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Identification of Human Adenovirus Early Region 1 Products by Using Antisera Against Synthetic Peptides Corresponding to the Predicted Carboxy Termini

SIU-POK YEE,[1] DAVID T. ROWE,[2] MICHEL L. TREMBLAY,[1] MARK McDERMOTT,[1] AND PHILIP E. BRANTON[1]*

Departments of Pathology[1] and Biology,[2] McMaster University, Hamilton, Ontario, Canada

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Synthetic peptides were prepared which corresponded to the carboxy termini of the human adenovirus type 5 early region 1B (E1B) 58,000-molecular-weight (58K) protein (Tyr-Ser-Asp-Glu-Asp-Thr-Asp) and of the E1A gene products (Tyr-Gly-Lys-Arg-Pro-Arg-Pro). Antisera raised against these peptides precipitated polypeptides from adenovirus type 5-infected KB cells; serum raised against the 58K carboxy terminus was active against the E1B 58K phosphoprotein, whereas serum raised against the E1A peptide immunoprecipitated four major and at least two minor polypeptides. These latter proteins migrated with apparent molecular weights of 52K, 50K, 48.5K, 45K, 37.5K, and 35K, and all were phosphoproteins. By using tryptic phosphopeptide analysis, the four major species (52K, 50K, 48.5K, and 45K) were found to be related, as would be expected if all were products of the E1A region. The ability of the antipeptide sera to precipitate these viral proteins thus confirmed that the previously proposed sequence of E1 DNA and mRNA and the reading frame of the mRNA are correct. Immunofluorescent-antibody staining with the antipeptide sera indicated that the 58K E1B protein was localized both in the nucleus and in the cytoplasm, especially in the perinuclear region. The E1A-specific serum also stained both discrete patches in the nucleus and diffuse areas of the cytoplasm. These data suggest that both the 58K protein and the E1A proteins may function in or around the nucleus. These highly specific antipeptide sera should allow for a more complete identification and characterization of these important viral proteins.

The early region 1 (E1) of human adenovirus type 5 (Ad5) is composed of two early transcription units, termed E1A (1.5 to 4.5% of the genome) and E1B (4.5 to 11%) (3, 5, 9, 14, 20). The translation products of this region are of particular interest for two reasons. First, the E1 region of Ad5 DNA is both necessary and sufficient for oncogenic transformation (13, 16), and thus these viral proteins probably play a role in the initiation and maintenance of transformation. Second, the E1A products act as positive regulators in the expression of other early adenovirus genes (4, 29). Thus, E1 polypeptides carry out diverse and important functions in infected and transformed cells.

Each E1 early transcription unit appears to produce at least three mRNA classes. Species of 1.1, 0.9, and 0.6 kilobases (kb) are transcribed from E1A, and analyses of these mRNAs and DNA sequencing data predict that polypeptides with molecular weights of about 32,000 (32K), 26K, and 16K should be produced (28). All of these proteins should have identical amino and carboxy termini, as they differ only in internal splice sites. The products of the 1.1- and 0.9-kb mRNAs should be 289 and 243 amino acids long, respectively, a difference of only about 16%. The E1B region produces 2.2-, 1.0-, and 0.5-kb mRNAs. The 0.5-kb species codes for the late Ad5 polypeptide IX (1). Recently it has been suggested that the 2.2-kb species codes for a 21K polypeptide (sometimes referred to as 19K), and, using a different start site and reading frame, for a protein with a predicted size of 55K (6). The 1.0-kb mRNA appears to code for a 21K protein believed to be identical to that produced by the 2.2-kb mRNA (6).

Several attempts have been made to identify the Ad5 E1 gene products, primarily by using either (i) immunoprecipitation with sera from animals bearing tumors induced by Ad5 or Ad5-transformed cells or (ii) in vitro translation of selected Ad5 mRNAs. A protein with an apparent molecular weight of 58K frequently has been detected (19, 22, 23, 31). This polypeptide is phosphorylated (31; P. Malette, S.-P. Yee, and

P. E. Branton, J. Gen. Virol., in press), and it is a product of the E1B region because it is synthesized from E1B-selected mRNA (17, 25, 39), is absent or greatly reduced in cells infected with group II E1B-defective host range mutants (21, 22), and is present in cells transformed by Ad5 DNA fragments containing the E1B region but is absent in cells transformed by smaller fragments (33; D. T. Rowe and F. L. Graham, unpublished data). The products of the E1A region have been much more difficult to identify because they are produced in small amounts and antitumor sera appear to have a low avidity for them (18, 22, 33). In vitro translation of E1A-selected mRNA has yielded a variety of products ranging from two to six polypeptides of about 30K to 60K (7, 12, 17, 18, 24, 30, 34, 35). Efforts to increase the amount of E1A proteins by treating infected cells with metabolic inhibitors have also yielded two to six polypeptides in the 30K to 60K range (15, 18, 34).

Small synthetic peptides have been used to create antisera which specifically recognize proteins containing these amino acid sequences as antigenic sites (38, 42). In the present study we prepared antisera against synthetic peptides corresponding to the carboxy termini of the 58K polypeptide and the E1A polypeptides as predicted from mRNA analyses and DNA sequencing data (6, 26, 27, 28, 41). Both of these antisera were active in immunoprecipitating viral polypeptides from Ad5-infected KB cells.

MATERIALS AND METHODS

Cell culture and virus infection. Human KB cells were grown in 150-mm plastic petri dishes (Nunc) as previously described (8). Cells were infected with Ad5 at a multiplicity of infection of 35 PFU per cell.

Radioactive labeling. Ad5- or mock-infected cultures were incubated from 7 to 11 h postinfection with 4 ml of medium lacking phosphate but containing 2 mCi of carrier-free $^{32}P_i$ (New England Nuclear Corp.) or with 4 ml of medium lacking methionine but containing 100 μCi of [^{35}S]methionine (Amersham Corp.; specific activity, 1,300 Ci/mmol).

