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Genetic Mapping of a Major Site of Phosphorylation in Adenovirus Type 2 E1A Proteins

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Adenovirus early region 1A (E1A) encodes two acidic phosphoproteins which are required for transactivation of viral transcription, efficient viral DNA replication in phase G_0-arrested human cells, and oncogenic transformation of rodent cells. Biochemical analysis of in vivo ^{32}P-labeled adenovirus type 2 E1A proteins purified with monoclonal antibodies demonstrated that these proteins were phosphorylated at multiple serine residues. Two-dimensional phosphotryptic peptide maps of wild-type and mutant E1A proteins were used to locate a major site of E1A protein phosphorylation at serine-219 of the large E1A protein. Although this serine fell within a consensus sequence for phosphorylation by the cyclic AMP-dependent protein kinases, experiments with mutant CHO cells defective in these enzymes indicated that it was not. Oligonucleotide-directed mutagenesis was used to substitute an alanine for serine-219. This mutation prevented phosphorylation at this site. Nonetheless, the mutant was indistinguishable from the wild type for early gene transactivation, replication on G_0-arrested WI-38 cells, and transformation of cloned rat embryo fibroblast cells.

Proteins encoded in early region 1A (E1A) of the human adenoviruses serve important functions in both replication and transforming ability. During a productive infection, E1A proteins stimulate the normal transcription of early viral genes (2, 27, 40). Although the action of these proteins is not absolutely required, it is necessary for maximum transcriptional activation (40, 48), possibly by facilitating the formation of transcriptional complexes (16). Much evidence indicates that transcriptional *trans* activation is indirect, resulting from an interaction with cellular proteins (13, 28). Mutagenic analysis of early adenovirus promoters suggests that E1A protein expression results in an increase in the activity of cellular transcription factors (reviewed by Kingston et al. [29]). The results of biochemical analyses of infected-cell extracts are consistent with this model for *trans* activation of genes transcribed by RNA polymerase III (22, 59a). Complete transformation of rodent cells by adenovirus requires the concerted action of E1A proteins and those encoded by early region 1B (E1B). Expression of E1A proteins alone results in partially transformed cells with unrestricted growth potential (see reviews Branton et al. [4] and Bernards and van der Eb [3]). E1A proteins can also cooperate with *ras* oncogenes to transform primary embryo cells (45). The realization that E1A proteins are prime effectors in regulating the transcriptional state of a virus-infected cell and establishing the transformed phenotype have stimulated much interest in the biochemical activity of these proteins, but as yet no biochemical activity has been described which can account for their physiological effects.

Two overlapping differentially spliced mRNAs, 12S and 13S, are expressed from early region 1A of adenoviruses types 2 and 5 (Ad2 and Ad5) during the early phase of a productive infection and in transformed cells (for a review, see Pettersson et al. [43]). These mRNAs share identical amino- and carboxy-terminal sequences. They differ only by 46 internal amino acids unique to the larger protein as a result of the differential splicing event (42). Both E1A proteins are phosphorylated (17, 59). Protein phosphorylation is known to modulate the enzymatic and biological activities of several proteins. Classic examples are provided by the serine-specific protein kinases (for a review, see Cohen [8]). Consequently, we have been interested in determining whether this modification modulates any of the known biological effects of the E1A proteins. In this work we analyzed wild-type and mutant E1A proteins by two-dimensional phosphotryptic peptide analysis. We deduce that serine-219 (with reference to the sequence of the larger E1A protein) is a major site of phosphorylation of both E1A proteins. We constructed a mutant, pm1330, which substituted an alanine for the wild-type serine at this position and analyzed its phenotype.

MATERIALS AND METHODS

Cell culture, viruses, and virus infections. HeLa and KB cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with newborn calf serum (NCS; 10% for plates and 5% for spinner cultures). Cells (line 293 [20]) were maintained in DMEM plus 10% fetal bovine serum (FBS) for plates and 5% NCS for spinner cultures. Secondary human fibroblast cells (line WI38) were maintained in DMEM plus 10% FBS, and growth was arrested in 0.2% NCS as described previously (52). CREF cells were maintained in DMEM plus 10% FBS (15). Cells were infected with virus at a multiplicity of infection (MOI) of 10 or 50 PFU/cell as indicated in the text and figure legends and grown in 2% NCS until harvested. Plaque assays were done on monolayers of HeLa and 293 cells. Cells were maintained as described above and seeded for 2 days prior to use for plaque assays onto 60-mm dishes. Cells were infected and overlaid with agar as described previously (58).

Ad5 strain *dl*309 is wild type in region E1A and was used as the wild-type control in these studies. The E1A mutants *dl*310, *dl*311, *dl*312, and *dl*313 are derivatives of *dl*309 and have been described previously (26). Ad2/5pm975 and *dl*1500

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are E1A mutants which express only the large or small E1A protein, respectively (38, 39). Mutant viruses were grown and titrated on 293 cells.

Radioactive labeling. Virus and mock-infected cultures were incubated in medium containing cytosine arabinoside (araC) for 36 to 38 h postinfection (p.i.) as described previously (17). At this time cells were washed three times in 0.15 M NaCl and incubated with 7 ml of phosphate-free medium plus 2% dialyzed NCS containing 2 mCi of carrier-free $^{32}P_i$ (ICN) for 2 h at 37°C.

