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Production of a Monospecific Antiserum Against the Early Region 1A Proteins of Adenovirus 12 and Adenovirus 5 by an Adenovirus 12 Early Region 1A–β-Galactosidase Fusion Protein Antigen Expressed in Bacteria

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Antisera were prepared against the amino acid sequences encoded within the N-terminal half of the adenovirus 12 (Ad12) early region 1A (E1A) gene. This was accomplished by construction of a plasmid vector which encoded the N-terminal 131 amino acids of Ad12 E1A joined in frame to the coding sequence of β-galactosidase. After induced synthesis in *Escherichia coli*, the Ad12 E1A–β-galactosidase fusion protein (12-1A-FP) was extracted with urea and used to raise antibodies in rabbits. The 12-1A-FP antisera immunoprecipitated major phosphoproteins of 39,000 and 37,000 apparent molecular weights from Ad12-transformed and infected cells. The 12-1A-FP antisera also immunoprecipitated E1A phosphoproteins from Ad5-transformed and infected cells. Immunospecificity of the 12-1A-FP antisera was demonstrated by the ability of 12-1A-FP antigen to block immunoprecipitation of E1A proteins. Furthermore, E1A proteins immunoprecipitated from in vivo-labeled cells comigrated with those translated in vitro by RNA that had been hybridization selected to E1A DNA.

Characterization of proteins encoded by regulatory genes is critical for a comprehensive understanding of gene expression. The early region 1A (E1A) gene of adenovirus plays a dynamic role during both cellular infection and transformation. Analysis of several E1A mutants of adenovirus 5 (Ad5) has revealed that there is a transcriptional dependence of early viral genes upon a product of the E1A gene (4, 24). From the E1A gene of Ad5, two major RNAs of 12S and 13S are transcribed, which share the same 5′ and 3′ termini but differ by the amount of intervening sequence removed by splicing (5, 10, 25, 41). The 12S and 13S mRNAs encode acidic proteins of 242 and 288 amino acids, respectively (32). The amino acid sequences of both E1A proteins are identical except for an internal stretch of 46 amino acids which is unique to the larger E1A protein (32). However, only the product of the larger acidic protein is required for modulation of early gene transcription (28, 34).

Not all mutations in the E1A gene of Ad5 exhibit the same pleiotropic effect. For example, the defects contained in the frameshift mutants H5*hr*1 and H5*in*500 and the nonsense mutant *hr*440 each prevent full-length synthesis of the larger E1A acidic protein (9, 34, 40). However, the H5*hr*1 mutant does not synthesize an E1B, E2, E3, or E4 transcript (4), whereas the *hr*440 mutant expresses E1B and E4 transcripts (40), and the H5*in*500 mutant produces wild-type levels of E1B and E3 transcripts but low levels of E2 and E4 transcripts (9). Taken altogether, these findings indicate that the protein product of the larger E1A transcript, in fact, does modulate expression of other early genes, but that, depending upon the location and nature of the mutation in the E1A DNA, manifestation of this effect will vary. These findings raise the intriguing possibility that the E1A protein product contains separate functional domains.

How the E1A protein activates expression of early viral genes during infection is unresolved. Feldman et al. (12) propose that the E1A gene product indirectly mediates adenovirus transcription by interacting with host cell components rather than by direct recognition of regulatory control regions within the DNA. This they argue since the pseudorabies virus immediate early gene product is a heterologous activator of adenovirus gene transcription in the absence of E1A function.

It is likewise unclear whether the E1A protein contains transforming functions independent of modulation of transcription during infection. In rodent cells, integration of both E1A and E1B genes (0 to 11 map units [mu]) is necessary for complete transformation (18, 20, 44), but direct linkage of E1A and E1B genes is not necessary (43). An argument for an independent transforming function of E1A can be made from the analysis of the E1A *hr* mutants *hr*440 and H5*in*500, which are capable of transcribing E1B yet are incapable of transformation (9, 40).

In general, antisera from animals bearing adenovirus-induced tumors do not strongly react with E1A proteins, nor can such antisera be used to identify them definitively. These limitations, in addition to the low concentrations of E1A proteins produced in both transformed and infected cells, have hampered attempts towards the characterization and isolation of E1A proteins. However, antisera specific to Ad5 E1A proteins have recently been produced by using synthetic peptides. These antisera have been used to identify and localize Ad5 E1A proteins within cells by immunofluorescent staining of both the nuclear matrix and cytoplasmic regions (13, 49).

We report here the production of the first monospecific antiserum directed against the E1A proteins of Ad12. Ad12 is highly oncogenic compared with Ad5 (14, 15). In fact, the tumorigenic potential of Ad12 in rats has been recently attributed to the ability of the larger of the two Ad12 E1A products to suppress expression of class I major histocom-

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patibility antigens (6, 39). Ad5 E1A gene products do not affect the level of class I antigens in Ad5-transformed rat cells (6, 39). It is of further interest that the E1A genes of Ad12 are able to transcomplement Ad5 during infection (47). Similar to the E1A gene of Ad5, two major forms of mRNA originate from region E1A of Ad12. The Ad12 E1A mRNAs differ internally from one another by utilizing one of two donor splice sites but share the same acceptor splice site. The 3′ ends of each Ad12 E1A transcript are identical, whereas the 5′ ends are heterogeneous since two or more initiation sites are utilized (37, 38). Prediction from the DNA sequence indicates that these two Ad12 E1A mRNAs encode proteins of 266 and 235 amino acids (33, 42).

We describe the E1A products synthesized in Ad12-transformed cells with an antiserum directed against an E1A-β-galactosidase fusion protein antigen synthesized in *Escherichia coli*. We further demonstrate the ability of this Ad12 E1A monospecific antiserum to immunoprecipitate E1A proteins from both Ad5-infected and transformed cells and discuss the usefulness of this cross-reactive antiserum.