Cell extracts and immunoprecipitation. Cells were washed with phosphate-buffered saline (PBS) and removed by scraping with a rubber policeman. The washed cell pellets were suspended in RIPA buffer, which consisted of 0.05 M Tris (pH 7.2) containing 150 mM NaCl, 0.1% (vol/vol) sodium dodecyl sulfate (SDS), 0.1% (vol/vol) sodium deoxycholate, 0.1% (vol/vol) Triton X-100, and 100,000 IU of aprotinin per ml, at a concentration of approximately 2×10^7 cells per ml. The lysate was vortexed vigorously and incubated on ice for 20 min. The insoluble material was removed by centrifugation at $12,500 \times g$ for 10 min at 4°C. To each 1 ml of cell extract was added an appropriate volume of antiserum and 250 μl of protein A-Sepharose beads (Pharmacia Fine Chemicals) suspended (1:10) in RIPA buffer. The mixture was incubated at 4°C with constant mixing for 2 to 3 h, and then the Sepharose beads with bound antibody were collected by centrifugation at $30 \times g$ for 30 s and washed three times with 1 ml of RIPA buffer and twice with 1 ml of 100 mM Tris (pH 7.0) containing 200 mM LiCl and 0.1% (vol/vol) 2-mercaptoethanol.

SDS-polyacrylamide gel electrophoresis. Immunoprecipitates were dissolved in 100 μl of sample buffer (100 mM Tris [pH 6.8] containing 2% SDS, 2% [vol/vol] 2-mercaptoethanol, and 10% [vol/vol] glycerol), the mixture was boiled for 2 min, the beads were removed by centrifugation, and 25 to 50 μl of the supernatant was analyzed by SDS-polyacrylamide gel electrophoresis using 12% polyacrylamide gels with a 5% stacking gel, as previously described (8).

Antitumor sera. Sera were collected from hamsters bearing tumors induced by various Ad5-transformed cell lines. These lines included 14B (43); 983-2, a cell line obtained by transformation of hamster cells with *Xho*I fragment C; and 945-C1 and 954-C4, cell lines derived by transformation of hamster cells with *Hin*dIII digests of Ad5 DNA (F. L. Graham, unpublished data). A combined serum containing equal volumes of these sera was used to detect viral antigens in infected cell extracts. For immunoprecipitations, 10 μl of combined serum was used per ml of cell extract.

Antisera to synthetic peptides. Synthetic peptides (see Fig. 1) were purchased from Bachem. Production was by sequential solid-phase synthesis (37), and purity was greater than 90%. The sequences of the synthetic peptides were corroborated by automated Edman analysis. For the preparation of antisera, the synthetic peptides were conjugated to bovine serum albumin (BSA) with bis-diazotized benzidine as described by Bassiri et al. (2). Briefly, 50 mg of BSA (Sigma Chemical Co.) was dissolved at 0°C, along with a 40 M excess of the peptide, in 10 ml of 0.16 M borate–0.13 M NaCl (pH 9.0). A 2-ml portion of bis-diazotized benzidine in 0.2 M HCl was added to the mixture with constant stirring at 0°C. The pH was adjusted to 7.0 with 0.5 M NaOH, and the reaction was allowed to continue for 2 h further at 4°C with constant stirring. The conjugate was stored at −70°C until use. Antisera were prepared in 5-month-old male New Zealand white rabbits, using 1 mg of the peptide-BSA conjugate which had been emulsified in 1 ml of complete Freund adjuvant. The rabbits were injected at 10 different intradermal and intramuscular sites, including footpads. The animals were boosted 4 weeks later with 1 mg of conjugate in 1 ml of incomplete Freund adjuvant. Serum was collected 7 to 10 days after each boost, clarified by centrifugation, and stored at −20°C. In most experiments, the sera were rendered peptide specific by the addition of BSA at 2.5 mg/ml. After 48 h at 4°C, the immune precipitate was removed by centrifugation at $100,000 \times g$ for 1 h. A 5-ml portion of the supernatant serum was then combined with 1 ml of PBS containing 10^8 HeLa cells which had been disrupted by sonication. After 48 h at 4°C, the precipitate was removed by centrifugation, and the supernatant was used as antipeptide serum. For immunoprecipitates, 20 μl of antipeptide serum (purified or unpurified) was used. Preimmune sera were collected from each rabbit.

Partial hydrolysis of proteins by digestion with staphylococcal V-8 protease. Proteins were analyzed by partial hydrolysis with V-8 protease according to the method of Cleveland et al. (10). Bands containing the appropriate polypeptides were cut out of dried poly-

acrylamide gels, using an autoradiogram as a template. The gel slices were cut into pieces, and the pieces were applied to a second 15% polyacrylamide gel with a 5% stacking gel in the presence of 50 μl of sample buffer containing 0, 1, 20, or 400 μg of staphylococcal V-8 protease per ml. The samples were electrophoresed until the dye front reached the stacking gel-resolving gel interface, at which time electrophoresis was stopped for 2 to 3 h. Electrophoresis was then continued as usual.

Tryptic peptide mapping. ^{32}P-labeled E1A proteins were separated by SDS-polyacrylamide gel electrophoresis, and the corresponding areas for 52K, 50K, 48.5K, and 45K polypeptides on dried, fixed gels were excised and diced into small pieces. The fragments were incubated at 37°C for 24 h in 3 to 4 ml of 0.05 M ammonium bicarbonate containing 50 μg of tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin per ml. The supernatant was removed and replaced with 2 to 3 ml of fresh trypsin, and incubation was continued for 12 to 16 h. The supernatants were pooled, oxidized on ice with fresh performic acid, combined with 5 volumes of water, and lyophilized. The peptides were dissolved in about 40 μl of 10% formic acid, spotted (usually 1,000 to 4,000 cpm) onto cellulose thin-layer plates (Polygram CEL300), and separated by the method of Scheidtmann et al. (32). Electrophoresis was carried out in the first dimension for 50 min at 1,000 V with formic acid-acetic acid-pyridine-water (6:1.25:0.25:92.5) (pH 1.9). The plates were rotated 90°, and a second dimension of separation was performed by ascending chromatography in *n*-butanol–pyridine–acetic acid–water (50:40:10:40). The positions of ^{32}P-labeled phosphopeptides were determined by autoradiography.