Cell extracts and immunoprecipitation. Cells were washed with cold 0.15 M NaCl, removed by scraping with a rubber policeman, and spun at 1,200 rpm for 7 min in an IEC DPR 6000 centrifuge. Cells were lysed with Nonidet P-40 and digested with micrococcal nuclease, DNase I, and RNase A as described previously (53). The cell extracts were spun in an Eppendorf microfuge for 15 min, and an amount of supernatant corresponding to 5×10^6 cells (100 μl) was diluted to 500 μl with RIPA buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 0.1% [vol/vol] sodium laurel sulfate [SDS], 0.1% sodium deoxycholate, 0.1% Triton X-100, 100,00 IU of trasylol per ml). E1A monoclonal antibody R7 (500 μg) (A. S. Tsukamoto, B. Ferguson, I. L. Weissman, and A. J. Berk, manuscript in preparation) coupled to the insoluble matrix Reactigel-6X (Pierce Chemicals) was added to this cell lysate and incubated with rocking at 4°C overnight. Reactigel-coupled monoclonal antibody was prepared by coupling 5 mg of purified monoclonal antibody to 1 ml of Reactigel by the suggested procedures of the manufacturer. The immunoprecipitates were spun in an Eppendorf microfuge for 2 min to pellet the resin. The resin was washed with 1× RIPA buffer made 1 M in NaCl, followed by a final wash with RIPA buffer. The pellet was resuspended in 150 μl of 1× gel sample buffer, boiled for 2 min, and analyzed by one-dimensional (1-D) SDS-polyacrylamide gel electrophoresis (31) followed by autoradiography.

2-D peptide maps, phosphoamino acid analysis, and phosphatase digestion. E1A proteins were located either on two-dimensional (2-D) gels of total infected-cell extracts or in SDS-polyacrylamide gels of immunoprecipitates by autoradiography and excised from the gel as described previously (30). The ^{32}P-labeled E1A proteins were eluted in 50 mM NH_4CO_3–0.1% SDS–5% β-mercaptoethanol for >14 h at 37°C. The eluted proteins were precipitated with 50 μg of carrier bovine serum albumin (BSA) by addition of 10% trichloroacetic acid (TCA) at 4°C for 30 min. The precipitate was spun for 15 min at 4°C in an Eppendorf microfuge, and the pellets were washed twice with −20°C acetone. For peptide analysis, samples were oxidized with performic acid on ice for 2 h as described previously (1), followed by total trypsin digestion with 30 μg of tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Worthington Diagnostics) for 12 to 16 h at room temperature. Then an additional 20 μg of TPCK-trypsin was added, and incubation was continued for 4 h. The samples were spotted in the center of a cellulose thin-layer chromatography (TLC) plate (10 by 10 cm) 1 cm from the bottom and electrophoresed in the first dimension in 1% ammonium carbonate [$(NH_4)_2CO_3$], pH 8.9, for 1 h at 400 V. Chromatography in the second dimension was performed in *n*-butanol–pyridine–glacial acetic acid–H_2O (15:10:3:12). The plates were dried and autoradiographed.

For phosphoamino acid analysis, acetone-washed E1A proteins were hydrolyzed in 100 μl of 6 M HCl for 2 h at 100°C. Samples were dried in vacuo, suspended in deionized distilled H_2O, and dried again. This was repeated twice to remove HCl. The samples were Cerenkov counted and suspended in a mixture of 1 μg each of phosphoserine, phosphothreonine, and phosphotyrosine and spotted at the bottom left corner of a cellulose TLC plate (20 by 20 cm). Electrophoresis in the first dimension was for 3 h at 400 V in acetic acid-formic acid-H_2O, pH 1.9 (78:25:897). Plates were dried and then electrophoresed in the second dimension in pyridine-acetic acid-H_2O, pH 3.5 (5:50:945) for 3 h at 400 V. Plates were dried, and unlabeled standards were visualized by ninhydrin staining before autoradiography.

Construction of the viral mutant pm1330. The E1A-containing bacteriophage M13 clone M13GG1 (39) was used as the single-stranded template for oligonucleotide-directed mutagenesis (60). The 20-mer oligonucleotide 5′-GCCCGACA*GCG*CCTGTGTCT-3′, corresponding to the Ad2 sequence from nucleotide (nt) 1322 to nt 1341 except with two base changes (italic letters), was synthesized by the procedure described previously (36). This oligonucleotide was used to prime the synthesis of mutant M13 phage as described previously (39), and the mutations in the resulting recombinant phage, M13GG1pm1330 (hereafter called pm1330), were confirmed by DNA sequencing (46). The plasmid BE5 contains Ad2 sequence from nt 1 to 3323 (18) inserted into the *Pst*I site of pBR322. The pm1330 point mutations were introduced into pBE5 by substitution of the *Sma*I-*Xba*I fragment (nt 1009 to 1336) with the equivalent fragment from pm1330 replicative form DNA. Procedures were those described by Maniatis et al. (34). Plasmid DNA containing the two point mutations (pBE5pm1330) was digested with *Pst*I and *Xba*I and ligated to the large *Xba*I fragment of *dl*309 (26, 27). The ligated DNA was transfected (56) into 293 cells (20) to introduce the pm1330 mutations into a complete viral genome (54). Individual plaques were picked and expanded on 293 cells. Adenovirus DNA was prepared as described by Hirt (21) except that 1 mg of pronase per ml was included in the initial lysis buffer and the plate was incubated at 37°C for 2 h before addition of NaCl to 1 M and incubation on ice. The Hirt DNA was digested with *Hph*I restriction endonuclease (Bio Labs) and resolved on 2% agarose gels. DNA was transferred as described by Southern (51), except to Biodyne nylon support. The Southern blots were probed overnight at 25°C with the end-labeled mutagenic oligonucleotide and autoradiographed, and the mutant pm1330 was identified by the presence of a new *Hph*I fragment of the expected size (482 base pairs) hybridizing strongly to the mutagenic oligonucleotide. Mutant pm1330 was plaque purified two more times on 293 cell monolayers and expended into a large stock on 293 cells. Infectivity was determined by plaque formation on 293 cell monolayers.

