Identification of Adenovirus 12-Encoded E1A Tumor Antigens Synthesized in Infected and Transformed Mammalian Cells and in *Escherichia coli*

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A 16-amino acid peptide, H₂N-Arg-Glu-Gln-Thr-Val-Pro-Val-Asp-Leu-Ser-Val-Lys-Arg-Pro-Arg-Cys-COOH (peptide 204), targeted to the common C-terminus of human adenovirus 12 (Ad12) tumor antigens encoded by the E1A 13S mRNA and 12S mRNA, has been synthesized. Antibody prepared in rabbits against peptide 204 immunoprecipitated two proteins of apparent *M_r* 47,000 and 45,000 from extracts of [³⁵S]methionine-labeled Ad12-early infected KB cells and a 47,000 protein from extracts of the Ad12-transformed hamster cell line, HE Cl9. Immunoprecipitation analysis of infected and transformed cells labeled with ³²P_i showed that both major Ad12 E1A T antigens are phosphoproteins. Immunofluorescence microscopy of Ad12-early infected KB cells with anti-peptide antibody showed the site of E1A protein concentration to be predominantly nuclear. E1A proteins were detected by immunofluorescence at 4 to 6 h postinfection and continued to increase until at least 18 h postinfection. Anti-peptide 204 antibody was used to analyze the proteins synthesized in *Escherichia coli* cells transformed by plasmids containing cDNA copies of the Ad12 E1A 13S mRNA or 12S mRNA under the control of the *tac* promoter (D. Kimelman, L. A. Lucher, M. Green, K. H. Brackmann, J. S. Symington, and M. Ptashne, Proc. Natl. Acad. Sci. U.S.A., in press). A major protein of ca. 47,000 was immunoprecipitated from extracts of each transformed *E. coli* cell clone. Two-dimensional gel electrophoretic analysis of immunoprecipitates revealed that the T antigens synthesized in infected KB cells, transformed hamster cells, and transformed *E. coli* cells possess very similar molecular weights and acidic isoelectric points of 5.2 to 5.4.

The human adenoviruses fall into five distinct groups based on DNA homology measurements, A through E (12a). Each group contains homologous transforming regions (22). The transforming regions of groups A (adenovirus type 12 [Ad12], Ad18, and Ad31), B (Ad3, Ad7, Ad11, Ad14, Ad16, and Ad21), and C (Ad1, Ad2, Ad5, and Ad6) have been localized in early region 1 (E1) in the left 1.3 to 11% of the viral genome. E1 comprises two transcription units, early region 1A (E1A) and E1B. The tumor (T) antigens encoded by adenovirus E1A are of particular interest since they appear to function in the activation of adenovirus early genes (2, 21) and cellular genes (18, 23, 32).

Ad12 (group A) is the most highly oncogenic adenovirus and induces tumors in newborn hamsters in as little as 2 to 3 weeks (9, 10, 13). The reason why Ad12 possesses a much higher oncogenic potential than other adenoviruses is of great interest. Recent studies have indicated that the T antigen(s) encoded by the Ad12 E1A 13S mRNA may function to specifically suppress the expression of class I transplantation antigens, thus providing a possible explanation for the high tumorigenicity of Ad12-transformed cells (3, 29).

In this paper, we describe the synthesis of the 16-amino acid peptide, peptide 204, encoded at the common C terminus of the Ad12 E1A 13S and 12S mRNA molecules. Antibody prepared against peptide 204 is shown to immunoprecipitate two major Ad12 E1A T antigens from infected and transformed mammalian cells and from *Escherichia coli*

cells transformed by plasmids containing cDNA copies of the Ad12 E1A 13S or 12S mRNA. Analysis by two-dimensional gel electrophoresis suggested that the T antigens synthesized in infected and transformed mammalian cells are closely related to the proteins synthesized in *E. coli* transformed by the cDNA-containing plasmids. The Ad12 E1A tumor antigen-specific anti-peptide antibodies provide specific reagents for the further study of the Ad12 E1A tumor antigens.