MATERIALS AND METHODS

Cells, viruses, and infection. The Ad12-transformed hamster cell line HA12/7 was previously described (11, 50). The Ad12-transformed BALB/c mouse cell line MO12/F10 was obtained from J. Williams, Carnegie Mellon University, Pittsburgh, Pa., and was maintained in minimal essential medium plus 5% fetal calf serum. The Ad5-transformed human cell line 293 was also previously described (19). The Ad5 virus was plaqued on HeLa cells and used to infect HeLa monolayer cells at a multiplicity of infection of 50 in the absence of drugs.

Growth and induction of Ad12 E1A–β-galactosidase fusion protein antigen in *E. coli*. *E. coli* LacIQ W3110 containing the plasmid pAd441, which encodes an Ad12 E1A–β-galactosidase fusion protein (12-1A-FP) as described below, was grown as an overnight culture in Casamino Acids (Difco Laboratories, Detroit, Mich.) medium containing (per liter): 5 g of Casamino Acids, 5.8 g of $NaHPO_4$, 3.0 g of KH_2PO_4, 0.5 g of NaCl, and 1.0 g of NH_4Cl. The mixture was brought to pH 7.2 to 7.6 with NaOH, autoclaved, and supplemented with 1 ml of sterile 0.2% vitamin B_1, 25 ml of 20% glucose, 1 ml of 1 M $MgSO_4$, and 50 mg of ampicillin. A sample of the overnight culture (5 ml) was added to 500 ml of Casamino Acids medium in a 2-liter Erlenmeyer flask and grown to an A_{600} of 0.3 at 37°C on a shaker. The culture was induced to synthesize the fusion protein by the addition of 119 mg of solid isopropyl-β-D-thiogalactopyranoside (IPTG) and continued rapid shaking for an additional 45 to 60 min.

Isolation of fusion protein antigen extract from *E. coli*. The induced *E. coli* isolates were pelleted by centrifugation at 8,000 rpm for 20 min at 4°C. The pellet was washed in phosphate-buffered saline (PBS) containing 25% glucose and was recentrifuged. The fusion protein was extracted in 10 M urea as described by Bikel et al. (7). The 10 M urea extract was dialyzed against PBS twice at room temperature (45 min each time) followed by exhaustive dialysis at 4°C.

Preparation of Ad12 E1A antisera. New Zealand white rabbits were first injected intradermally with 300 μg of the 12-1A-FP antigen extract in complete Freund adjuvant and then boosted at 2- to 4-week intervals with 300 μg of the 12-1A-FP in incomplete Freund adjuvant. Rabbits were ear bled 7 to 10 days after injection. Sera were adsorbed with a dialyzed 10 M urea extract from untransformed *E. coli* cells (5 to 10 mg of protein extract per ml of serum) at 0°C for 1 to 2 days, and the immune complex was pelleted at 40,000 rpm in a Ty65 rotor for 45 min at 4°C. The supernatant was readsorbed with a sonicated extract derived from either uninfected HeLa cells or non-adenovirus-transformed hamster cells (10^8 cells in 1 ml of PBS per 5 ml of serum), which was incubated at 0°C for 1 to 2 days and repelleted at 40,000 rpm for 45 min at 4°C. This final supernatant, referred to as the 12-1A-FP antiserum, was used for immunoprecipitation.

Labeling of cells and preparation of cell extracts. Cell monolayers were labeled with [^{35}S]methionine by incubation in methionine-free medium for 1 h followed by the addition of fresh methionine-free medium containing 50 μCi of [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.) per ml and continued incubation for 1 to 4 h. Cells were also labeled with carrier-free $^{32}P_i$ (New England Nuclear Corp., Boston, Mass.) first by incubation in phosphate-free medium for 1 h and then by the addition of fresh phosphate-free medium containing 125 μCi of $^{32}P_i$ per ml for 1 to 4 h. The labeled cells were rinsed in PBS, removed with a rubber policeman, pelleted, and washed once in PBS. In all subsequent steps, the buffers and extract were maintained at 4°C. The washed cell pellet was suspended in 3 volumes of 1 mM $MgCl_2$–1 mM KCl–10 mM Tris (pH 8.1), containing 100 kallikren IU of aprotinin per ml and 1 mM phenylmethylsulfonyl fluoride, and kept on ice for 5 min followed by rapid freezing in a dry ice-methanol bath (B. Atkinson, C. S. Ernst, B. E. D. Ghrist, M. Herlyn, D. Herly, A. Ross, M. Blaszczyk, Z. Steplewski, and H. Koprowski, Cancer Res., in press). The cell suspension was thawed and centrifuged at 40,000 rpm for 45 min at 4°C in a Ty65 rotor. The supernatant (fraction I) was removed to a new Eppendorf tube. The pellet was lysed in RIPA buffer (50 mM Tris, pH 7.2; 150 mM NaCl; 0.1% [wt/vol] sodium dodecyl sulfate [SDS]; 0.1% [wt/vol] sodium deoxycholate [NaDOC]; 0.1% [vol/vol] Triton X-100; 100 kallikren IU of aprotinin per ml; 1 mM phenylmethylsulfonyl fluoride) at a ratio of 3×10^7 cells per ml of RIPA buffer. The lysed pellet was vortexed, kept on ice for 20 min, and then briefly sonicated and centrifuged at 40,000 rpm in a Ty65 rotor at 4°C for 45 min. This supernatant (fraction II) was placed into a new Eppendorf tube, and the pellet was discarded. By this procedure, fraction I represented the cell cytosol, and fraction II represented the cell nuclei, membranes, and organelles. Fractions I and II were each precleared by the addition of 150 μl of a 10% suspension of *Staphylococcus aureus* (Staph A, Bethesda Research Labs, Bethesda, Md.) on ice for 30 to 60 min followed by centrifugation in a microfuge at 4°C for 15 min. The numbers of trichloroacetic acid-precipitable counts were determined for both precleared fractions. Fractions I and II were either used directly or stored at −80°C.