Preparation of early RNA and S1 nuclease mapping. Cytoplasmic RNA was harvested from 500-ml suspension cultures of HeLa cells at 6 h p.i. with *dl*309, pm1330, *dl*310, or *dl*312 at 20 PFU/cell as previously described (2). Cytoplasmic RNA (100 μg) was hybridized to ^{32}P-labeled probes specific for E1, E2, E3, or E4 and digested with S1 nuclease as described previously (41). The S1-protected fragments were denatured, fractionated by electrophoresis on 8 M urea–5% polyacrylamide gels for 2,400 V-h, dried, and autoradiographed with intensifying screens.

Transformation assays on CREF cells. Transformation assays were done essentially as described previously (15). Cloned rat embryo fibroblast (CREF) cells were seeded into 60-mm dishes at 3×10^5 cells per plate. Two days later, when cells were 80% confluent, one plate was trypsinized and counted, and the remaining plates were infected at an MOI of 10 with wild-type or mutant Ad2 or Ad5 virus. At 3 h p.i.

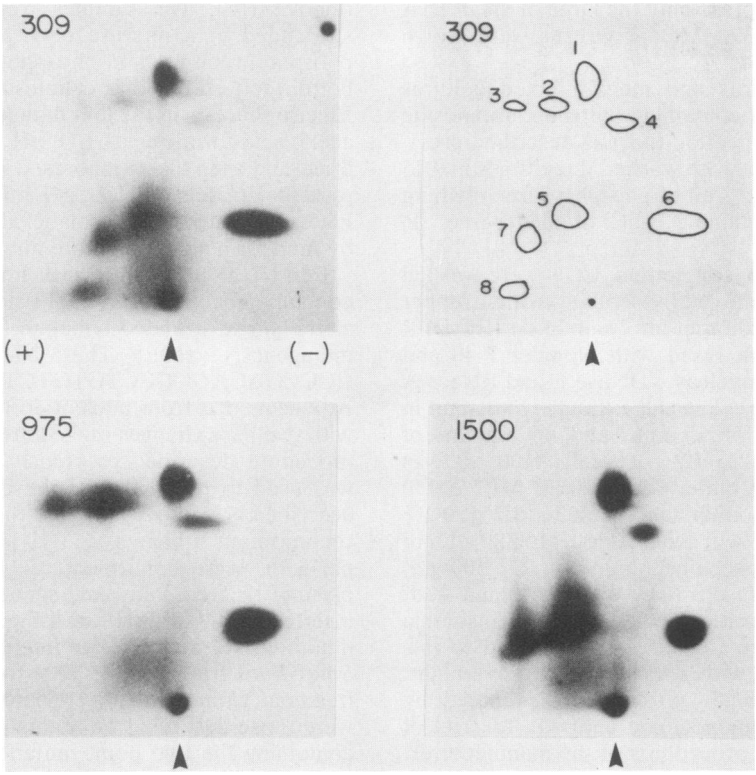

FIG. 1. 2-D phosphotryptic peptide maps of E1A proteins. HeLa cells were infected with *dl*309, pm975, or *dl*1500 at an MOI of 10 in the presence of araC. At 36 h p.i., the cells were pulse-labeled for 2 h with $^{32}P_i$, total cell extracts were prepared, and E1A proteins were immunoprecipitated and run on SDS-polyacrylamide gels as described in the text. E1A proteins from wild-type and mutant-virus-infected cells were eluted from gel slices, precipitated with TCA containing 50 μg of BSA as carrier protein, oxidized with performic acid, digested with TPCK-trypsin, concentrated by lyophilization, and analyzed on cellulose TLC plates (10 by 10 cm). Electrophoresis was carried out for 1 h at 400 V in 1% ammonium carbonate (pH 8.9), with the origin (arrows) at center baseline and the cathode to the right. Plates were dried and then chromatographed in an ascending buffer of *n*-butanol–pyridine–acetic acid–H_2O (15:10:3:12) from bottom to top. Plates were autoradiographed with intensifying screens to shorten exposure times. The upper right panel is a schematic drawing of the eight phosphotryptic fragments observed for wild-type E1A proteins.

the plates were split 1:5, and at about 72 h p.i. (before reaching confluence), the medium was changed to SMEM plus 2% FBS, 5% calf serum, and 0.1 mM $CaCl_2$. The medium was replaced every 3 to 4 days for 4 to 6 weeks until foci began to appear. Foci were counted at 5 and 6 weeks, at which time no new foci appeared. Individual transformed foci were picked with glass cloning cylinders, expanded, and assayed for growth in soft agar as described previously (38).

RESULTS

2-D phosphotryptic peptide analysis of isolated E1A proteins. To determine whether there was a major site(s) of E1A protein phosphorylation, we performed 2-D TLC of ^{32}P-labeled peptides generated by exhaustive trypsin digestion of isolated E1A proteins. HeLa cells were infected with *dl*309 (which has a wild-type E1A sequence [26]), the E1A deletion mutant *dl*312 (27), or mock infected, treated with araC to increase E1A protein concentration (17), and labeled with $^{32}P_i$ from 36 to 38 h p.i. Cell extracts were prepared under conditions which solubilize >90% of the E1A proteins (53). Immunoprecipitates of these extracts were made with an E1A monoclonal antibody (A. Tsukamoto, B. Ferguson, I. L. Weissman and A. J. Berk, unpublished results) covalently coupled to an insoluble matrix. The immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis and autoradiography. Bands corresponding to the E1A proteins (apparent molecular weight 45,000 to 52,000 [45K to 52K] were observed in *dl*309- but not in mock- or *dl*312-infected cells (data not shown). These bands were excised from the gel along with the corresponding region from the *dl*312-infected cell sample, eluted, exhaustively digested with trypsin, and analyzed by 2-D TLC (Fig. 1). Seven (designated peptides 1 through 7) of the eight phosphorylated tryptic fragments observed in this experiment (schematically depicted in Fig. 1) were reproducibly observed, although their relative intensities varied between experiments. No phosphorylated species were observed in the sample generated from *dl*312-infected cells, except for labeled material which remained associated with the origin (not shown). Although the relative intensities of the tryptic fragments varied from sample to sample, the predominant spot always corresponded to the peptide designated 6 in Fig. 1. The upper left panel of Fig. 5 shows a short exposure of a typical phosphotryptic peptide map of *dl*309 E1A proteins which emphasizes our conclusion that peptide 6 is the predominant phosphorylated peptide. Most of the phosphotryptic peptide maps shown throughout this paper were exposed for longer periods to demonstrate the other phosphopeptides more clearly and in some cases to show the absence of peptide 6. In these longer exposures the difference in intensity between peptide 6 and the other