MATERIALS AND METHODS

Peptide synthesis, coupling of peptides to carrier protein, and preparation of anti-peptide antibody. Peptide 204, which encodes a 16-amino acid sequence at the common C terminus of the Ad12 E1A T antigens (H₂N-Arg-Glu-Gln-Thr-Val-Pro-Val-Asp-Leu-Ser-Val-Lys-Arg-Pro-Arg-Cys-COOH), was synthesized manually by the solid-phase procedure (1), by methods recently described (12). The peptide was coupled to keyhole limpet hemocyanin (KLH) through the sulfhydryl group of the cysteine at the C terminus. Antibody was raised in 2-month-old New Zealand white rabbits. The induction of anti-peptide antibody was monitored by enzyme-linked immunosorbent assay and by immunoprecipitation analysis of [³⁵S]methionine-labeled extracts of Ad12-infected KB cells (12).

Preparation of [³⁵S]methionine-labeled and ³²P_i-labeled whole-cell extracts of Ad12-early infected KB cells and Ad12-transformed cells. Ad12 (strain Huie) was grown in a suspension culture of KB cells in Eagle minimal essential medium (MEM) containing 5% horse serum (14). Ad12-early infected

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KB cells were labeled with [^{35} S]methionine in the presence of 1- β -D-arabinofuranosylcytosine (araC) (8). KB cells (200 ml ; 4×10^5 cells per ml) were centrifuged and infected with 4 to 50 PFU of purified Ad12 stock per cell in 1/20 the initial volume of MEM. After 1 h at 37°C , cells were diluted to one-half the initial volume, and $20 \text{ }\mu\text{g}$ of araC per ml was added. Additional araC ($20 \text{ }\mu\text{g}/\text{ml}$) was added at 15 and 26 h postinfection. At 30 h postinfection, the cells were centrifuged, washed twice with warm methionine-free MEM containing araC, and resuspended in 50 ml of methionine-free MEM containing $20 \text{ }\mu\text{g}$ of araC per ml, 2% dialyzed horse serum, and 1 mCi of [^{35} S]methionine ($1,100 \text{ Ci}/\text{mmol}$; New England Nuclear Corp.). After incubation at 37°C for 8 to 10 h, the cells were centrifuged and washed twice with cold phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride and 0.1% Trasylol (Aprotinin; $10,000$ Kallikrein Inactivation Units per ml; Mobay Chemical Corp.). The pellet was solubilized by sonication in 5.0 ml of whole-cell sonication buffer (20 mM Tris hydrochloride [pH 7.4], 10% glycerol, 50 mM NaCl, 5 mM EDTA, 1 mM β -mercaptoethanol, 1% deoxycholate, 1% Nonidet P-40, 0.5 M urea) and centrifuged at $100,000 \times g$ for 1 h at 4°C in a Beckman 50 Ti rotor. The supernatant fluid, containing ca. 2×10^8 trichloroacetic acid-insoluble cpm/ml, was used for immunoprecipitation analysis.

The Ad12-transformed hamster cell line, HE Cl9, was grown as described previously (17). When monolayer cultures (75 cm^2) were 70 to 80% confluent they were washed twice with methionine-free Dulbecco modified MEM containing 2% dialyzed fetal bovine serum (labeling medium) and then preincubated for 1 h at 37°C in labeling medium. The medium was removed, and the cells were incubated for 4 h at 37°C in 2 ml of labeling medium containing $400 \text{ }\mu\text{Ci}$ of [^{35} S]methionine. The monolayer was washed twice with PBS containing 1 mM phenylmethylsulfonyl fluoride and 0.1% Trasylol, sonicated in 2 ml of whole-cell sonication buffer, and clarified at $100,000 \times g$ as described above.

The cells were labeled with $^{32}\text{P}_i$ (ICN Pharmaceuticals Inc., Irvine, Calif.) as follows. Monolayer cultures of Ad12-infected KB cells or HE Cl9 cells (75 cm^2) were washed twice with phosphate-free MEM containing 2% dialyzed fetal bovine serum (labeling medium) and preincubated in the labeling medium for 1 h. Two milliliters of fresh labeling medium containing 1 mCi of carrier-free $^{32}\text{P}_i$ was then added, and incubation was continued for 4.5 h at 37°C . Monolayers were washed twice with PBS containing 1 mM phenylmethylsulfonyl fluoride and 0.1% Trasylol. The cells were lysed with 3 ml of whole-cell sonication buffer and processed as described above.

