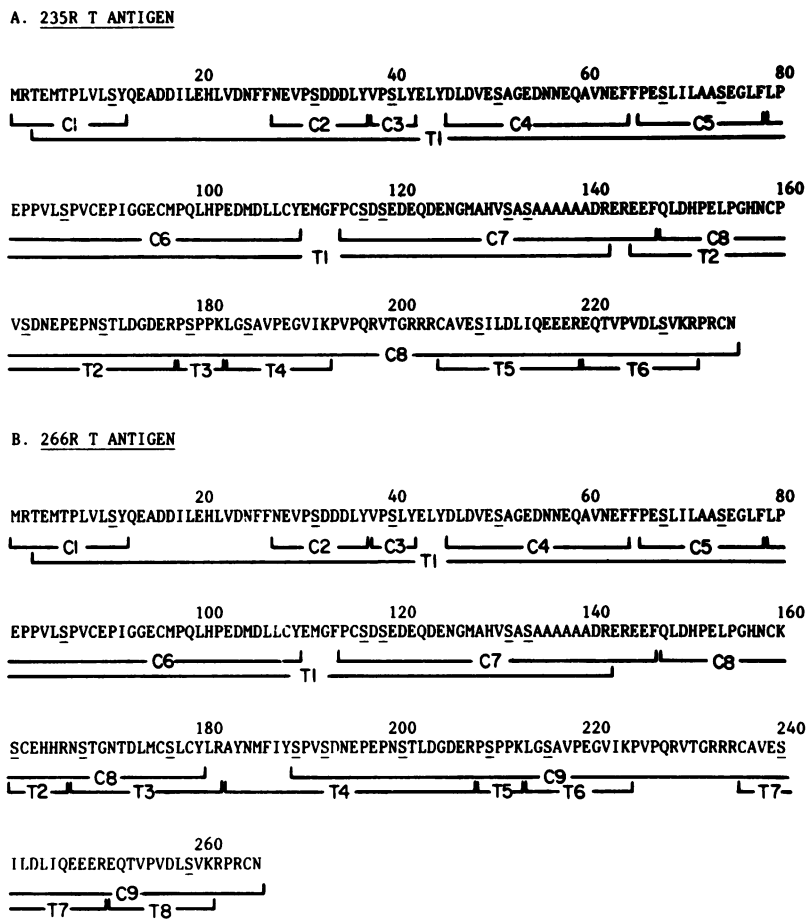

FIG. 7. Potential tryptic and chymotryptic phosphopeptides containing serine in the Ad12 E1A 235R (A) and 266R (B) T antigens. The location of tryptic (T) and chymotryptic (C) peptides with serine residues that could be phosphorylated are indicated in brackets and numbered in sequence from the N terminus. Serine residues (S) are underlined. Amino acids are represented by the single letter code.

(digestion intermediates) or incomplete recovery of certain peptides (see reference 26); (iii) further phosphate metabolism of the phosphorylated T antigen in vivo, i.e., phosphorylation or dephosphorylation, which could lead to overrepresentation or underrepresentation of certain phosphopeptides; and (iv) heterogeneity of phosphorylation of T-antigen molecules in vitro due to topological modifications during isolation of some T-antigen molecules from *E. coli*. We have numbered only those spots that appear to be reproducibly detected in all maps. (Although spot 8 in map A of Fig. 8B is of low intensity, other in vivo chymotryptic preparations show spot 8 in higher intensity, e.g., maps A and C in Fig. 9B.)

There are nine chymotryptic peptides in the 266R T antigen with potential phosphoserine residues; these are located between amino acid residues 1 and 12, 28 and 37, 38 and 42, 46 and 64, 66 and 78, 79 and 110, 115 and 147, 148 and 180, and 190 and 266 (Fig. 7B). The phosphopeptide map of the in vitro-labeled 266R T antigen (Fig. 8B, map D) is similar to that of the in vitro- and in vivo-labeled 235R T antigen (Fig. 8B, maps A, B, and C). This is not surprising, since seven of the nine chymotryptic peptides with serine residues in the 266R T antigen are shared with the 235R T antigen.