Immunoprecipitation. Immunoprecipitation of [^{35}S]methionine-labeled proteins was performed with 30×10^6 trichloroacetic acid-precipitable counts of precleared lysate from fractions I and II. For ^{32}P-labeled proteins, 5×10^6 to 10×10^6 trichloroacetic acid-precipitable counts of lysate from each fraction were used. Separation of the lysate into these two fractions significantly reduced the level of nonspecific proteins that were immunoprecipitated.

The precleared lysates were incubated with 12-1A-FP antisera at a final dilution of 1:20 on ice for 2 to 24 h. Fifty microliters of Staph A was added to the immunoprecipitation reaction and incubated for 15 to 30 min on ice. The Staph A antibody complex was pelleted for 1 min at room temperature in an Eppendorf microfuge. The supernatant was discarded, and the pellet was washed and vortexed four times in RIPA buffer (described above). The SDS sample buffer (0.065 M Tris-hydrochloride [pH 6.8], 2% SDS, 10% glycer-

ol, 5% 2-β-mercaptoethanol, 0.001% bromophenol blue [26]) was added to the Staph A antibody complex pellet, vortexed intermittently for 30 min, and boiled for 5 min. The Staph A was pelleted at room temperature for 2 to 3 min, and the labeled proteins in the supernatant were size fractioned in 15% SDS-polyacrylamide gels (acrylamide-to-bis ratio, 30:0.8) as described by Laemmli (26) and visualized by fluorography (8). In some instances, protein A (a 20% suspension [wt/vol] in PBS [Pharmacia Fine Chemicals, Inc., Piscataway, N.J.]) was used in place of Staph A. The procedure for using protein A was identical to that for using Staph A except that 100 μl was added to the immunoprecipitation reaction mixture which was then rotated at 4°C overnight.

Hybridization selection and cell-free translation. RNA was phenol extracted from transformed cell lines. For Ad12-transformed cells, vanadyl ribonucleoside complexes were used in the phenol extraction (3). DNA restriction fragments generated from plasmids were isolated by the method of Vogelstein and Gillespie (45). Hybridization selection of mRNA to specific DNA restriction fragments was performed as described by Ricciardi et al. (35). Cell-free translation of hybridization-selected mRNAs was in the rabbit reticulocyte system (31).

Plasmids and DNA. Plasmid pLG400 (21) contains the LacI and LacZ fusion gene of *E. coli* and is referred to below as LacZ. Plasmid pAd418, which contains full-length Ad12 E1A cDNA joined to the TAC promoter, was prepared by D. Kimelman, L. A. Lucher, K. H. Brackmann, M. Green, and M. Ptashne (unpublished data). The plasmid pLA-1 contains 0 to 9.4 mu of Ad5 DNA and was obtained from F. Tamanoi, Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y. Plasmid pSVG-12 contained the *Eco*RI fragment C of Ad12 (0 to 16.5 mu) and was derived by inserting this Ad12 fragment within the *Bam*HI (linkered) to the *Eco*RI site of pSV-gpt (29).

RESULTS

Construction of the Ad12 E1A–β-galactosidase plasmid vector. The plasmid pAd441 contains sequences corresponding to the amino-terminal half of the Ad12 E1A coding region which are joined 5′ and in-frame to sequences which encode β-galactosidase (Fig. 1). The 132 amino acids encoded within the E1A DNA of pAd441 are shared completely by the two viral E1A proteins of Ad12, which contain 235 and 266 amino acids (33, 42) and which are translated from overlapping mRNAs (37, 38) (Fig. 1). Preceding the Ad12 E1A–β-galactosidase fused coding regions is a 250-base pair (bp) insert (designated T in Fig. 1) which contains the Lac ribosomal binding site as well as the TAC promoter. The TAC promoter is a hybrid of the Lac and TRP promoters and is 10 times more powerful than the Lac promoter alone (2a). The pAd441 vector also contains the ampicillin resistance gene of pBR322 (designated A in Fig. 1).

The construction of pAd441 was accomplished by isolation of the *Pst*I-*Pvu*II fragment from pAd418 (Kimelman et al., unpublished data), which contains the TAC promoter sequence joined to full-length Ad12 E1A cDNA (Fig. 1). The E1A cDNA of pAd418 extends 4 bp 5′ of the initiator AUG codon of E1A and the *Pvu*II site is 400 bp downstream from the AUG. To form pAd441, we essentially joined the *Pst*I-*Pvu*II fragment of pAd418 to the *Pst*I-*Bam*HI fragment of pLG400 (21) which contained the LacZ gene encoding β-galactosidase (Fig. 1). To achieve ligation of these two DNA fragments, the *Pst*I-*Bam*HI fragment of pLG400 was derived by restriction of pLG400 with *Bam*HI followed by a reaction with the Klenow fragment of DNA polymerase to fill in protruding ends (46) and, finally, restriction by *Pst*I. The pAd441 vector was transfected into a LacZ⁻ strain of *E. coli*, and the isolated ampicillin-resistant colonies, which turn red on MacConkey-Lactose plates, indicated positive expression of β-galactosidase (27).