Immunofluorescence. Ad5- and mock-infected cells growing on cover slips were harvested at 12 h postinfection and fixed with methanol-acetone (1:1) at −20°C for 15 min. The cells were washed three times with PBS and then treated for 30 min with either antipeptide serum or preimmune rabbit serum. After another three washes with PBS, the cells were treated for 30 min with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Cappel Laboratories) diluted 1:20 in PBS immediately before use. The antipeptide sera used in these studies had been purified and absorbed with BSA and HeLa cell lysate as described above. The antisera against the carboxy-terminal peptides for the Ad5 E1A proteins and for the Ad5 58K protein were diluted 1:10 and 1:5, respectively, in PBS. Fluorescence was recorded on Kodak Ektachrome 400 slide film.

RESULTS

Preparation of antisera against specific E1 proteins. Because the amino acid sequences of Ad5 E1 proteins could be predicted from analyses of mRNA and sequencing of viral DNA (6, 26, 27, 28, 41), it was possible to produce synthetic peptides which could be used to generate antisera with activity against these viral proteins. Synthetic peptides were therefore prepared which corresponded to the carboxy termini of the E1B 58K protein and of the E1A gene products (Fig. 1). For the 58K protein, the peptide (designated 58-C) consisted of six amino acids at the carboxy terminus with a tyrosine residue added at the amino end to serve both as a means to couple the peptide to the BSA carrier and as a site for iodination, if needed. For the E1A proteins, the peptide (designated E1A-C) consisted of the five amino acids at the carboxy terminus with the sixth residue, a cysteine, being replaced with a spacer glycine residue to avoid formation of peptide dimers. Again, an amino terminal tyrosine residue was added for coupling and iodination. These peptides were coupled to BSA, and the complex was used to immunize rabbits as described above.

Carboxy Terminus of E1A Polypeptides

NH_2....asp-leu-ser-cys-lys-arg-pro-arg-proCOOH

E1A Synthetic Peptide (E1A-C)

NH_2tyr-gly-lys-arg-pro-arg-proCOOH

Carboxy Terminus of 58K

NH_2....phe-gly-ser-ser-asp-glu-asp-thr-aspCOOH

58K Synthetic Peptide (58-C)

NH_2tyr-ser-asp-glu-asp-thr-aspCOOH

FIG. 1. Carboxy-terminal sequences and synthetic peptides. The carboxy termini of the Ad5 E1B 58K species and of the Ad5 E1A gene products were deduced from viral DNA and mRNA sequencing data (6, 26, 27, 28, 41). The synthetic peptides corresponding to the carboxy termini were synthesized by a sequential solid-phase system (37).

Immunoprecipitation of Ad5 polypeptides with antisera raised against the synthetic peptides. Sera obtained from rabbits immunized with the synthetic peptides were tested for their ability to immunoprecipitate Ad5 polypeptides. Extracts from Ad5- and mock-infected cells labeled with [^{35}S]methionine from 7 to 11 h postinfection were precipitated either with combined hamster antitumor serum or with antiserum raised against the E1A-specific peptide (antiserum designated E1A-C1) or against the E1B 58K-specific peptide (antiserum designated 58-C1). The antitumor serum precipitated polypeptides from Ad5-infected cells with apparent molecular weights of 58K, 29K, and 19K and a collection of proteins at 40K to 50K (Fig. 2, lane D). Previous studies (21, 22; D. T. Rowe, P. E. Branton, and F. L. Graham, manuscript in preparation), using various host range mutants and cells transformed by specific fragments of Ad5 DNA, have suggested that the 58K and 19K polypeptides are coded for by the E1B region and that the 40K to 50K polypeptides are coded for by E1A. The origin of the 29K polypeptide is still not known. None of these polypeptides was precipitated from mock-infected cells (Fig. 2,

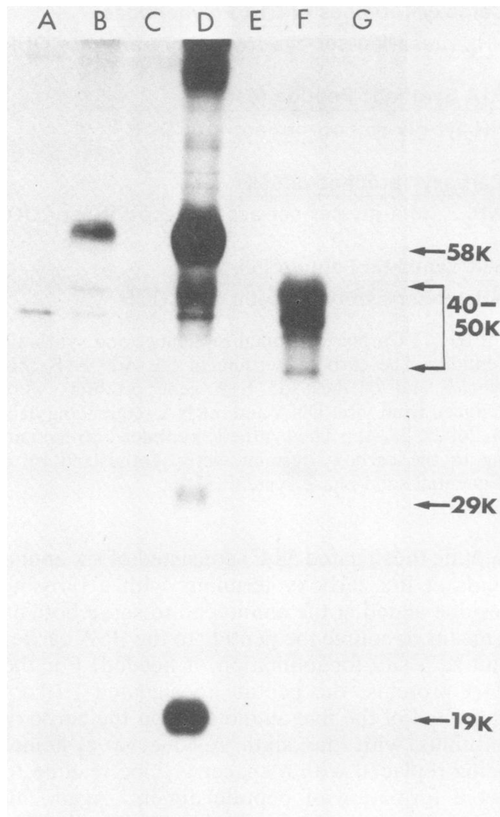

FIG. 2. Immunoprecipitation of Ad5 E1 polypeptides by antitumor and antipeptide sera. Ad5- and mock-infected cells were incubated with [^{35}S]methionine from 7 to 11 h postinfection, and whole-cell extracts were immunoprecipitated with normal rabbit preimmune serum, combined hamster antitumor serum, or antipeptide serum 58-C1 or E1A-C1. Lanes: A, preimmune rabbit serum with Ad5-infected cells; B and C, 58-C1 serum with Ad5-infected and control cells, respectively; D and E, combined antitumor serum with Ad5-infected and control cells, respectively; F and G, E1A-C1 serum with Ad5-infected and control cells, respectively.

lane E). The rabbit 58-C1 antipeptide serum precipitated almost exclusively a 58K polypeptide (Fig. 2, lane B). Little material was precipitated by normal rabbit preimmune serum (Fig. 2, lane A). The rabbit E1A-C1 antipeptide serum (Fig. 2, lane F) precipitated a collection of polypeptides from Ad5-infected cells, with apparent molecular weights ranging from about 35K to 50K, which had migration properties similar to those of the E1A 40K to 50K gene products seen with antitumor serum. The E1A-C1 serum precipitated little from mock-infected cells (Fig. 2, lane G).