Phosphorylation patterns of cytoplasmic and nuclear forms of Ad12 E1A 47K and 45K T antigens. The SV40 large T antigen appears to be differentially phosphorylated in the cytoplasm and nucleus of SV40-infected cells (27). To determine whether different subclasses of phosphorylated Ad12 E1A T antigen are present in the nucleus and cytoplasm, Ad12-infected KB cells were metabolically labeled with $^{32}P_i$ for 6 h at 24 h postinfection in the presence of araC. The 45K and 47K T antigens were isolated from the nuclear and cytoplasmic fractions by immunoprecipitation and gel electrophoresis, digested with trypsin or chymotrypsin, and peptide mapped. Virtually identical patterns of tryptic phosphopeptides were obtained with the 45K T antigen from the cytoplasm (Fig. 9A, map A) and nucleus (Fig. 9A, map B) and with the 47K T antigen from the cytoplasm (Fig. 9A, map C) and nucleus (Fig. 9A, map D). These data suggest that the T antigens are phosphorylated at the same sites in the C-terminal half of the molecule. In addition, very similar patterns of chymotryptic phosphopeptides were found with 45K T antigen from the cytoplasm (Fig. 9B, map A) and nucleus (Fig. 9B, map B), and with 47K T antigen from the cytoplasm (Fig. 9B, map C) and nucleus (Fig. 9B, map D). Thus, the distribution of phosphorylated serine residues in the N-terminal half of the T-antigen molecule also appears to be the same in the nucleus and the cytoplasm.

DISCUSSION

As we show here, the Ad12 E1A 235R and 266R T antigens produced in *E. coli* are not phosphorylated in *E. coli*, although they are extensively phosphorylated in Ad12-

infected and -transformed mammalian cells. In addition, we find that the *E. coli*-produced E1A T antigens do not possess autophosphorylating protein kinase activity, such as that reported for several RNA tumor virus transforming proteins (11, 30, 33). Since phosphorylation at specific sites may be essential for biological function, we have studied the phosphorylation of the *E. coli*-produced Ad12 E1A T antigens in vitro by using a kinase isolated from KB cells. The kinase was purified about 300-fold and appears to be a cyclic AMP-independent, Ca^{2+}-independent protein kinase requiring only Mg^{2+} and ATP for activity. Analysis of two-dimensional tryptic and chymotryptic peptide maps indicates that mainly the same amino acid sites are phosphorylated in vitro and in vivo. Serine but not threonine or tyrosine residues present in the T-antigen molecules are phosphorylated in Ad12-infected cells and in the in vitro phosphorylation reaction mixture. There are 17 and 21 serine residues in the 235R and 266R T antigen molecules, respectively. As many as six tryptic phosphopeptides and eight chymotryptic peptides that contain phosphoserine residues were detected, suggesting that the number of phosphorylated sites is large. However, the data do not establish that individual T-antigen molecules are phosphorylated at the same sites, since phosphorylation could be heterogeneous; i.e., different serine residues could be phosphorylated in different T-antigen molecules.

We did not detect different subclasses of phosphorylated Ad12 E1A T antigens in the nucleus and cytoplasm. Thus, the phosphorylation program of the E1A T antigens may differ from that of the SV40 large T antigen in which additional sites are phosphorylated in the cell nucleus (27). However, in our experiments, long $^{32}P_i$ labeling periods (6 to 8 h) were used. Under these conditions, it is possible that newly phosphorylated T antigen is further phosphorylated and that cytoplasmic T antigen represents mainly further phosphorylated species normally found in the cytoplasm or derived by leakage from the nucleus. Further experiments with pulse-labeling protocols are needed to evaluate these possibilities.

We have detected the phosphorylation of solely serine residues in the E1A 235R and 266R T antigens, although it is possible that the phosphorylation of a single threonine or tyrosine residue would be masked by a large background of phosphorylated serine. Phosphorylation of serine is particularly interesting in view of the modification of biological activity established in several cases by phosphorylation with serine kinases (6). The exclusive phosphorylation of serine appears unusual by comparison with that of other virus-coded transforming proteins thus far analyzed. For example, several retrovirus transforming proteins are tyrosine-specific protein kinases that are autophosphorylated at one or more tyrosine residues and further phosphorylated at specific serine residues, presumably by host cell protein kinases (3, 7, 8). The SV40 T antigen is phosphorylated at multiple serine and threonine residues (26, 32). The polyomavirus middle T antigen is phosphorylated at multiple serine and threonine residues (25, 29) as well as at tyrosine residues (29).