Expression of 12-1A-FP from *E. coli*. *E. coli* cells which contained the plasmid pAd441 were induced to synthesize 12-1A-FP by the addition of IPTG to the growing culture. To assay for induced synthesis, we fractionated total *E. coli* protein in SDS-polyacrylamide gels (26), and the proteins were visualized by Coomassie blue stain. A protein of 130,000 molecular weight (130K) was the only novel protein synthesized at a high level in induced *E. coli* (Fig. 2, lane 2) compared with uninduced *E. coli* (lane 1). This 130K protein was the size predicted from the DNA sequences of Ad12 E1A and β-galactosidase contained within pAd441. The N-terminal sequence of the 12-1A-FP antigen, purified from a polyacrylamide gel, was analyzed by automated Edman degradation (23) and indicated that the induced protein

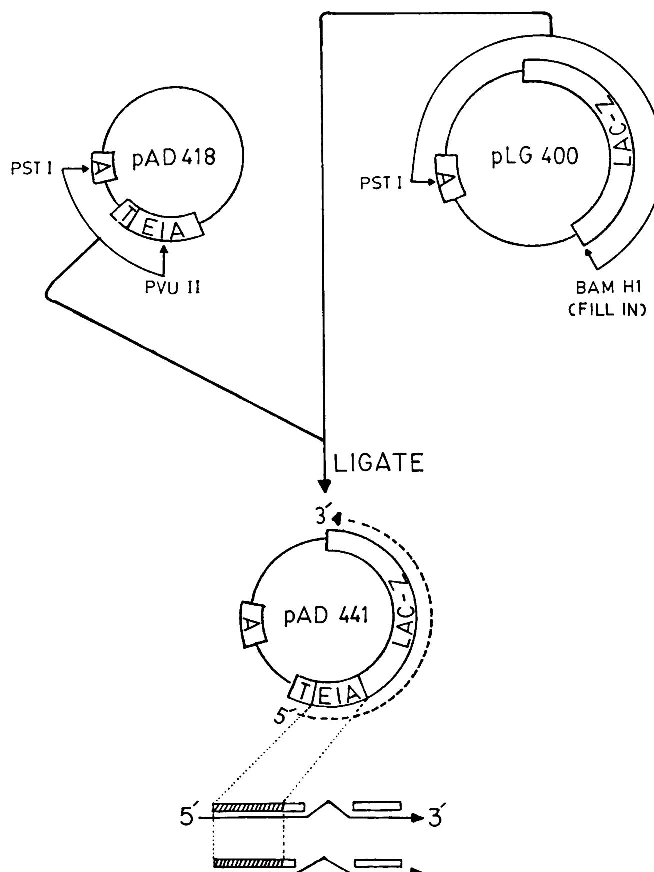

FIG. 1. Construction of the Ad12 E1A–β-galactosidase expression vector plasmid pAd441. The plasmid pAd441 encodes 132 amino acids corresponding to the amino-terminal end of the Ad12 E1A coding region. These sequences are joined in-frame to the gene of *E. coli* which encodes β-galactosidase (LacZ). The location of the coding sequences of Ad12 E1A in pAd441 with respect to corresponding viral mRNAs is shown in the lowermost part of the figure. The pAd441 vector also contains an ampicillin resistance gene (A) and a 250-bp sequence (T) containing the TAC promoter and a ribosomal binding site. Construction of pAd441 was achieved by joining the smaller *Pst*I-*Pvu*II fragment of pAd418 (Kimelman et al., unpublished data) to the larger *Pst*I-*Bam*HI fragment of pLG400 (21). Details of this construction are discussed in the text.

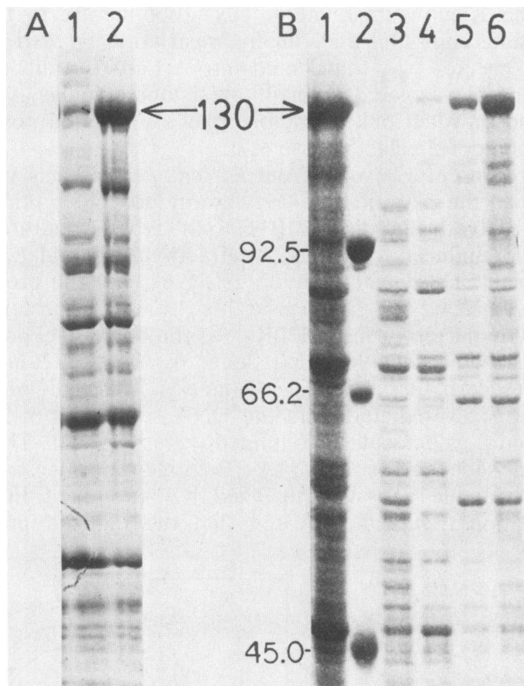

FIG. 2. Fractionated *E. coli* proteins. (A) *E. coli* proteins fractionated in a polyacrylamide gel showing induction of 12-1A-FP. *E. coli* cells containing the pAd441 expression vector (Fig. 1) were induced to synthesize 12-1A-FP by the addition of IPTG as described in the text. After induction, the *E. coli* cells were pelleted and lysed in SDS-Laemmli sample buffer (26), fractionated in a 7.5% SDS-polyacrylamide gel, and stained with Coomassie blue dye. Lane 1, no IPTG added; lane 2, IPTG added. The arrow points to the 130K fusion protein. (B) *E. coli* proteins fractionated in an SDS-polyacrylamide gel after induction of 12-1A-FP and extraction by urea. *E. coli* cells containing the pAd441 vector (Fig. 1) were induced to synthesize 12-1A-FP. The *E. coli* cells were pelleted, and the proteins were extracted by using various urea concentrations as described by Bikel et al. (7) and in the text. The urea-extracted proteins were fractionated in a 7.5% SDS-polyacrylamide gel (26) and stained with Coomassie blue. Fractionation of proteins from induced *E. coli* cells containing pAd441 was as follows: lane 1, not extracted by urea; lane 4, extracted in 2 M urea; lane 5, extracted in 5 M urea; and lane 6, extracted in 10 M urea. Lane 3, 10 M urea-extracted proteins from induced *E. coli* cells which do not contain pAd441. Lane 2, marker proteins as follows: phosphorylase B (92.5K); bovine serum albumin (66.2K); and ovalbumin (45K). The position of the 130K fusion protein 12-1A-FP is indicated by the arrow.

initiated with the same six N-terminal amino acids as viral Ad12 E1A (data not shown). The induced 130K fusion protein represented about 25% of the total protein of the *E. coli* isolates.