phosphotryptic peptides was less obvious because the exposures were beyond the linear response range of the film.

From analysis of viral mutants *dl*1500 and pm975, which express only the 243- or 289-amino-acid protein, respectively (38, 39), distinct peptides could be specifically attributed to one or the other protein (Fig. 1). Peptides 2 and 3 were unique to the 289-amino-acid protein, whereas peptides 5 and 7 corresponded to the 243-amino-acid protein. Peptides 1, 4, and 6 were common to both proteins. Peptide 8 was not observed reproducibly and consequently could not be assigned to one or the other E1A protein. Peptide 6 was the predominant phosphorylated peptide in each protein. Significantly, this peptide was positively charged at pH 8.9, which is unusual for phosphorylated tryptic peptides. These analyses were performed on E1A proteins isolated at late times p.i. in the presence of araC. We have not done phosphotryptic peptide maps of E1A proteins isolated early p.i. However, the mobility of E1A proteins in 2-D gels is very similar whether they are isolated early (8 h) or late (38 h) p.i. (A. Tsukamoto and A. J. Berk, unpublished results). This result suggests that E1A protein phosphorylation is similar at early and late times.

The observation of two unique phosphotryptic peptides in the digest of the 243-amino-acid protein was somewhat surprising. Since the larger protein contained 46 more amino acids than the smaller protein, one would expect that the amino acids present in two tryptic peptides generated from each side of the inserted region in the larger protein would be contained in a single unique tryptic peptide generated by cleaving the smaller protein on either side of the insertion site. Consequently, if the same sites were phosphorylated in the sequences which are shared between the two closely related proteins, we would have expected the map of the smaller protein to differ from that of the larger protein by the loss of two tryptic peptides and the presence of one unique tryptic peptide. The observation of two unique phosphotryptic peptides in the 243-amino-acid protein could be the result of partial trypsin cleavage, phosphorylation at alternative sites in the unique tryptic peptide, or phosphorylation of the 243-amino-acid protein at a site which is not phosphorylated in the equivalent position of the large protein. Current results do not allow us to distinguish among these possibilities.

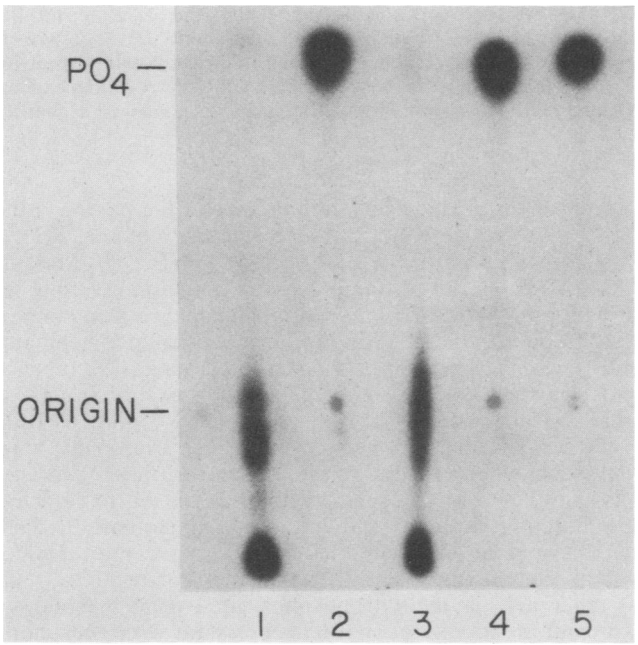

FIG. 2. Susceptibility of E1A proteins to phosphatase. E1A proteins 289 (lanes 1 and 2) and 243 (lanes 3 and 5) were labeled in vivo with $^{32}P_i$ and isolated from 2-D gels of total cell protein. Labeled proteins were eluted from gel slices and then TCA-precipitated with 50 μg of BSA carrier protein, performic acid oxidized, and digested with TPCK-trypsin. Digests were then lyophilized and left untreated (lanes 1 and 3) or digested with calf intestinal phosphatase (1.0 U; Boehringer-Mannheim) in 20 μl of 50 mM NH_4HCO_3 for 30 min at 45°C. Portions containing about 500 cpm were then subjected to electrophoresis for 120 min at 400 V on thin-layer cellulose in pyridine-acetic acid-H_2O (5:50:945). $^{32}P_i$ (lane 5) was run as a standard. Labeled species were visualized by autoradiography.