Preparation of [^{35} S]methionine-labeled extracts of *E. coli* cells transformed by plasmids pAd418 and pAd416. Plasmids pAd418 and pAd416, which contain cDNA copies of the Ad12 E1A 13S mRNA and 12S mRNA, respectively (25), under the control of the *tac* promoter, were constructed as described by D. Kimelman, L. A. Lucher, K. H. Brackmann, J. S. Symington, M. Ptashne, and M. Green (Proc. Natl. Acad. Sci., in press). Clones of pAd418- and pAd416-transformed *E. coli* W3110i⁹ were grown at 37°C to an absorbance at 550 nm of 0.6 to 0.8 in M9 CAT medium (M9 medium supplemented with 0.2% Casamino Acids, $1.2 \text{ }\mu\text{g}$ of thiamine per ml, and $50 \text{ }\mu\text{g}$ of ampicillin per ml), centrifuged at 4°C for 10 min at $2,000 \times g$, and washed twice with methionine-free labeling medium (M9 medium containing $50 \text{ }\mu\text{g}$ each of L-threonine, L-leucine, L-proline, L-arginine, L-histidine, L-isoleucine, and L-valine per ml, instead of Casa-

mino Acids, and $50 \text{ }\mu\text{g}$ of ampicillin per ml). Cells were suspended in labeling medium at two-fifths the initial volume and incubated at 37°C for 30 min. Isopropyl- β -D-thiogalactoside (Bethesda Research Laboratories, Gaithersburg, Md.) was added to 1 mM, and the cells were further incubated for 60 min at 37°C . [^{35} S]methionine was added to $5 \text{ }\mu\text{Ci}/\text{ml}$, and the cells were incubated for 5 min at 37°C , centrifuged, and resuspended at one-fifth the initial volume in *E. coli* sonication buffer (50 mM Tris hydrochloride [pH 7.9], 25% sucrose, 1% Nonidet P-40, 0.5% sodium deoxycholate, 2 mM dithiothreitol, 5 mM EDTA, 0.1% Trasylol, 1 mM phenylmethylsulfonyl fluoride). Cells were sonically disrupted and then clarified by centrifugation at $6,000 \times g$ for 30 min at 4°C , and the supernatant fluid was stored at -20°C until used for immunoprecipitation analysis.

Immunoprecipitation analysis of [^{35} S]methionine-labeled and $^{32}\text{P}_i$ -labeled cell extracts. Anti-peptide 204 serum was clarified immediately before use by centrifugation at $100,000 \times g$ for 5 min in a Beckman airfuge. Portions of whole-cell extract containing 0.5×10^7 to 1.0×10^7 trichloroacetic acid-insoluble [^{35} S]methionine or $^{32}\text{P}_i$ cpm, prepared as described above, were immunoprecipitated with $10 \text{ }\mu\text{l}$ of anti-peptide 204 or preimmune rabbit serum. The immunoprecipitates were processed and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (15). For peptide competition experiments, $5 \text{ }\mu\text{g}$ of peptide 204 or of an unrelated peptide (peptide 19 [Glu-His-Phe-Leu-Pro-Leu-Arg-Asn-Ile-Cys]) per ml were included in the immunoprecipitation reaction (12).

Two-dimensional gel electrophoretic analysis of whole-cell extracts and immunoprecipitates prepared from [^{35} S]methionine-labeled cells. Whole-cell extracts were prepared from [^{35} S]methionine-labeled Ad12-infected and -transformed cells, as described above. About 7×10^6 cpm of each preparation were dialyzed against 50 mM NH_4HCO_3 at room temperature, lyophilized, dissolved in 40 to $80 \text{ }\mu\text{l}$ of lysis buffer, and loaded on an isoelectric focusing gel (12.5 cm by 4 mm) as previously described (4, 24). Immunoprecipitates were prepared with anti-peptide 204 antibody from extracts of [^{35} S]methionine-labeled Ad12-infected KB cells, HE Cl9 cells, or *E. coli* cells transformed by pAd416 or pAd418 as described above. The pellet of immunoprecipitated proteins was processed and loaded on an isoelectric focusing gel essentially as described by Jochemsen et al. (20), as follows. The immunoprecipitate was extracted with $40 \text{ }\mu\text{l}$ of 0.2% sodium dodecyl sulfate–9.5 M urea–5% 2-mercaptoethanol–2% ampholine by brief incubation at 56°C . After clarification by centrifugation at $10,000 \times g$ for 5 min, Nonidet P-40 was added to 2%, and the eluted proteins were loaded on an isoelectric focusing gel. After focusing, the gel was electrophoresed in the second dimension on a 10% polyacrylamide gel (width, 13 cm ; length, 10 cm ; thickness, 1.5 mm). Gels were soaked sequentially for 30 min each time in 10% acetic acid–water–1 M sodium salicylate (5), dried on Whatman 3MM paper, and exposed to Kodak X-Omat film at -70°C .