Extraction of the bacterial fusion protein and production of antisera. Extraction of the 12-1A-FP was accomplished by utilizing urea as described by Bikel et al. (7). As seen from the Coomassie blue-stained SDS-polyacrylamide gel (Fig. 2B), extraction of the 12-1A-FP in 10 M urea (lane 6) was more than twice as efficient as extraction in 5 M urea (lane 5), and extraction in 2 M urea (lane 4) was hardly effective. The 130K protein could not be extracted by 10 M urea from *E. coli* isolates which were induced but did not contain the pAd441 expression vector (Fig. 2B, lane 3). It is noteworthy that the 12-1A-FP failed to be extracted by nonionic detergents since >95% of the 130K protein remained in the cell pellet (data not shown). Extraction by 10 M urea (Fig. 2B, lane 6) resulted in a ca. 25% purification of the 12-1A-FP compared with total unextracted protein from induced *E. coli* samples (Fig. 2B, lane 1). Furthermore, removal of urea by dialysis in PBS reinstated β-galactosidase activity of the 12-1A-FP comparable with that of commercial β-galactosidase as measured by the *O*-nitrophenyl-β-D-galactopyranoside assay (27; data not shown). As much as 17 mg of 12-1A-FP could be extracted from 1 liter of induced *E. coli* cells. The 10 M urea extract containing the 12-1A-FP antigen was dialyzed in PBS and introduced into rabbits from which antisera were obtained. These antisera, referred to as the 12-1A-FP antisera, were used to immunoprecipitate proteins from adenovirus-transformed and -infected cells.

Bacterial fusion protein antisera immunoprecipitated two major E1A proteins from Ad12-transformed cells. The 12-1A-FP antisera were used to identify E1A proteins which are synthesized in the Ad12-transformed hamster cell HA12/7, which has been reported to express the Ad12 genome extensively (1). The 12-1A-FP antisera immunoprecipitated two major proteins with apparent molecular weights of 39K and 37K from [^{35}S]methionine-labeled HA12/7 cells (Fig. 3A, lane 1). A minor protein of 36K was also immunoprecipitated. In all experiments with HA12/7 cells, as well as other Ad12-transformed hamster and mouse cells, only the 39K and 37K proteins were consistently detected in both the cell cytosol fraction (fraction I) and the cell fraction containing nuclei, membranes, and organelles (fraction II). To prove that these proteins were immunospecific precipitation products of the 12-1A-FP antisera, we added 12-1A-FP antigen with the antisera to the labeled cell lysates. Immunoprecipitation of the 39K, 37K, and 36K proteins was inhibited as the amount of 12-1A-FP antigen in the reaction increased (Fig. 3A, lanes 2 to 4). Indeed, the profile of labeled proteins from a completely inhibited immunoprecipitation reaction (Fig. 3A, lane 4) was identical to one in which only preimmune serum was added to the [^{35}S]methionine-labeled cell lysate (Fig. 3A, lane 5). The series of high-molecular-weight bands (Fig. 3A, lane 1), which were also inhibited by the addition of 12-1A-FP antigen (lanes 3 and 4), likely represents tightly associated nonspecific cellular proteins that are coprecipitated with the 39K and 37K E1A proteins.

We further established that for each protein immunoprecipitated from Ad12-transformed cells by the 12-1A-FP antisera, there was a corresponding translation product synthesized in vitro by RNA that had been hybridization selected to Ad12 E1A DNA. When total cytoplasmic RNA from either the Ad12-transformed hamster cell HA12/7 or mouse cell MO12/F10 was first hybridization selected to DNA which contained sequences exclusive to Ad12 E1A (pAd441 DNA; Fig. 1) and was then translated in a cell-free system, three proteins of 39K, 37K, and 36K were detected (Fig. 3B, lanes 8 and 1, respectively). In addition, a minor E1A protein of 22K was also detected as an in vitro translation product. For comparison, mRNA from HA12/7 cells was hybridization selected to a *Pvu*II fragment of Ad12 DNA (1,906 to 3,623 bp) which exclusively contained E1B sequences. Proteins translated from hybridization-selected E1B mRNAs in the cell-free system were of 58K and 28K (see on longer exposure) and of 19K and 17K apparent molecular weight (Fig. 3B, lane 7). Both Ad12 E1A and E1B proteins were translated from mRNA that had been hybridization selected to the entire transforming region (*Eco*RI-C; 0 to 16.5 mu) of Ad12 (Fig. 3B, lane 6).