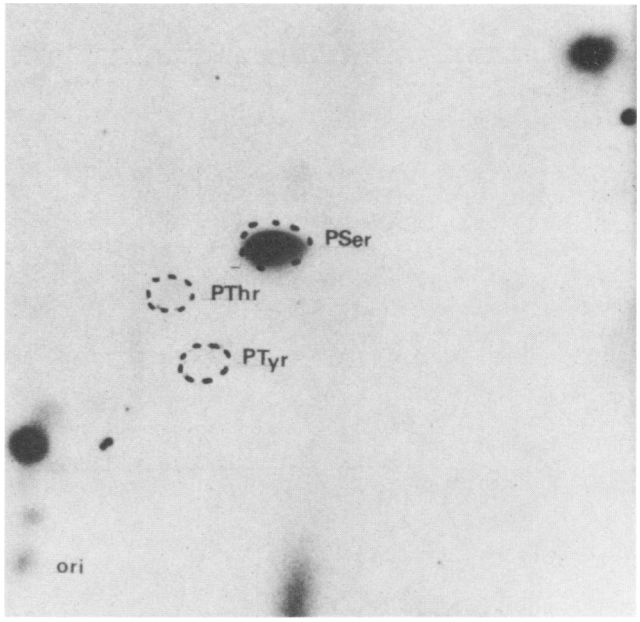

FIG. 3. Phosphoamino acid analysis of E1A proteins. E1A proteins were labeled in vivo with $^{32}P_i$ and isolated from 2-D gels of total cellular protein. Proteins were eluted from gel slices and TCA-precipitated with 50 μg of BSA carrier protein. TCA pellets were washed twice with cold acetone, dried, and hydrolyzed in 6 N HCl for 2 h at 100°C. Hydrolysates were diluted with H_2O, concentrated by lyophilization, suspended in H_2O containing 1 μg (final concentration) of unlabeled p-Ser, p-Thr, and p-Tyr standards, and analyzed on cellulose TLC plates (20 by 20 cm). First-dimension electrophoresis was for 3 h at 400 V in acetic acid-formic acid-H_2O, pH 1.9 (78:25:897), with the origin at the lower left and the anode to the right. Plates were dried and then electrophoresed in the second dimension in pyridine-acetic acid-H_2O, pH 3.5 (5:50:945), for 3 h at 400 V from bottom (cathode) to top (anode). Unlabeled standards were stained with ninhydrin and labeled species were visualized by autoradiography, with intensifying screens used to decrease exposure time to 3 days.

Determination of the phosphate linkage and the phosphorylated amino acid. To define the type(s) of phosphate linkage in E1A proteins, isolated ^{32}P-labeled E1A proteins were digested with trypsin and calf intestinal phosphatase, and the products were analyzed by thin-layer electrophoresis. All phosphate was released as free P_i (Fig. 2), indicating that most or all protein phosphorylation is in simple phosphate-ester linkages (35). We next determined the phosphorylated amino acids by acid hydrolysis and analysis of the products by two-dimensional thin-layer electrophoresis. The predom-

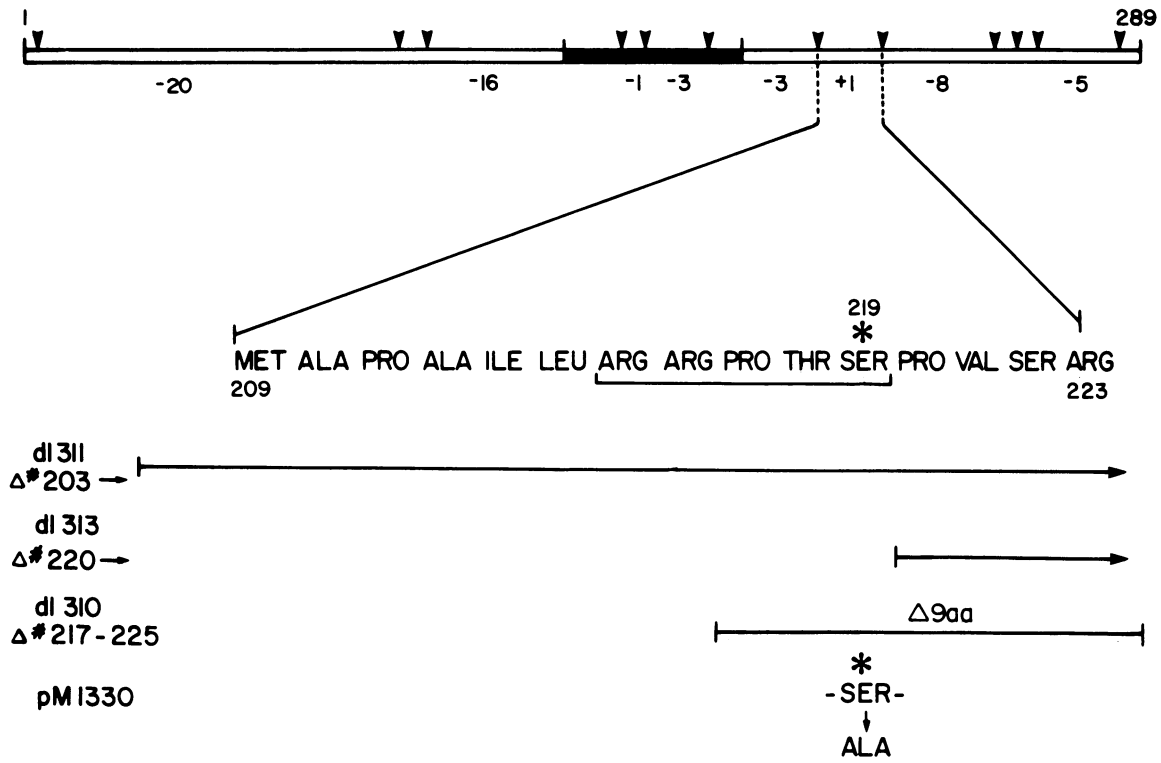

FIG. 4. Structure of the E1A 289-amino-acid protein. The sequence of the 289-amino-acid E1A protein is shown as an open bar; the 46-amino-acid unique region is indicated by the solid bar. Arrows indicate trypsin cleavage sites, and numbers below the bar represent the calculated net charge of serine-containing tryptic peptides (assuming one phosphate group per tryptic fragment). The amino acid sequence of the only positively charged tryptic peptide (amino acids 209 through 223) is indicated. Trypsin is not expected to cleave between the two arginine residues (see the text). Serine-219, marked by an asterisk, falls within a consensus sequence (underlined) for cAMP-dependent protein kinases. Below this, the carboxy-terminal E1A mutants *dl*310, *dl*311, and *dl*313 are shown with their respective amino acid sequence deletions. Also shown is the oligonucleotide-generated mutant pm1330.

inant phosphorylated species was phosphoserine; a minor amount of phosphothreonine was detected, but no phosphotyrosine (Fig. 3).