In summary, within the limits of our analysis, it appears that the purified kinase phosphorylates the Ad12 E1A T

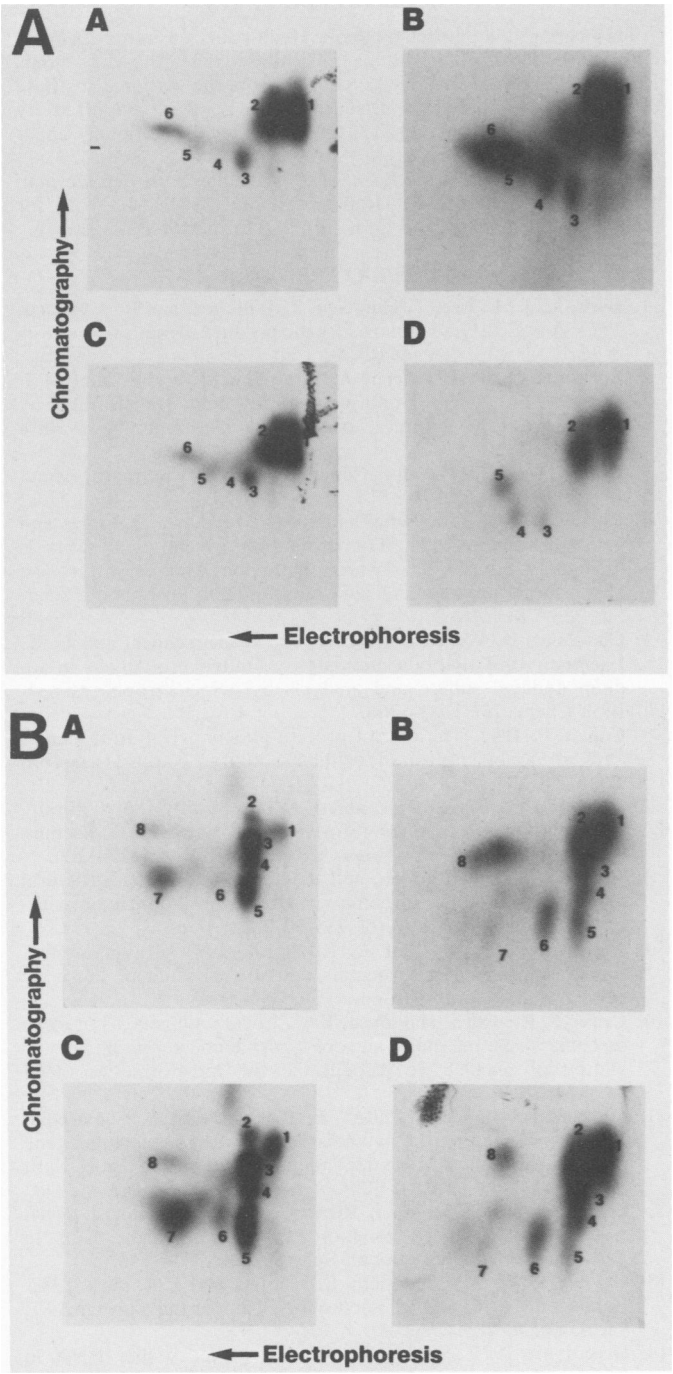

FIG. 8. Two-dimensional phosphopeptide maps of tryptic and chymotryptic digests of phosphorylated Ad12 E1A T antigens. Proteolytic digestion and peptide mapping were performed as described in Materials and Methods. (A) Tryptic digests. Map A, 45K T antigen from $^{32}P_i$-labeled Ad12 early infected cytoplasm; map B, purified *E. coli*-produced 235R T antigen phosphorylated in vitro with [γ-^{32}P]ATP by purified kinase; map C, 47K T antigen from $^{32}P_i$-labeled Ad12 early infected cytoplasm; map D, purified *E. coli*-produced 266R T antigen phosphorylated in vitro with [γ-^{32}P]ATP by purified kinase. (B) Chymotryptic digests. Map A, 45K T antigen from $^{32}P_i$-labeled Ad12 early infected cytoplasm; map B, purified *E. coli*-produced 235R T antigen phosphorylated in vitro with [γ-^{32}P]ATP by purified kinase; map C, 47K T antigen from $^{32}P_i$-labeled Ad12 early infected cytoplasm; map D, purified *E. coli*-produced 266R T antigen phosphorylated in vitro with [γ-^{32}P]ATP by purified kinase.