Indirect immunofluorescence staining. Subconfluent monolayers of KB cells growing on 12-mm circular cover slips (Bellco Glass, Inc., Vineland, N.J.) in 24-well plastic trays were infected 1 day after seeding with 80 PFU of Ad12 per cell. Cells were cultured in the presence or absence of $20 \text{ }\mu\text{g}$ of araC per ml and fixed at various times postinfection. HE Cl9 cells were cultured on cover slips for 2 days before fixing. Cells were fixed in methanol for 10 min at -20°C (KB cells) or in methanol followed by acetone for 10 min (HE Cl9 cells). Cells on cover slips were prewet in PBS and incubated

for 1 h in a humidity chamber with 20 μ l of antipeptide 204 rabbit serum or normal rabbit serum diluted 1:5 in PBS. For the competition assay, 10 μ g of peptide was included per 20 μ l of serum, and the antibody plus peptide was incubated for 10 min at 4°C before use. After antibody treatment, cells were washed three times in PBS and incubated in the dark with fluorescein-conjugated goat anti-rabbit immunoglobulin G (Pel Freez) which had been diluted 1:5 and centrifuged at $100,000 \times g$ in a Beckman airfuge for 5 min immediately before use. After 1 h of incubation, they were washed three times in PBS and mounted on microscope slides in a drop of Aqua-Mount (Lerner Laboratories, New Haven, Conn.). Fluorescence was observed with a Nikon Diaphot-TMD microscope equipped with an epifluorescence attachment. Photographs were taken at a magnification of $\times 50$ on Kodak Tri X pan film with a 5-s exposure. Diafine developer (Acufine, Inc., Chicago, Ill.) was used.

RESULTS

Preparation of antipeptide antibody targeted to a 16-amino acid sequence encoded at the common C terminus of the major Ad12 E1A T antigens. Based on the amino acid sequence deduced from the DNA sequence of the Ad12 E1A coding region (25, 31), we have synthesized peptide 204 (H₂N-Arg-Glu-Gln-Thr-Val-Pro-Val-Asp-Leu-Ser-Val-Lys-Arg-Pro-Arg-Cys-COOH). Peptide 204 consists of the penultimate 16-amino acid sequence at the common C terminus of the T antigens encoded by the Ad12 E1A 13S and 12S mRNAs (7).

FIG. 1. Immunoprecipitation of extracts of [³⁵S]methionine-labeled Ad12-early infected KB cells by antibody raised against peptide 204. Labeled extracts were immunoprecipitated, and the immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography as described in the text. Peptide competition was performed by including 5 μ g of peptide per ml in the immunoprecipitation reaction. Lanes: a, immunoprecipitation by antipeptide 204 serum; b, immunoprecipitation by antipeptide 204 antibody in the presence of peptide 204; c, immunoprecipitation in the presence of an unrelated peptide, peptide 19; d, immunoprecipitation by preimmune rabbit serum. The lane at the right contains molecular weight markers of Me-¹⁴C-labeled bovine serum albumin (69K), ovalbumin (46K), carbonic anhydrase (30K), and lactoglobulin A (18.4K).

FIG. 2. Immunoprecipitation of extracts of [³⁵S]methionine-labeled Ad12-transformed hamster cell line, HE Cl9, by antibody raised against peptide 204. Immunoprecipitation analysis was performed as described in the legend to Fig. 1. Lanes: a, immunoprecipitation by antipeptide 204 antibody; b, immunoprecipitation by antipeptide 204 antibody in the presence of 5 μ g of peptide 204 per ml; c, immunoprecipitation in the presence of 5 μ g of peptide 19 per ml. The lane at the right contains molecular weight markers as described in the legend to Fig. 1.