The above analyses reveal that the E1A proteins possess multiple phosphorylation sites, as previously reported by Yee et al. (59). However, a major phosphorylation site appears to be a serine of a positively charged tryptic peptide which is common to both the 243- and 289-amino-acid proteins. We examined the sequence of serine-containing tryptic peptides of the E1A proteins predicted by the primary amino acid sequences deduced from cDNA clones (42) (Fig. 4). Only the C-terminal portion of the E1A proteins between amino acids 209 and 223 (of the larger protein) could likely give rise to a positively charged serine-containing tryptic fragment. (Note that the rate of trypsin cleavage within the sequence Arg-Arg-Pro at amino acids 215 to 217 is expected to be extremely slow [7].) This peptide contained two serine residues, at positions 219 and 222. An interesting characteristic was that serine-219 fell within a recognition sequence for cyclic-AMP (cAMP)-dependent protein kinases (44). Fortuitously, a number of viral mutants with deletions in this region of the E1A proteins had been isolated previously (26, 27). These mutants were used to further characterize phosphotryptic peptide 6. The lower part of Fig. 4 indicates the portion of the protein deleted in each of the relevant mutants.

Analysis of mutant E1A proteins with deletions in the carboxy terminus. 2-D phosphotryptic peptide maps were done for the mutant E1A proteins synthesized in HeLa cells infected with *dl*1310, *dl*311, and *dl*313 and the parent virus used for the construction of these deletion mutants, *dl*309, which is wild type in the E1A region (26) (Fig. 5). Mutant *dl*311 carries an out-of-frame deletion mutation resulting in an E1A protein lacking the wild-type sequence from amino acid 203 on. *dl*310 carries an in-frame deletion of nine amino acids encompassing both serine-219 and serine-222 (amino acids 217 through 225 in the 289-amino-acid protein). E1A proteins from both of these mutants lacked peptide 6. This result is consistent with peptide 6 resulting from phosphorylation of either or both serine-219 and serine-222 of the wild-type E1A proteins. *dl*313 carries an out-of-frame deletion mutation in amino acid 220, retaining serine-219. This mutant yields a positively charged phosphotryptic peptide with altered mobility relative to the wild-type peptide 6 and no other new or altered phosphorylated tryptic peptides. This shift in mobility of spot 6 is consistent with phosphorylation of serine-219 in both wild-type and *dl*313 E1A proteins. The shift in *dl*313 peptide 6 mobility was expected due to the new sequence juxtaposed to serine-219 as a consequence of the deletion. Thus, these tryptic peptide maps of mutant E1A proteins suggest that serine-219 is a major E1A protein phosphorylation site in wild-type virus.

Oligonucleotide-directed mutagenesis of serine-219. To further test whether serine-219 is phosphorylated in wild-type E1A proteins, oligonucleotide-directed mutagenesis was used to generate a conservative amino acid change at this position. This was achieved by constructing a 20-mer oligonucleotide which would generate two base pair changes:

T → G at nt 1330 (to change a serine codon to an alanine codon) and A → G at nt 1332 (to facilitate identification of the mutant by generating a new *Hha*I site and eliminating an *Hph*I site) in the Ad2 sequence (18). These base pair changes were introduced into a bacteriophage M13 clone of E1A by the methods of Zoller and Smith (60). The mutant E1A region was then inserted into the adenovirus genome by the method of Stow (54). This mutant virus stock, designated pm1330, was plaque purified three times and analyzed by Southern blotting to confirm its purity.

Phosphotryptic peptide analysis of pm1330. E1A proteins isolated from pm1330-infected cells were analyzed as described for the previous peptide maps. The substitution of an alanine for serine-219 results in the loss of peptide 6 without the appearance of another phosphorylated peptide species (Fig. 5). On the basis of these results we infer that serine-219 is phosphorylated in wild-type E1A proteins. We conclude that the mutation in pm1330 prevents phosphorylation of peptide 6.

Analysis of the phenotype of pm1330. E1A functions were analyzed in cells infected with the mutant pm1330. Transcriptional *trans* activation by pm1330 E1A proteins was analyzed by measuring the concentration of early viral RNAs induced in infected HeLa cells. Independent experiments showed no difference in the level of cytoplasmic RNA (as measured by S1 nuclease analysis) for E1B, E2, E3, or E4 at 6 h p.i. between pm1330- and *dl*309-infected HeLa cells (data not shown). We also analyzed 6-h RNA from *dl*310, which has an in-frame deletion encompassing the serine mutated in pm1330. Again, no difference in early mRNA levels was detected for this mutant, as reported previously (48), indicating that deletion of this major phosphorylation site does not interfere with the transcription-stimulating ability of the E1A proteins.