To study further the specificities of the antipeptide sera, extracts from $^{32}P_i$-labeled cells were immunoprecipitated. It was already known that 58K is a phosphoprotein (31; Malette et al., in press). The antitumor serum (Fig. 3, lane D) and the 58-C1 antipeptide serum (Fig. 3, lane B) both precipitated a ^{32}P-labeled 58K species from infected cells. No such phosphoprotein was detected by immunoprecipitation of extracts from mock-infected cells (Fig. 3, lanes C and E) or by use of preimmune serum (Fig. 3, lane A). The antitumor serum also precipitated two other phosphoproteins from infected cells, a major species of about 52K and a heterogeneous species of about 40K to 50K (Fig. 3, lane D). The E1A-C1 serum also precipitated a group of phosphoproteins from infected cells, ranging from about 40K to 50K (Fig. 3, lane F). No labeled polypeptides were detected by immunoprecipitation of extracts from uninfected cells with E1A-C1 serum (Fig. 3, lane G). To study these species more carefully, ^{32}P-labeled polypeptides precipitated by hamster 983-2 antitumor serum and by E1A-C1 serum were separated on gels

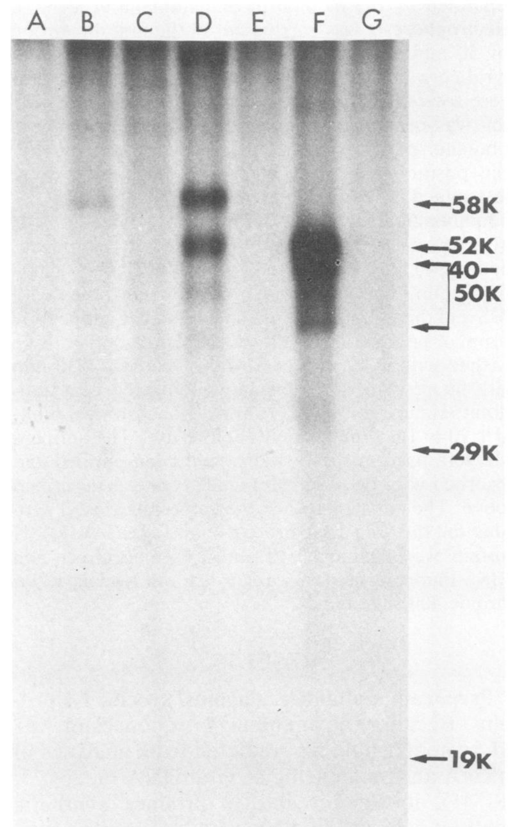

FIG. 3. Immunoprecipitation of ^{32}P-labeled Ad5 E1 polypeptides by antitumor and antipeptide sera. The procedure and contents of lanes were as described in the legend to Fig. 2, except cells were labeled with $^{32}P_i$ instead of [^{35}S]methionine.

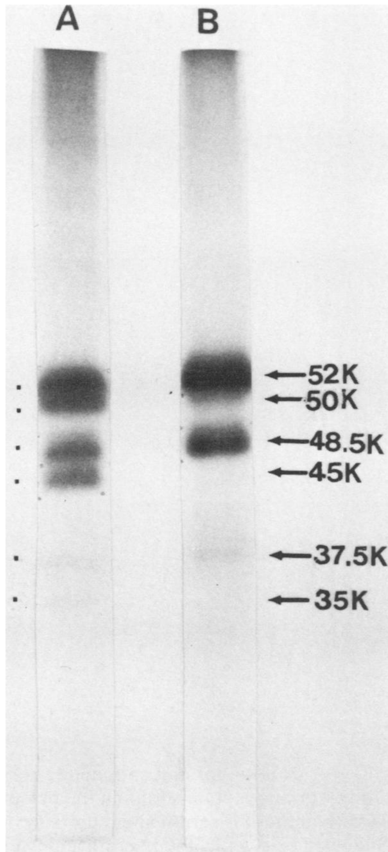

FIG. 4. Comparison of phosphoproteins precipitated by 983-2 hamster antitumor and E1A-C1 sera. ^{32}P-labeled Ad5-infected cells were immunoprecipitated with E1A-C1 serum (lane A) or antitumor serum (lane B). The samples were analyzed on a 12% SDS-polyacrylamide gel containing an acrylamide/bisacrylamide ratio of 30:0.2 instead of the normal ratio of 30:0.8. In addition, the gel was electrophoresed for 1 h further after the dye front had reached the bottom of the gel.

which would better resolve proteins of 30K to 50K. The E1A-C1 serum clearly precipitated 52K, 50K, 48.5K, and 45K phosphoproteins and two minor species of 37.5K and 35K (Fig. 4, lane A). The hamster antitumor serum recognized only the 52K, 48.5K, and 37.5K species (Fig. 4, lane B).

Another way of identifying proteins which were specifically immunoprecipitated by the antipeptide sera was to precipitate extracts in the presence of various concentrations of synthetic peptide. The precipitation of proteins with antigenic sites specified by these sera should be blocked by the peptides. Neither the 58-C nor the E1A-C peptides had any effect on the precipitation of the 58K and the 40K to 50K polypeptides by the hamster antitumor serum (Fig. 5, lanes A–D and I–L). The 58-C peptide had no effect on precipitation of the 35K to 52K complex by E1A-C1 antipeptide serum, nor did the E1A-C peptide affect 58K precipitation by 58-C1 antipeptide serum (data not shown). However, increasing concentrations of the 58-C peptide blocked the precipitation of the 58K protein by 58-C1 serum (Fig. 5, lanes E–H), and the E1A-C peptide blocked the precipitation of the 35K to 52K complex by E1A-C1 serum (Fig. 5, lanes M–P). In addition to these major viral species, several other polypeptides were detected in the immunoprecipitates, often at low levels. The presence of most of these was unaffected by the addition of the peptides, and thus they may represent nonspecific contaminants of the precipitation. However, the level of some of the minor species was reduced by the presence of the peptides, and thus these proteins could either contain the antigenic site specified in the peptide or be specifically bound to the Ad5 proteins containing these sites.