Peptide 204 was coupled to keyhole limpet hemocyanin through the sulfhydryl group of the C terminal cysteine residue, and antibody was raised to the peptide-protein conjugate in two rabbits. Both rabbits were positive for the presence of antipeptide antibody in 2 months as determined by enzyme-linked immunosorbent assay.

Immunoprecipitation by antipeptide 204 antibody of Ad12 E1A T antigens from extracts of Ad12-infected KB cells and an Ad12-transformed hamster cell line. Ad12-infected KB cells were incubated in medium containing araC to block viral DNA synthesis and to maintain the early stages of productive infection. Infected cells were labeled with [³⁵S]methionine at 30 to 36 h postinfection. By using this protocol, the synthesis of E1A proteins has been reported to be amplified in Ad5-infected HeLa cells (8). Whole-cell extracts were prepared and immunoprecipitated with antibody raised against peptide 204. Two prominent proteins of apparent M_r 's of 47,000 (47K) and 45K were precipitated by antipeptide 204 antibody (Fig. 1a) but not by preimmune rabbit serum (Fig. 1d). Antipeptide 204 antibody did not immunoprecipitate the 47K and 45K proteins from extracts of mock-infected cells (data not shown). Evidence for the specificity of immunoprecipitation with antipeptide 204 antibody was obtained by peptide competition experiments. Peptide 204, when included in the immunoprecipitation reaction at a concentration of 5 μ g/ml, completely blocked the precipitation of the 47K and 45K proteins (Fig. 1b). The inclusion of an unrelated peptide in the immunoprecipitation reaction had no effect on the immunoprecipitation of 47K and 45K proteins (Fig. 1c). These data suggest that the 47K and 45K

FIG. 3. Immunoprecipitation of extracts of ^{32}P -labeled Ad12-early infected KB cells by antibody raised against peptide 204. Immunoprecipitation analysis was performed as described in the legend to Fig. 1. Lanes: a, immunoprecipitation by anti-peptide 204 antibody; b, immunoprecipitation by anti-peptide 204 antibody in the presence of peptide 204; c, immunoprecipitation by anti-peptide 204 antibody in the presence of peptide 19; d, immunoprecipitation by preimmune rabbit serum.

FIG. 4. Immunoprecipitation of extracts of ^{32}P -labeled Ad12-transformed cell line HE Cl9 by antibody raised against peptide 204. Immunoprecipitation analysis was performed as described in the legend to Fig. 1. Lanes: a, immunoprecipitation by anti-peptide 204 antibody; b, immunoprecipitation by anti-peptide 204 antibody in the presence of peptide 204; c, immunoprecipitation by anti-peptide 204 antibody in the presence of peptide 19; d, immunoprecipitation by preimmune rabbit serum. The lane at the right contains molecular weight markers as described in the legend to Fig. 1.

proteins represent the two major T antigens encoded by the Ad12 E1A 13S and 12S mRNAs.

The Ad12-transformed cell line, HE Cl9, contains multiple copies of all or nearly all of the Ad12 genome (17). Immunoprecipitation analysis was performed with extracts of [^{35}S]methionine-labeled HE Cl9 cells, using anti-peptide 204 antibody. A major protein with an apparent M_r of 47K was immunoprecipitated (Fig. 2a) (the minor proteins immediately flanking the 47K protein in the gel appear to be cellular contaminants since their immunoprecipitation was not blocked by peptide 204 in Fig. 2b). The 45K T antigen that was observed in Ad12-infected cells (Fig. 1a) was not readily detected in the immunoprecipitate prepared from HE Cl9 cells. Possibly the 45K T antigen was present at very low levels. Preimmune rabbit serum did not precipitate the 47K T antigen from HE Cl9 cells (data not shown). Peptide 204 blocked the immunoprecipitation of the 47K species (Fig. 2b), whereas an unrelated peptide had no effect (Fig. 2c).