To define whether the elimination of this phosphorylation site affected any of the growth properties of the virus, we examined the plaquing efficiency and viral replication of pm1330. The plaque-forming activity of pm1330 on HeLa cell monolayers was the same as on 293 cells, a cell line which can complement mutations in E1A and E1B due to the constitutive expression of these proteins (20). Thus, the mutation does not affect E1A functions required for plaque formation on HeLa cell monolayers. The mutant, pm975, which expresses only the larger 289-amino-acid E1A protein (39), replicated as efficiently as the wild type on HeLa cells. However, on G_0-arrested WI38 cells (secondary human lung fibroblasts), the lack of the 243-amino-acid protein became apparent. pm975 viral DNA replication was significantly depressed compared with that of the wild type, and the size of the virus burst was greatly diminished (52). We examined the ability of pm1330 to replicate in serum-starved, G_0-arrested WI38 cells and found that this mutant replicated with the same kinetics as its wild type E1A parent *dl*309 (data not shown).

The transforming activity of the mutant was examined in focus-forming assays on CREF cells (15). Mutants pm1330 and *dl*310 transformed with the same frequency as *dl*309 (Table 1). The transforming activity of *dl*310 on CREF cells has been reported to be equal to that of *dl*309 (48).

Foci of cells transformed by *dl*309, *dl*310, and pm1330 were picked and assayed for colony formation in soft agar. Cells transformed by pm1330 and *dl*310 formed colonies of similar size and at a similar frequency to *dl*309-transformed cells. Thus, eliminating the phosphorylation of serine-219 does not impair the transforming ability of these viruses.

E1A protein phosphorylation in CHO cells defective in cAMP-dependent protein kinases. The major phosphorylation site at serine-219 is located three amino acids to the carboxyl side of two basic residues: *Arg-Arg*-Pro-Thr-*Ser*-219. A serine residue two or three amino acids downstream from two basic residues has been cited as a potential substrate for cAMP-dependent protein kinases (44). Chinese hamster ovary (CHO) cells mutant in cAMP-dependent protein kinase type I and greatly reduced for type II have been isolated (reviewed in Gottesman [19]). Phosphorylation of serine-219 in wild-type E1A proteins was examined in these

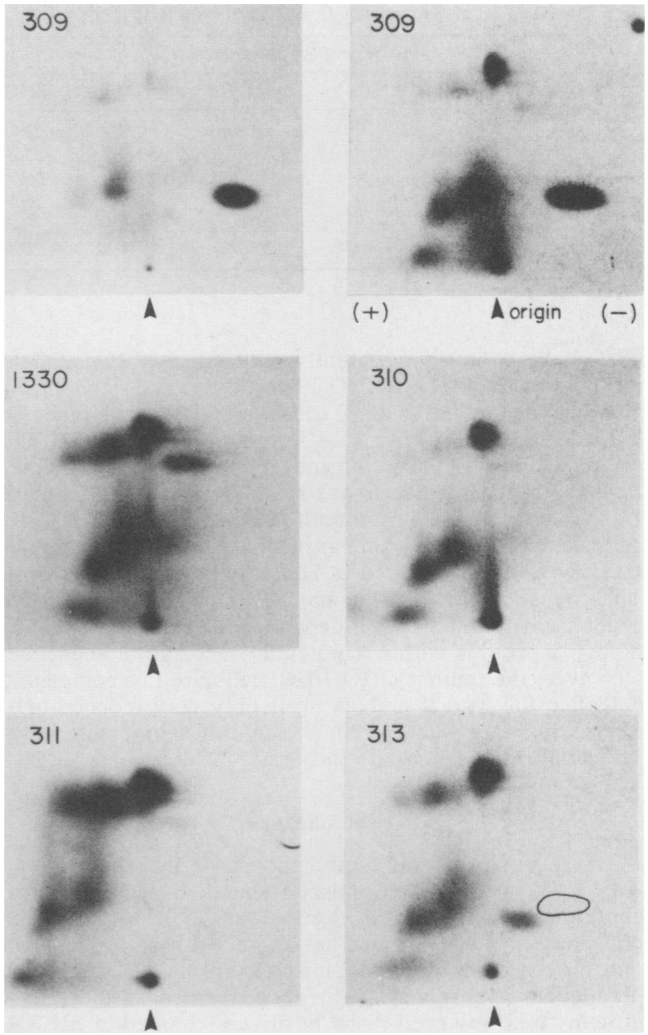

FIG. 5. 2-D phosphotryptic peptide maps of wild-type and mutant E1A proteins. HeLa cells were infected at an MOI of 10 for *dl*309, *dl*311, and pm1330 and an MOI of 50 for *dl*313 in the presence of araC. E1A proteins were isolated and peptide maps performed as described in the Fig. 1 legend. An equal number of Cerenkov counts of purified E1A proteins was spotted onto the TLC plates. The upper left panel (*dl*309) represents a short exposure of a peptide map to demonstrate the relative intensity of peptide 6 (refer to schematic drawing in Fig. 1). The *dl*309 peptide map in the upper right is an overexposure which coincides with the exposure time of the other four maps of E1A mutants. This was done to demonstrate a decrease or absence of phosphorylation in peptide 6 and also to show that no new phosphorylated peptides appeared in these mutants. The stenciled spot in the *dl*313 map indicates the position of peptide 6 in the wild type, *dl*309, to show the altered migration of this phosphopeptide in *dl*313. Arrows indicate the origin.

TABLE 1. Transformation assays on CREF cells

Expt	Avg no. of foci/dish at wk 6 p.i.				
	Uninfected	*dl*312	*dl*309	pm1330	*dl*310
1	0	0	36.4	30.2	36.0
2	0	0	37.0	39.2	31.4
3	0	0	26.0	17.8	ND[a]
4	0	0	19.4	21.4	ND
5	0	0	28.5	ND	23.6
6	0	0	35.0	ND	26.4

[a] ND, Not done.

CHO cells, which have greatly reduced cAMP-dependent protein kinase activity (CHO 10260).