Most importantly, the Ad2 E1A proteins produced in vivo comigrated in the same SDS-polyacrylamide gel with the corresponding E1A proteins synthesized in vitro. This was

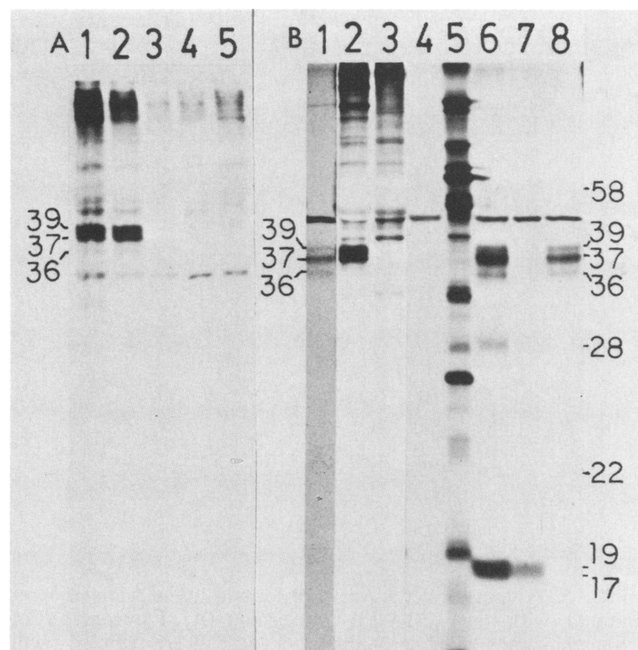

FIG. 3. Fluorograph of [^{35}S]methionine-labeled proteins from Ad12-transformed cells. (A) Proteins immunoprecipitated by 12-1A-FP antisera in the presence of the 12-1A-FP–antigen. The HA12/7-transformed cells were labeled with 50 μCi of [^{35}S]methionine per ml for 2 h, and proteins of the cell lysate (fraction I, cytosol) were immunoprecipitated with 12-1A-FP antisera as described in the text. Where indicated, increasing amounts of the extract containing the 12-1A-FP antigen (Fig. 2B, lane 6) were incubated for 10 min with the 12-1A-FP antisera before the immunoprecipitation reaction. Labeled proteins were separated in 15% SDS-polyacrylamide gels (26). Immunoprecipitation of [^{35}S]methionine-labeled proteins from HA12/7 cells with 12-1A-FP antisera was as follows: lane 1, without 12-1A-FP antigen; lane 2, with 2 μg of 12-1A-FP antigen; lane 3, with 5 μg of 12-1A-FP antigens; lane 4, with 10 μg of 12-1A-FP antigen. Lane 5, immunoprecipitation of [^{35}S]methionine-labeled proteins with preimmune sera and no 12-1A-FP antigen. The apparent molecular weights of the proteins are shown at the left. (B) Comparison of E1A proteins translated in vitro by hybridization-selected RNA with proteins labeled in vivo and immunoprecipitated by the 12-1A-FP antiserum. Extracted RNA from Ad12-transformed cells was hybridization selected to the specific restriction fragments containing Ad12 DNA (35) and in vitro translated in the rabbit reticulolysate cell-free system (31). Labeled proteins were separated in a 15% SDS-polyacrylamide gel (26). The Ad12-transformed cells were labeled with 50 μCi of [^{35}S]methionine per ml for 1 to 4 h, and in vivo-labeled proteins of the cell lysate (fraction II) were immunoprecipitated with the 12-1A-FP antisera as described in the text. [^{35}S]methionine-labeled proteins translated in vitro by RNA from HA12/7 cells were hybridization selected to the following: Ad12 *Eco*RI-C DNA (0 to 16.5 mu; E1A + E1B) (lane 6); an Ad12 *Pvu*II fragment (1,906 to 3,623 bp; E1B) (lane 7); pAd441 DNA (404 to 911 bp; E1A) (lane 8); and RNA from MO12/F10 cells hybridization selected to pAd441 DNA (E1A) (lane 1). Proteins translated in the cell-free system with RNA from Ad5-infected HeLa cells isolated during late infection serve as molecular weight markers (lane 5). Lane 4, cell-free reaction with no added mRNA. [^{35}S]methionine proteins from in vivo-labeled MO12/F10 cells were immunoprecipitated by 12-1A-FP antisera (lane 2) and preimmune sera (lane 3). The apparent molecular weights are shown at the left and right. Lane 1 was overexposed.

true for several Ad12-transformed hamster and mouse cell lines. For example, analysis of the MO12/F10 cells shows that the 39K and 37K E1A proteins translated in vitro from hybridization-selected RNA (Fig. 3B, lane 1) comigrated with proteins of the same size that had been labeled in vivo and immunoprecipitated by the 12-1A-FP antisera (lane 2); the minor 22K E1A protein detected as a translation product was not observed by immunoprecipitation. It is also important that the Ad12 E1A proteins from the MO12/F10-transformed mouse cells (Fig. 3B, lanes 1 and 2) are the same apparent size as those from the HA12/7-transformed hamster cells (lane 8).

Ad12 E1A proteins were phosphorylated in vivo. The Ad12 E1A proteins which had been immunoprecipitated from [^{35}S]methionine-labeled, transformed cells were also shown to be phosphorylated in vivo. Phosphoproteins of 39K, 37K, and 36K were immunoprecipitated from ^{32}P-labeled HA12/7-transformed cells by the 12-1A-FP antisera (Fig. 4, lane 1). No phosphoproteins were immunoprecipitated by preimmune sera (Fig. 4, and lane 3). A partial inhibition of the immunoprecipitation reaction by coaddition of 12-1A-FP antigen extract with 12-1A-FP antisera to the labeled cell lysate helped to dramatize that the 37K immunoprecipitation product is the predominant Ad12 E1A phosphoprotein in HA12/7-transformed cells (Fig. 4, lane 2). Complete inhibition of the immunoprecipitation reaction (Fig. 4, lane 2) was achieved by using a greater amount of 12-1A-FP antigen (data not shown). In similar experiments (data not shown), the Ad12 E1A proteins from infected cells were also shown to be phosphorylated.