Phosphorylation of the Ad12 E1A T antigen in Ad12-infected and -transformed cells. Recent reports have provided evidence that the Ad5 E1A T antigens are phosphorylated (8, 33). To determine whether Ad12 E1A T antigens are phosphorylated, we metabolically labeled Ad12 early infected cells with $^{32}\text{P}_i$. Whole-cell extracts were prepared and immunoprecipitated with anti-peptide 204 antibody. Both the 47K and 45K T antigens are phosphorylated in Ad12-productively infected cells (Fig. 3a). Preimmune rabbit serum did not precipitate either T antigen (Fig. 3d). Peptide 204 blocked the immunoprecipitation of ^{32}P -labeled T antigens (Fig. 3b), whereas an unrelated peptide did not (Fig. 3c). Immunoprecipitation analysis was performed also with ^{32}P -labeled HE Cl9 transformed hamster cells. The 47K T

FIG. 5. Immunoprecipitation of extracts of [^{35}S]methionine-labeled *E. coli* W3110i cells transformed by pAd416 or pAd418, using antibody raised against peptide 204. Immunoprecipitation analysis was performed as described in the legend to Fig. 1. Lanes: a, immunoprecipitation of extracts of pAd416-transformed cells by anti-peptide 204 antibody; b, immunoprecipitation of extracts of pAd418-transformed cells by anti-peptide 204 antibody; c, immunoprecipitation of pAd418-transformed cell extracts by preimmune serum. The lane at the right contains molecular weight markers as described in the legend to Fig. 1.

antigen present in HE C19 is phosphorylated (Fig. 4a and c). The immunoprecipitation of the phosphorylated 47K T antigen was blocked by peptide 204 (Fig. 4b), and normal rabbit serum did not precipitate the protein (Fig. 4d). Again, no evidence was found for the presence of the 45K T antigen in HE C19 cells.

Immunoprecipitation by anti-peptide 204 antibody of Ad12 E1A T antigens from extracts of *E. coli* cells transformed by two Ad12 E1A cDNA-containing plasmids. We have recently constructed two plasmids, pAd418 and pAd416, that contain cDNA copies of the Ad12 E1A 13S mRNA and 12S mRNA, respectively, under the control of the *tac* promoter (Kimelman et al., in press). Clones of *E. coli* W3110i^a cells transformed by pAd418 and pAd416 synthesize proteins of apparent M_r 's of 47K that are absent from nontransformed cells (Kimelman et al., in press). Immunoprecipitation analysis was performed to determine the relationship between the new proteins synthesized in transformed *E. coli* cells and the Ad12 E1A T antigens detected in Ad12-infected and -transformed mammalian cells. Cultures of pAd418- and pAd416-transformed *E. coli* cells were induced by treatment with 1 mM isopropyl- β -D-thiogalactoside and labeled with [³⁵S]methionine, and cell extracts were prepared and analyzed by immunoprecipitation with anti-peptide 204 antibody. A major 47K protein was precipitated from extracts of both pAd416 (Fig. 5a)- and pAd418 (Fig. 5b)-transformed *E. coli*

cells but not from nontransformed *E. coli* cells. A minor 27K protein was also detected in the immunoprecipitates prepared from pAd416- and pAd418-transformed *E. coli* cells (Fig. 5a and b).

Two-dimensional gel electrophoresis of immunoprecipitates from Ad12-infected KB cells, Ad12-transformed hamster cells, and pAd418- and pAd416-transformed *E. coli* cells. The Ad2 (4, 16, 19), Ad5 (8, 26), and Ad12 (30) E1A T antigens are resolved by two-dimensional gel electrophoresis at a unique acidic portion of the gel. From two to six E1A T antigens have been observed with apparent M_r values ranging from 37K to 58K and with pI values ranging from 4.5 to 6.0 (4, 8, 15, 19, 33). In preliminary two-dimensional gel electrophoresis studies with [³⁵S]methionine-labeled cell extracts we detected 45K to 47K acidic proteins in Ad12 early-infected and transformed cells. Further two-dimensional gel electrophoresis studies were then performed with immunoprecipitates obtained with anti-peptide 204 antibody and extracts of [³⁵S]methionine-labeled Ad12-early infected KB cells, Ad12-transformed hamster cells, and pAd418- and pAd416-transformed *E. coli*. Two major spots with apparent M_r values of 45K and 47K and with pI values of 5.4 and 5.2, respectively, were resolved in the immunoprecipitate from Ad12-infected KB cells. A 47K protein with a pI of 5.2 was detected in the immunoprecipitate from HE C19 cells (Fig. 6B). The immunoprecipitates prepared from pAd416- and pAd418-trans-