Comparison of viral proteins precipitated by antitumor and antipeptide sera by partial hydrolysis with staphylococcal V-8 protease. To prove that the proteins precipitated by the antipeptide sera were in fact the same virus-specific polypeptides recognized by antitumor serum, partial hydrolysis of ^{32}P-labeled proteins was performed with staphylococcal V-8 protease. One major phosphopeptide was formed with the 58K species precipitated by both the 58-C1 serum and the antitumor serum (Fig. 6). Hydrolysis of the 52K protein precipitated by the antitumor serum and the E1A-C1 serum produced identical patterns of multiple phosphopeptides (Fig. 7). Thus, the antipeptide sera precipitated the same virus-specific polypeptides recognized by the hamster antitumor serum.

Analysis of the relationship among putative E1A polypeptides by tryptic peptide mapping. To determine the relationship among the major phosphoproteins precipitated by E1A-C1 serum, ^{32}P-labeled 52K, 50K, 48.5K, and 45K polypeptides were separated by SDS-polyacrylamide gel electrophoresis, and the individual species were excised from the gel and subjected to tryptic phosphopeptide analysis (Fig. 8). A comparison of the 52K and 48.5K polypeptides showed that they had several phosphopeptides in common (peptides 1a, 1b, 2a, 2b, 3, and 4). Similarly, 50K and 45K shared several phosphopeptides (peptides 2b, 3, and 4). Comparisons of the 52K and 50K polypeptides (data not shown) indicated that the peptides designated 1b, 2b, 3, and 4 comigrated. Thus all four polypeptides were related, as would be expected if all were E1A products. Several differences were also apparent. The major phosphopeptide, designated 6, migrated slightly differently with each protein.

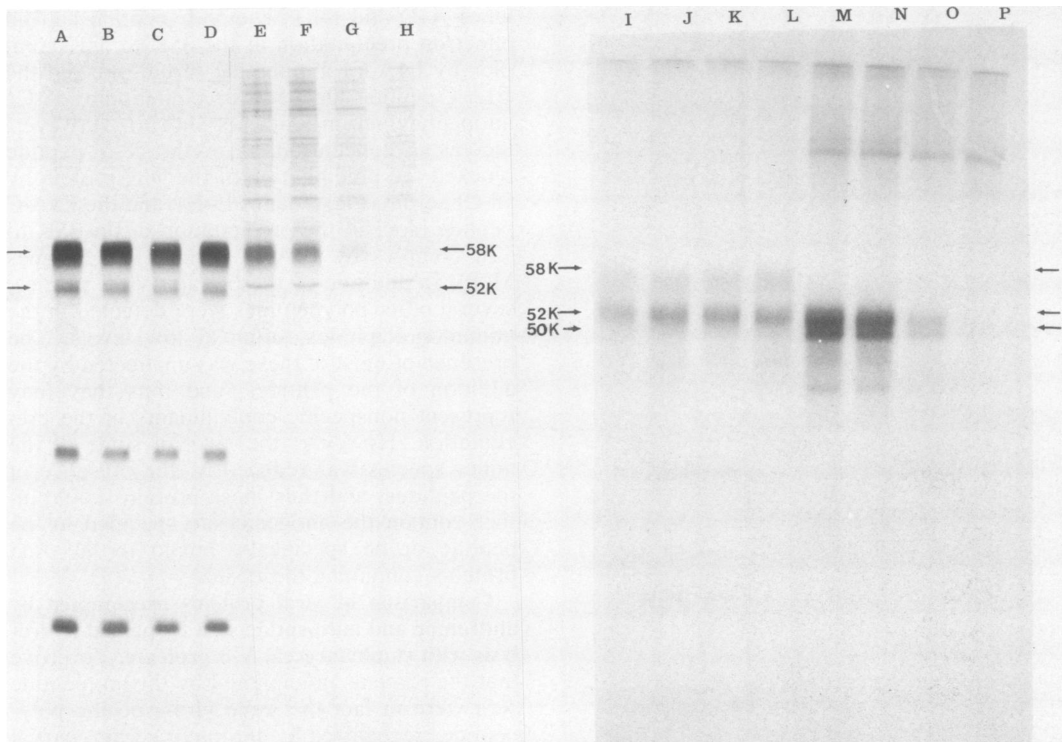

FIG. 5. Effect of synthetic peptides on precipitation of viral proteins by antitumor and antipeptide sera. ^{35}S- or ^{32}P-labeled Ad5-infected cells were precipitated with either antitumor or antipeptide serum in the presence of various amounts of synthetic peptides, as follows: ^{35}S-labeled cells with antitumor serum and 0 μg (lane A), 0.1 μg (lane B), 1.0 μg (lane C), and 10 μg (lane D) of 58-C peptide; ^{35}S-labeled cells with 58-C1 serum and 0 μg (lane E), 0.1 μg (lane F), 1.0 μg (lane G), and 10 μg (lane H) of 58-C peptide; and ^{32}P-labeled cells with antitumor serum (lanes I–L) or E1A-C1 antipeptide serum (lanes M–P) and the same respective amounts of E1A-C peptide as described for 58-C.

Thus it was not clear whether this species represented a different peptide in each case or was modified in various ways for each protein. It was also not clear whether peptides 1a and 2a were unrelated to the more minor species 1b and 2b or represented identical peptides which had been oxidized to different levels. Phosphopeptide 5 was seen only with the 48.5K polypeptide, and peptide 1b was not detected in the 45K polypeptide. Also, peptides 1a and 2a were not well resolved in the 50K and 45K polypeptides.