Wild-type and CHO 10260 cells were infected and labeled as for HeLa cells. The E1A proteins were isolated and prepared for 2-D phosphotryptic peptide maps as before. Phosphotryptic peptide 6 was readily apparent in maps of E1A proteins isolated from both wild-type and mutant CHO cells, in approximately similar intensity relative to the other labeled peptides observed in HeLa cells (data not shown). These results imply that the major E1A phosphorylation site is not a substrate for cAMP-dependent protein kinases, even though it resides within a potential recognition sequence. However, the mutant CHO 10260 cells are not completely defective for type II activity; it is only very reduced (49). Therefore, it remains possible that this minor activity is sufficient to phosphorylate this site.

DISCUSSION

The results presented here demonstrate that the Ad2 and Ad5 E1A proteins are phosphorylated at multiple sites, confirming the results of Yee et al. (59). Most of the phosphate label was found in serine, with a low level of phosphothreonine and no phosphotyrosine detected. The predominant phosphorylated tryptic fragment of these E1A proteins was observed to be positively charged at pH 8.9. Inspection of E1A protein sequences predicted from the cDNA sequences (42) revealed one region of these acidic proteins likely to give rise to a positively charged phosphate-containing tryptic peptide. Analysis of E1A proteins with deletions in this region (48) suggested that serine-219 and possibly serine-221 were sites of phosphorylation in the wild-type proteins. With oligonucleotide-directed mutagenesis, serine-219 was replaced with an alanine. This alanine-for-serine change in mutant pm1330 resulted in E1A proteins which did not yield the major, positively charged phosphotryptic peptide. None of the other seven E1A protein phosphotryptic peptides detected were affected by this mutation. From these results we infer that serine-219 is phosphorylated in the wild-type E1A proteins. We conclude that the alanine substitution at position 219 prevents phosphorylation in this region of the protein.

It seems unlikely that serine-221 is also phosphorylated in the wild-type proteins, since this would introduce two additional negative charges into the tryptic peptide. Also, mutation at position 219 completely prevented phosphorylation of this tryptic peptide. However, it remains possible that the alanine substitution at position 219 prevents a phosphorylation which normally occurs at position 221 in the wild-type protein. Mutant pm1330 had no observable defects in its ability to induce transcription of viral early genes to replicate in HeLa cells or G_0-arrested WI38 cells. In addition, pm1330 was similar to the wild type in its ability to transform CREF cells in vitro. These results are perfectly consistent with the report of Shenk et al. (48) that *dl*310 (nine-amino-acid in-phase deletion removing both serine-219 and -221) replicates and transforms like the wild type.

The proteins encoded by E1A and E1B cooperate via some as yet undefined mechanism to produce a fully transformed cell in vitro. There have been reports of a protein kinase activity associated with immunoprecipitates of adenovirus transforming proteins, in particular the 55K antigen from E1B (5, 6, 32). To determine whether E1B proteins affect E1A phosphorylation, we examined the phosphotryptic peptides generated from E1A proteins synthesized by viral mutants defective in the two major E1B proteins of 21K and 55K (D. Barker and A. J. Berk, unpublished data). We observed (Tsukamoto and Berk, unpublished observations) the wild-type pattern of E1A phosphotryptic peptides for each of these mutants, suggesting that if a kinase activity is associated with either of the E1B proteins, the E1A proteins are not a substrate for it. Evidence is also presented here that this major site of E1A phosphorylation is not phosphorylated by cAMP-dependent protein kinases. Data supporting both of these conclusions come from the results of Lucher et al. (33). They used a partially purified protein kinase from uninfected human KB cells to phosphorylate in vitro Ad12 E1A proteins synthesized in *Escherichia coli*. The kinase activity is cAMP independent, phosphorylates at serine residues, and appears to faithfully phosphorylate the same sites in vitro as in vivo in Ad12-infected cells. Thus, it appears (33) that a cellular serine-specific protein kinase is responsible for E1A protein phosphorylation.

Several known parameters of E1A protein function were tested on the phosphorylation mutant pm1330. No differences were seen between this mutant and wild-type virus, suggesting that the major site of phosphorylation is dispensable for the pleiotropic effects of this protein. Examination of the role of protein phosphorylation has been carried out for several other transforming genes and indicates that protein phosphorylation modulates the biological activity of some proteins but not others. For example, deletion of the major site of tyrosine phosphorylation in the $p130^{gag\text{-}fps}$ of Fujinami sarcoma virus downmodulates its biochemical and biological activity. Although the mutant protein is not defective in its transforming ability or its tyrosine-specific kinase activity, there is a marked delay in the appearance of transformed foci and the protein kinase specific activity is reduced fivefold compared with the wild type (57). Another example is given by the large T antigen of simian virus 40, in which the phosphorylation state seems to correlate with the ability to bind to viral DNA (47). On the other hand, deletion of the two major sites of phosphorylation in $pp60^{v\text{-}src}$ of Rous sarcoma virus does not affect either its kinase activity or its transforming ability (10, 50). Similarly, the polyomavirus middle T antigen principle phosphotyrosine residue appears to be dispensable for transforming activity (37). However, both of these proteins contain multiple minor phosphorylation sites, and perhaps one of these minor sites regulates some function.

Recently, Ferguson et al. (14) purified Ad2 E1A proteins synthesized in *E. coli*. When these proteins are microinjected into *Xenopus laevis* oocytes they are posttranslationally modified before transport to the nucleus (43a). Currently, phosphorylation is the only posttranslational modification established for the E1A proteins. We also know that the E1A proteins are phosphorylated at several other serine residues, one of which may regulate its activity.

Alternatively, it is possible that elimination of the major site of phosphorylation does affect some function of these proteins which we have not assayed here. Since no in vitro biochemical activities have been defined for the E1A protein, we may not be able to detect a possible subtle effect of the pm1330 mutation. To establish this will require further understanding of E1A protein activity.

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