FIG. 6. Two-dimensional gel electrophoresis of immunoprecipitates prepared from extracts of [³⁵S]methionine-labeled Ad12-infected KB cells, HE C19 cells, and pAd418- and pAd416-transformed *E. coli* cells by anti-peptide 204 antibody. The immunoprecipitates were analyzed individually or after mixing as depicted in the figure. Only the portion of the gel containing the region to which the acidic Ad12 E1A proteins migrate is shown.

formed *E. coli* cells each contained a major 47K protein with a pI value of 5.4 or 5.2 (Fig. 6D and E); a minor 27K protein at a pI of 5.7 was also observed in each immunoprecipitate (Fig. 6D and E). Samples of the immunoprecipitates from pAd418- and pAd416-transformed cells were mixed and subjected to two-dimensional gel electrophoresis. Two spots corresponding in pI values to the 47K proteins detected in each transformed *E. coli* clone were observed (Fig. 6F). Immunoprecipitates prepared from Ad12-infected KB cells and from pAd416-transformed *E. coli* cells were mixed and analyzed. Only two spots were observed (Fig. 6C), suggesting that the Ad12 T antigens synthesized in *E. coli* and in Ad12-infected KB cells possess very similar mobilities and pI values.

Subcellular localization of the E1A proteins in early Ad12-infected KB cells by indirect immunofluorescence and confirmation of antibody specificity. KB cells were infected with Ad12, maintained in the presence of araC to amplify early proteins, and fixed at 24 h postinfection. Cells were stained by the indirect immunofluorescence technique, using anti-peptide 204 antibody. This produces a bright fluorescence in virtually all of the cells (Fig. 7A), primarily in the cell nucleus. The specificity of the anti-peptide antibody was tested by competition with peptide 204 which was included during the antibody incubation. As was the case with immunoprecipitation, the inclusion of peptide 204 abolished the effect of the anti-peptide 204 antibody. The cells exhibited only a faint staining (Fig. 7B), indistinguishable from that found when normal rabbit serum was used on infected cells or from that found when anti-peptide 204 antibody was used on mock-infected cells (data not shown).

Indirect immunofluorescent localization of Ad12 E1A proteins in KB cells during the course of infection without metabolic inhibitors. KB cells growing on cover slips were infected with Ad12 virus without the addition of metabolic inhibitors and fixed with methanol at intervals after infection. Cells were stained as described above. No differences between the anti-peptide 204 antibody (Fig. 8A)- and normal serum (data not shown)-treated cells were observed at 4 h postinfection. At 6 h postinfection, there was a detectably greater general fluorescence seen in the anti-peptide 204 antibody-treated cells (data not shown). By 8 h postinfection, anti-peptide 204 antibody-treated cells showed a distinctly greater fluorescence, with a nuclear concentration being evident in many cells (Fig. 8B). By 12 h postinfection, the nuclear concentration was obvious in all cells that were not too rounded for clear distinction of the nucleus (data not shown). By 18 h postinfection, there was a further increase in the intensity of the nuclear fluorescence in cells stained with anti-peptide 204 antibody (Fig. 8C). When cells infected for 18 h were stained with normal rabbit serum, there was only a faint cytoplasmic glow, and no fluorescence was observed in the nucleus (Fig. 8D).

DISCUSSION

We have synthesized a peptide (peptide 204) containing the sequence of the 16 penultimate amino acids at the common C terminus of the T antigens encoded by the Ad12 E1A 13S and 12S mRNAs. The peptide was coupled to keyhole limpet hemocyanin and used to raise antibodies in rabbits against the Ad12 E1A T antigens. We show here that the anti-peptide antibody precipitated two major proteins of 47K and 45K from Ad12-early infected KB cells and a major 47K protein from the Ad12-transformed hamster cell line,

FIG. 7. Subcellular localization of E1A T antigens in the nucleus of Ad12-early (araC) infected KB cells by anti-peptide 204 antibody, and demonstration of the specificity of the antibody. Cells were fixed at 24 h postinfection and stained by the indirect immunofluorescent technique with (A) antibody raised against peptide 204 and (B) antibody raised against peptide 204 incubated with peptide 204.