Localization of Ad5 proteins by immunofluorescence. The antipeptide sera were used to stain infected and mock-infected cells by indirect fluorescent-antibody staining. Cells growing on cover slips were fixed at 10 h postinfection and then treated with either 58-C1 or E1A-C1 serum and goat anti-rabbit immunoglobulin G serum. Neither of these sera stained mock-infected KB cells very well (Fig. 9A and C). The 58-C1 serum stained Ad5-infected cells in both the nucleus and the cytoplasm (Fig. 9B). The cytoplasmic fluorescence was concentrated in the perinuclear region, and the remaining cytoplasm was only weakly stained. Thus, assuming that most of the fluorescence was due to the staining of the 58K polypeptide, these data suggest that at early times after infection the major portion of the 58K species is in or surrounding the nucleus. The E1A-C1 serum also stained both the nucleus and the cytoplasm (Fig. 9D). The nuclear staining was largely in concentrated speckled areas, whereas the cytoplasmic staining was weaker and diffuse. Assuming that the fluorescent staining was specific for E1A polypeptides, these data suggest that these proteins may function within the nucleus.

DISCUSSION

Antisera against synthetic peptides corresponding to specific regions of Ad5 E1 proteins were used to study viral polypeptides. The 58-C1 serum, which was raised against a peptide corresponding to the last six amino acids at the proposed carboxy terminus of the large E1B gene product, precipitated a 58K polypeptide

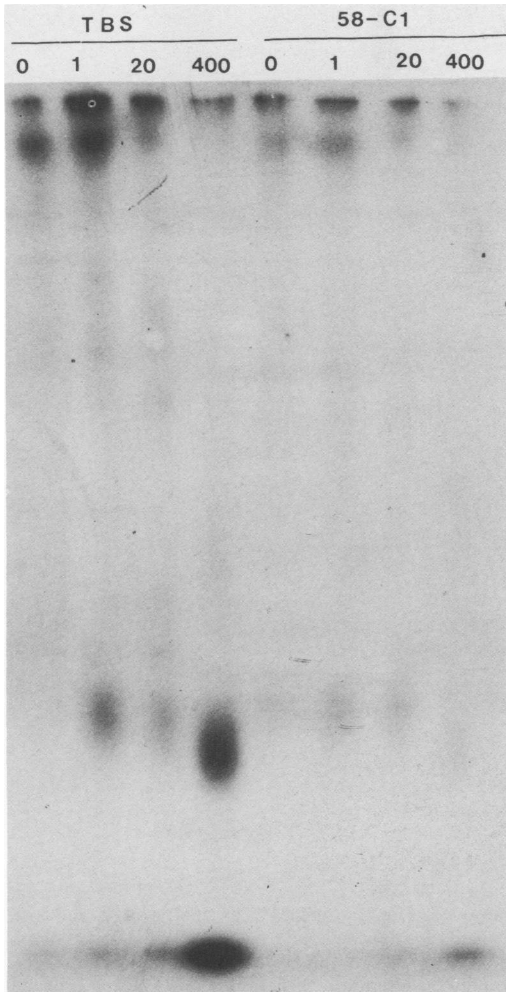

FIG. 6. Partial hydrolysis of the 58K polypeptide precipitated by antitumor of 58-C1 serum with staphylococcal V-8 protease. ^{32}P-labeled Ad5-infected cells were immunoprecipitated with antitumor serum or 58-C1 serum, and the labeled 58K protein precipitated by each was removed from an SDS-polyacrylamide gel and rerun in the presence of 0, 1, 20, or 400 μg of staphylococcal V-8 protease per ml as described in the text. TBS, Combined hamster antitumor serum (tumor-bearing serum).

which was shown by partial hydrolysis with V-8 protease to be the E1B 58K species recognized by antitumor serum. These data thus confirmed that the proposed sequence of the E1B DNA and mRNA and the reading frame of the mRNA (6, 26, 27) are correct. The E1A-C1 serum, raised against a peptide corresponding to the last five amino acids at the proposed carboxy terminus of the E1A gene products, precipitated six polypeptides, of which three were proteins recognized by the antitumor serum. These data therefore confirmed the proposed sequence of the E1A region and the reading frame of the mRNA (28, 41). The ability of these sera to precipitate the viral proteins was somewhat remarkable, as the synthetic peptides were quite short. However, both peptides were rich in hydrophilic amino acids, making them likely antigenic sites in the complete viral proteins. The small size of the peptides could even render the sera more specific, as the low number of epitopes would make it less likely that the sera would cross-react with unrelated proteins.

All of the viral proteins precipitated by the antipeptide sera were phosphorylated. It had been known for some time that the E1B 58K

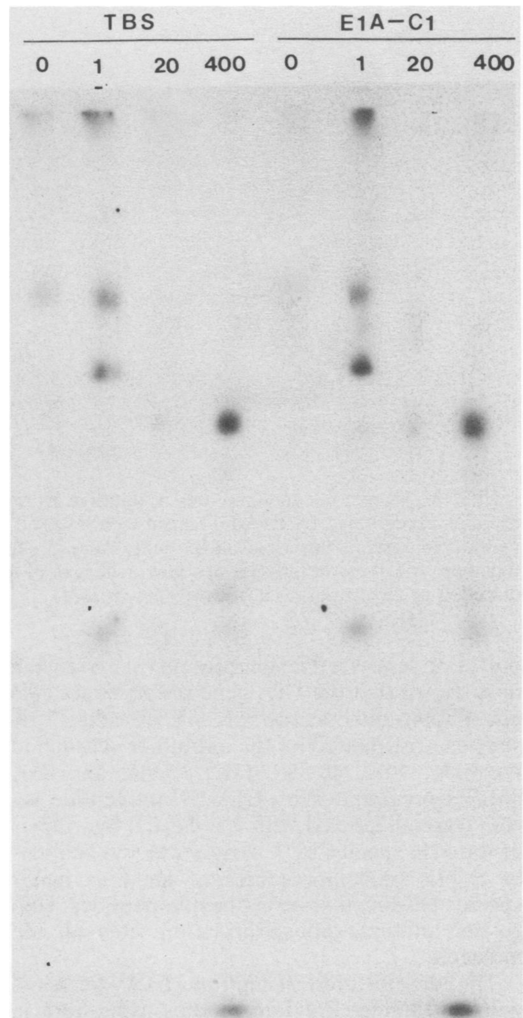

FIG. 7. Partial hydrolysis of the 52K polypeptide precipitated by antitumor or E1A-C1 serum with staphyloccal V-8 protease. An analysis similar to that described in the legend to Fig. 6 was carried out on the ^{32}P-labeled 52K protein precipitated by antitumor (TBS) and E1A-C1 sera.