12-1A-FP antisera immunoprecipitated E1A proteins from Ad5-transformed and infected cells. The E1A coding region

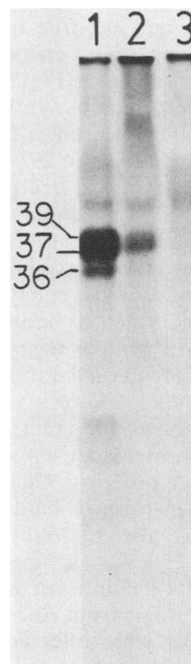

FIG. 4. Autoradiograph of ^{32}P-labeled proteins from Ad12-transformed cells immunoprecipitated by the 12-1A-FP antisera. HA12/7-transformed cells were labeled with ^{32}P (125 μCi/ml for 4 h), and the cell extract was fractionated as described in the text. Fraction I lysate (cytosol) was used here. Labeled proteins were separated in a 15% SDS-polyacrylamide gel (26) and visualized on Kodak XAR film. Immunoprecipitation of ^{32}P-labeled proteins was by the following: the 12-1A-FP antiserum (lane 1); the 12-1A-FP antiserum incubated in the presence of 10 μg of the 12-1A-FP antigen extract as described in the legend to Fig. 3A (lane 2); preimmune sera (lane 3). The apparent molecular weights of the immunoprecipitated proteins are indicated to the left of lane 1.

of Ad12 contained in the vector pAd441 (Fig. 1) shares limited homology with the corresponding N-terminus coding region of Ad5 E1A. A direct comparison of the amino acid sequences of Ad12 E1A (33, 42) and Ad5 E1A (32) indicates that the highest homology is between amino acid residues 40 through 80 of both Ad5 and Ad12 E1A in pAd441. On the basis of this homology, the 12-1A-FP antisera were tested for their ability to immunoprecipitate Ad5 E1A proteins. Lysates were prepared from 293 cells that had been labeled with [^{35}S]methionine. The 293 cells are human embryonic kidney cells that were transformed by Ad5 DNA (19) and express Ad5 E1A and E1B gene products (2, 34). Reaction of the 12-1A-FP antisera with either cell fraction I lysate (cytosol) or fraction II lysate (nuclei, membranes, and organelles) resulted in the immunoprecipitation of two groups of proteins ranging from 38 to 36K and 35 to 33K (Fig. 5A, lanes 2 and 4, respectively). Preimmune sera failed to immunoprecipitate these Ad5 proteins (Fig. 5A, lanes 3 and 5). For comparison, the corresponding E1A proteins translated in vitro by RNA from 293 cells that had been hybridization selected to a restriction fragment which contained Ad5 E1A DNA are shown in Fig. 5A, lane 1, and are indicated by both sets of arrows. In a previous study (34), the E1A proteins translated in vitro from hybridization-selected RNAs were reported as 51K and 48K. In this experiment, the apparent molecular weights of the two Ad5 E1A proteins are dramatically smaller as a result of altering the ratio of bis to acrylamide.

From the DNA sequence, the Ad5 E1A gene is known to encode only two major E1A proteins (32). Furthermore, by hybridization selection and cell-free translation, each E1A mRNA from 293 cells appears to encode a single protein as shown previously (34) and above (Fig. 5A, lane 1). Thus, the several [^{35}S]methionine products synthesized in vivo (Fig. 5A, lanes 2 and 4) could represent a population of various modified forms of E1A proteins in 293 cells. Indeed, lysates prepared from 293 cells that had been labeled with ^{32}P and reacted with the 12-1A-FP antisera appeared to immunoprecipitate only two Ad5 E1A proteins (Fig. 5B, lane 4; upper and lower arrows). These E1A phosphoproteins were detected in both fractions I and II of the cell lysate (Fig. 5B, lanes 1 and 4, respectively) and failed to be immunoprecipitated in the presence of 12-1A-FP antigen (Fig. 5B, lanes 2 and 5) or when preimmune serum was substituted for the 12-1A-FP antiserum (Fig. 5B, lanes 3 and 6).

These results demonstrated that antibodies directed against the Ad12 E1A proteins are capable of strongly cross-reacting with Ad5 E1A proteins. Of further interest, E1A proteins from Ad2-transformed hamster cells were also immunoprecipitated by the 12-1A-FP antisera (data not shown).

In addition, the 12-1A-FP antisera were used to immunoprecipitate the E1A proteins from Ad5-infected cells. In this experiment, HeLa cells were infected at a multiplicity of infection of 50 in the absence of drugs and were labeled at 8 h postinfection with [^{35}S]methionine or $^{32}P_i$. When the [^{35}S]methionine-labeled proteins from the cytosol fraction (fraction I) were incubated with 12-1A-FP antisera, proteins of 38K and 36K were immunoprecipitated (Fig. 6, lane 4; upper and lower arrows, respectively). These in vivo-labeled proteins comigrated with the two known Ad5 E1A proteins translated in vitro by hybridization-selected RNA (34; Fig. 6, lanes 5 and 9). Furthermore, the [^{35}S]methionine-labeled proteins were not immunoprecipitated from uninfected cells (lane 1), by preimmune antisera (lane 2), or in the presence of the 12-1A-FP antigen (lane 3). Moreover, ^{32}P-labeled E1A