HE Cl9. In addition, the anti-peptide antibody immunoprecipitated a major 47K and minor 27K protein from *E. coli* cells transformed by plasmids containing the cDNA clones encoding the Ad12 E1A 12S and 13S mRNAs (25); the 27K protein could be a degradation product of the 47K protein or an Ad12 E1A protein synthesized in *E. coli* by initiation at a downstream ATG codon. A close relationship between the

FIG. 8. Indirect immunofluorescent staining of E1A proteins in Ad12-infected KB cells as a function of time postinfection. Cells were infected without metabolic inhibitors, fixed at intervals, and stained with (A to C) antibody raised against peptide 204 or (D) normal rabbit serum. Times postinfection are (A) 4 h, (B) 8 h, and (C and D) 18 h.

Ad12 E1A T antigens synthesized in infected and transformed mammalian cells and those synthesized in transformed *E. coli* cells is supported by the findings that the proteins immunoprecipitated from extracts of four different cell types by antibody to peptide 204 possess very similar molecular weights and isoelectric points.

The conclusion that the immunoprecipitated proteins are the Ad12 E1A T antigens is supported by: (i) the specific inhibition of the immunoprecipitation reaction by peptide 204; (ii) the apparent M_r of 45K to 47K which is similar to that observed for Ad2-, Ad5-, and Ad12-encoded E1A T antigens (4, 8, 15, 19, 20, 26, 30, 33); (iii) the characteristic acidic isoelectric point observed by two-dimensional gel electrophoresis; (iv) the phosphorylation of the proteins immunoprecipitated from Ad12-infected and -transformed mammalian cells (8, 33); (v) the immunofluorescent staining of Ad12-infected KB cells by antipeptide 204 antibody, with the specificity of the staining being confirmed by the blocking of immunofluorescence with peptide 204; and (vi) the fluorescence associated with the nucleus, since a nuclear

localization of E1A protein has been suggested by cell fractionation and immunofluorescence studies (6, 11, 27, 33).

The immunofluorescence studies presented here provide evidence for the identity of the proteins that are immunoprecipitated by antipeptide antibody and the Ad12 E1A proteins that are concentrated in the nucleus of Ad12-infected KB cells. There is a movement of E1A protein to the cell nucleus with or without the blocking of DNA synthesis by infection in the presence of araC. When no metabolic inhibitors were used, the concentration of E1A proteins in the nucleus was pronounced by about 6 to 8 h postinfection and increased throughout infection. A nuclear localization of Ad12 E1A T antigens was also observed in the Ad12-transformed hamster cell line, HE Cl9 (unpublished data). This agrees with recent findings on Ad5-infected cells analyzed with antipeptide antibody directed to Ad5 E1A T antigens (6, 33).

One would expect to find the same major E1A T antigens in Ad12-infected human KB cells as in HE Cl9 cells that contain most if not all of the Ad12 genome (17). Appreciable levels of the smaller Ad12 E1A T antigen, the 45K protein,

were not detected. Perhaps this protein is synthesized in low levels in HE Cl9 cells. Alternatively, it is possible that the regulation of splicing in hamster cells is different from that in human cells and that the 12S mRNA represents only a minor species in HE Cl9 cells.

The variable number of different species of E1A T antigens that are translated in vitro and in vivo from Ad2 and Ad5 13S and 12S mRNA species is not understood. Different laboratories have detected from two to six proteins by one- and two-dimensional gel electrophoresis, representing one to three proteins encoded by each mRNA (4, 8, 15, 19, 26, 33). It is not clear whether this variability represents posttranslational modifications that are dependent upon the metabolic state of the infected cell, in vitro translation conditions, or possible artifacts that occur during the processing of cell extracts for analysis.

Very recently, anti-peptide antibody has been prepared against a 13-amino acid sequence encoded at the unique region of the Ad5 E1A 13S mRNA (6) and against a 6-amino acid sequence at the C termini of the Ad5 E1A 13S and 12S mRNAs (33). The antibody directed to the Ad5 E1A 13S mRNA unique region detected a major 46K protein, by the protein immunoblot method, but would not immunoprecipitate the Ad5 T antigen from extracts of infected cells. The anti-peptide antibody targeted to the C terminus of the Ad5 E1A 13S and 12S mRNAs immunoprecipitated four major proteins of 52K, 50K, 48.5K, and 45K; the 52K, the 48.5K, and a minor 37.5K species are products of the 13S mRNA, and the 50K, the 45K, and a minor 35K species are products of the 12S mRNA (28, 33).

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