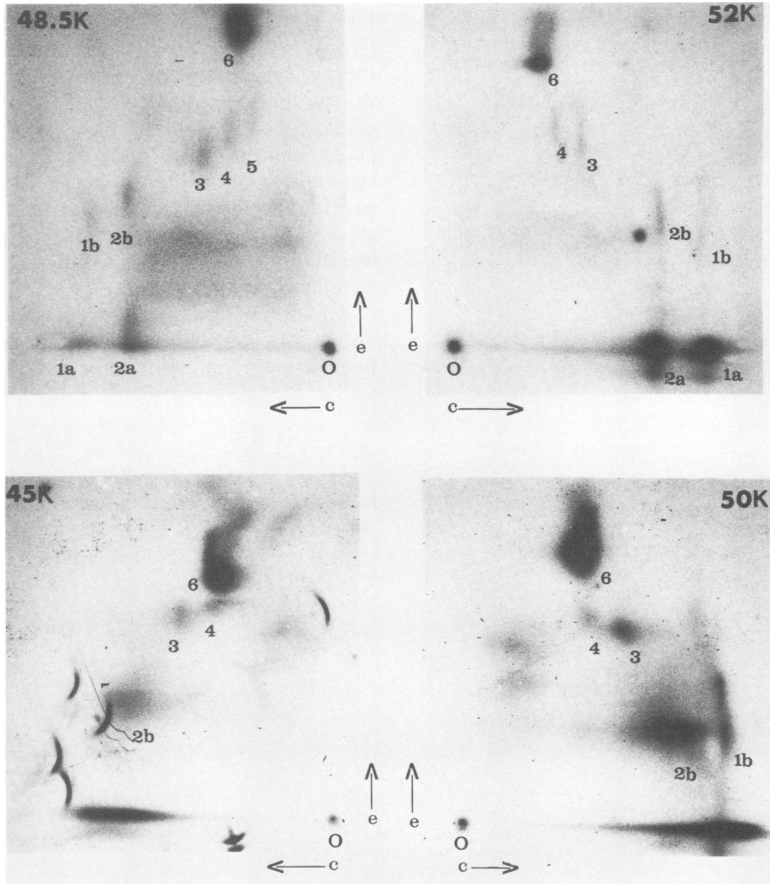

FIG. 8. Tryptic phosphopeptides of putative E1A polypeptides. The ^{32}P-labeled 52K, 50K, 48.5K, and 45K proteins precipitated by E1A-C1 serum were excised from an SDS-polyacrylamide gel and subjected to tryptic peptide analysis as described in the text. Pairs of samples (52K and 48.5K, and 50K and 45K) were analyzed together, and thus the profiles are mirror images of each other. O, Origin; c, direction of chromatography; e, direction of electrophoresis (toward the cathode).

polypeptide was a phosphoprotein (31). We have now shown that the E1A gene products are also phosphoproteins, as the 52K, 48.5K, and 37.5K species precipitated by the antitumor serum and the 52K, 50K, 48.5K, 45K, 37.5K, and 35K species precipitated by E1A-C1 antipeptide serum were all labeled with ^{32}P. Partial hydrolysis of the 52K species by V-8 protease and analysis of tryptic phosphopeptides of the four major species produced several phosphopeptides, suggesting multiple phosphorylation sites on the molecule.

The identification of multiple E1A species is quite intriguing. Previous studies using both in vitro translation of E1A-selected mRNA and analysis on two-dimensional gels of proteins made in infected cells pretreated with cycloheximide to augment the amount of E1A gene products had suggested that a cluster of about six acidic proteins is produced from the E1A region (18, 34). These species were related, as determined by tryptic peptide maps, and they possessed sequences which suggested that they were the products of the 1.1- and 0.9-kb E1A mRNAs (34). Using in vitro translation of E1A-selected mRNA, another group had detected four acidic proteins in the 40K to 50K range (7). More recently, however, using both in vitro translation (30) and analysis of cytosine arabinoside-treated cells (15), only two E1A products were identified, one each from the 1.1- and 0.9-kb mRNAs. Because these approaches could suffer from artifacts due to the in vitro methods or to the effects of inhibitors, our results obtained by short-term in vivo labeling of cells early after infection are of particular interest. Two major polypeptides, of 52 K and 48.5K, and a minor species, of 37.5K, were precipitated by the antitumor serum, whereas four major species, of 52K, 50K, 48.5K, and 45K, and two

minor species, of 37.5K and 35K, were recognized by the antipeptide serum E1A-C1. At present, neither the reasons for the differences between the sera nor the origin of the multiple polypeptides is understood. The 52K and 48.5K species could be the products of two different E1A mRNAs. It is also possible that these proteins are coded for by a single mRNA and that for unknown reasons the antitumor serum recognizes only these products. In a previous study in which six E1A polypeptides were observed, three species were shown to result from each of the 1.1- and 0.9-kb mRNAs (34). We now have data, obtained by using various Ad5 mutants, which suggest that the 52K, 48.5K, and 37.5K proteins are coded for by the 1.1-kb mRNA and that the 50K, 45K, and 35K proteins are coded for by the 0.9-kb mRNA (D. T. Rowe, S.-P. Yee, F. L. Graham, and P. E. Branton, Virology, in press). Multiple species could result from a single mRNA for a number of reasons. Differences in migration on gels could result from post-translational modifications, including phosphorylation or acetylation, from initiation at AUG sequences downstream from the normal initiation site, or from proteolytic cleavage of the gene product. If the latter is the case, it must occur at the amino terminus, as the E1A-C1 antipeptide serum is specific for the carboxy terminus. Experiments are now in progress to examine these various possibilities. The preliminary data presented in Fig. 8 do suggest that there may be phosphorylation differences between the 52K and 48.5K species and between the 50K and 45K species. It is also of interest that these E1A proteins migrate with apparent molecular weights which are considerably higher than those predicted from the amino acid sequence. It is possible that some type of post-translational modification, such as phosphorylation, or some other feature of the structure of these proteins, such as the high proline content (28), could cause them to migrate aberrantly on SDS-polyacrylamide gels. We have noted that their mobility on gels varied considerably with the gel conditions and that changes in the concentration of acrylamide or cross-linker altered their apparent molecular weights by as much as 5K. The nominal molecular weights presented in this report were the highest that we have measured for these proteins.