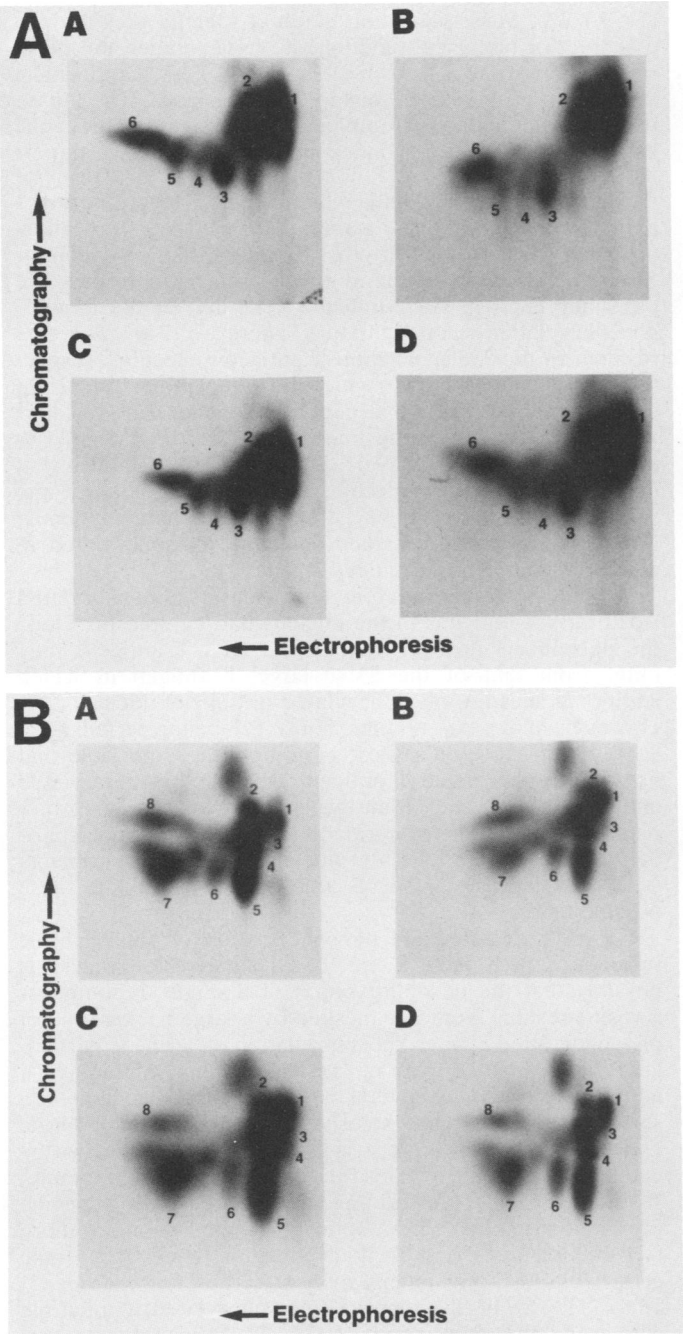

FIG. 9. Two-dimensional phosphopeptide maps of tryptic and chymotryptic digests of phosphorylated Ad12 E1A T antigens isolated from the nucleus and cytoplasm of infected cells. Proteolytic digestion and peptide mapping were performed as described in Materials and Methods. (A) Tryptic digests. Maps A and B, 45K T antigen from $^{32}P_i$-labeled Ad12 early infected cytoplasm and nucleus, respectively. Maps C and D, 47K T antigen from $^{32}P_i$-labeled Ad12 early infected cytoplasm and nucleus, respectively. (B) Chymotryptic digests. Maps A and B, 45K T antigen from $^{32}P_i$-labeled Ad12 early infected cytoplasm and nucleus, respectively. Maps C and D, 47K T antigen from $^{32}P_i$-labeled Ad12 early infected cytoplasm and nucleus, respectively.

antigens mainly at the same amino acid sites in vitro as are phosphorylated in vivo. Whether phosphorylation at specific sites is essential for the pleiotropic functions of the E1A T antigens is not known. Thus far, no in vitro biochemical test for T-antigen function has been established. The possibility of phosphorylating *E. coli*-produced T antigen in vitro encourages studies to define the biochemical nature of E1A T-antigen function.

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