No evidence has been obtained so far to suggest that the Ad5 E1A 0.6-kb mRNA produces a polypeptide in vivo. This mRNA is

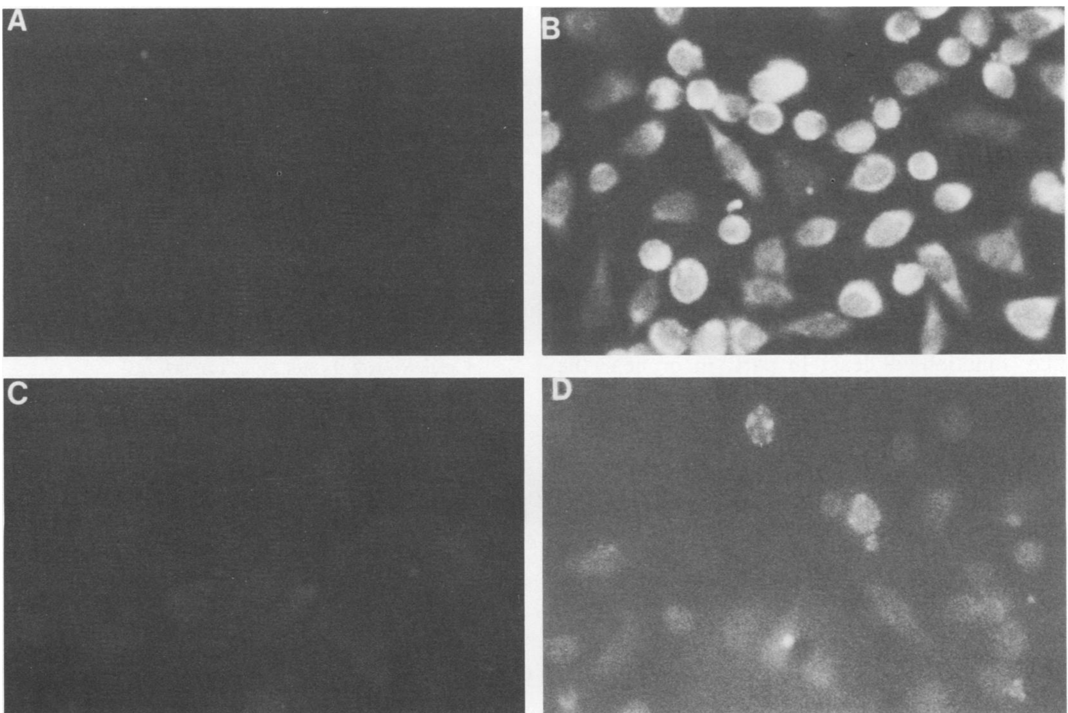

FIG. 9. Fluorescent-antibody staining with antipeptide sera. Ad5-infected (B and D) and mock-infected (A and C) cells were fixed and stained at 12 h postinfection, using the indirect fluorescent-antibody staining technique with the antipeptide sera 58-C1 (A and B) and E1A-C1 (C and D).

made in small quantities early after infection and in much higher amounts later (36). The product of the 0.6-kb mRNA by in vitro translation is a 14K (35) or a 28K (40) protein. No Ad5 polypeptide of appropriate size, produced with these kinetics, has been found with the E1A-C1 serum in experiments described herein or in other studies carried out at various times after infection (data not shown). Thus, either no protein is made in vivo from the 0.6-kb mRNA or the viral protein is not recognized by this serum. The latter could result if the carboxy terminus is not exposed in this polypeptide or if the reading frame of the 0.6-kb mRNA is different from that of the 1.1- and 0.9-kb mRNAs. Recent sequencing data obtained with Ad7 (11) and Ad2 (A. Virtanen, personal communication) suggest that the 0.6-kb mRNA does in fact have a different reading frame after the splice than the larger E1A mRNAs.

The antipeptide sera also allowed us to examine the location of the 58K protein and the E1A proteins in infected cells by immunofluorescence. Because it is possible that the antipeptide sera cross-react with cellular proteins, it is not possible to make absolute judgements on the intracellular locations of these viral proteins. However, if one assumes that the major activities of these sera are against the Ad5 E1 polypeptides, then one can make some tentative conclusions based on the immunofluorescence data. At early times after lytic infection, the 58K species, as detected by 58-C1 serum, was found predominantly in the perinuclear region of the cytoplasm and to some extent within the nucleus. The E1A proteins, as detected by E1A-C1 serum, were found in discrete patches within the nucleus and in diffuse areas of the cytoplasm. Results obtained in cell fractionation studies (D. T. Rowe, F. L. Graham, and P. E. Branton, submitted for publication) were also in agreement with these fluorescent-antibody data. Thus, both the 58K protein and the E1A proteins may function, at least in part, in or near the nucleus. This observation is of some interest as the E1A proteins apparently play a role in regulating transcription in infected cells (4, 29).

Because the antipeptide sera are highly specific, they should be of considerable value in purifying Ad5 proteins from cell extracts by immunoaffinity chromatography. The synthetic peptides were found to compete effectively with the viral proteins for binding with the antibody, and thus these peptides may be used to elute antibody-bound proteins from columns. This type of purification would be efficient and rapid, and it should preserve the biological activity of the proteins and therefore aid in the study of biological function in vitro with highly purified material.

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