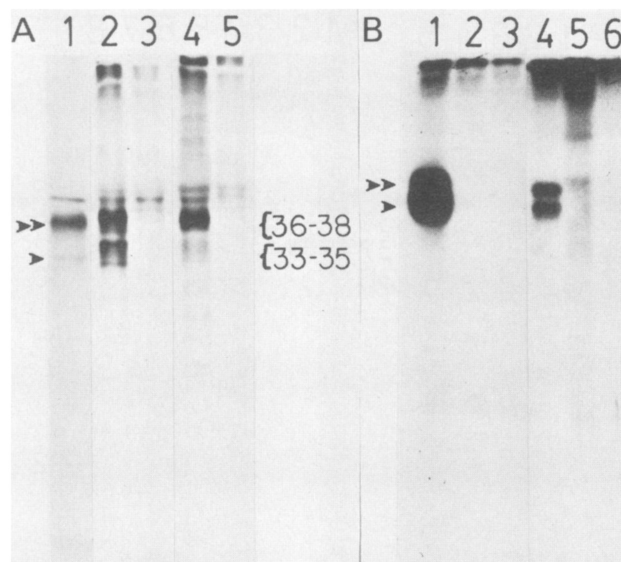

FIG. 5. Proteins from the Ad5-transformed 293 cells immunoprecipitated with the 12-1A-FP antiserum. (A) Fluorograph of [^{35}S]methionine-labeled proteins. 293 cells were labeled with [^{35}S]methionine from which the fractionated cell lysates were immunoprecipitated with the 12-1A-FP antisera. Labeled proteins were fractionated in a 15% SDS-polyacrylamide gel (26). Immunoprecipitation of [^{35}S]methionine-labeled proteins from fraction I lysate (cytosol) is shown as follows: lane 2, by 12-1A-FP antisera; lane 3, by preimmune sera. Immunoprecipitation of [^{35}S]methionine-labeled proteins from fraction II lysate (membrane, nuclei, and organelles) is shown as follows: lane 4, by 12-1A-FP antisera; lane 5, by preimmune sera. Lane 1, labeled proteins translated from RNA isolated from 293 cells that had been hybridization selected to a fragment of Ad5 DNA (0 to 9.4 mu; E1A and E1B) and translated in a cell-free system as previously described (35). The arrows to the left of lane 1 identify the positions of the larger (upper arrows) and smaller (lower arrows) E1A proteins translated in vitro, and the apparent molecular weights of immunoprecipitated proteins are shown at the right. (B) Autoradiograph of ^{32}P-labeled proteins. The 293 cells were labeled with ^{32}P, and the cell lysate was fractioned as described in the text. The labeled proteins were separated in a 15% SDS-polyacrylamide gel and visualized on Kodak XAR film. ^{32}P-labeled proteins from fraction I lysate (cytosol) were immunoprecipitated by the following: the 12-1A-FP antisera (lane 1); the 12-1A-FP antisera incubated in the presence of 10 μg of the 12-1A-FP antigen extract as described in the legend to Fig. 3A (lane 2); preimmune sera (lane 3). Lanes 4, 5, and 6 are the same as lanes 1, 2, and 3, respectively, except that fraction II lysate (membrane, nuclei and organelles) was used in place of fraction I. Twice as many trichloroacetic acid-precipitable counts were used in lanes 1 to 3 compared with lanes 4 to 6. The positions of the immunoprecipitated proteins are indicated by the upper and lower arrows.

proteins in both fraction I (cytosol) and fraction II (nuclei, organelles, and membranes) lysates of Ad5-infected cells (Fig. 6, lanes 6 and 8, respectively) comigrated with [^{35}S]methionine-labeled E1A proteins (Fig. 6, lanes 4 and 9). These Ad5 E1A phosphoproteins were not immunoprecipitated from uninfected cells (Fig. 6, lane 7). These results again dramatize the highly specific and strong cross-reactive nature of the 12-1A-FP antisera to immunoprecipitate E1A proteins of Ad5.

DISCUSSION

The E1A gene of adenovirus performs a crucial function in both infected and transformed cells. During infection of human cells, a product of the E1A gene modulates expres-

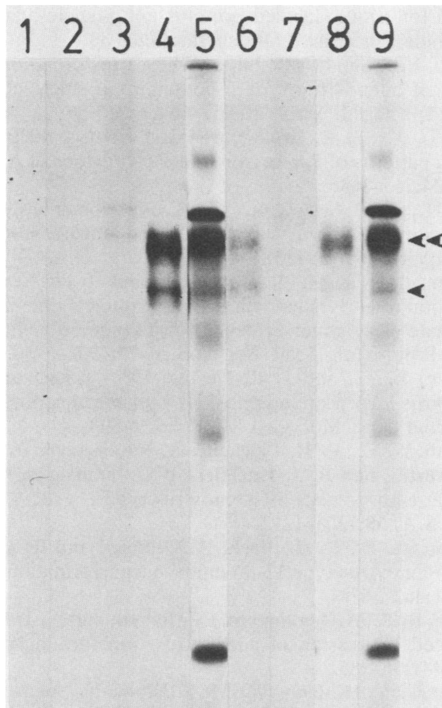

FIG. 6. Fluorograph of E1A proteins from Ad5-infected cells labeled with [^{35}S]methionine or ^{32}P and immunoprecipitated by the 12-1A-FP antisera. Ad5 E1A and E1B proteins translated in vitro by hybridization-selected RNAs were used to compare and confirm the identity of the immunoprecipitated products. Labeled proteins were separated in a 15% SDS-polyacrylamide gel (26). Extracted RNA from Ad5-transformed 293 cells was hybridization selected (35) to a 0 to 9.4 mu fragment (E1A and E1B) of Ad5 DNA, derived from the pLA1 plasmid, and in vitro translated in the rabbit reticulocyte cell-free system (31). HeLa cells were infected at a multiplicity of infection of 50 in the absence of drugs and were in vivo labeled at 8 h postinfection with [^{35}S]methionine or ^{32}P$_i$ as described in the text. The cell extracts were fractionated into a cytosol fraction (fraction I) lysate and a nuclei, membrane, and organelle (fraction II) lysate, as described in the text, and immunoprecipitated with the 12-1A-FP antisera. Immunoprecipitated [^{35}S]methionine-labeled proteins were from the cytosol fraction of the following: uninfected HeLa cells by the 12-1A-FP antisera (lane 1); infected HeLa cells by preimmune sera (lane 2); infected HeLa cells by 12-1A-FP antisera incubated in the presence of 10 μg of 12-1A-FP antigen extract as described in the legend to Fig. 3 (lane 3); infected HeLa cells by the 12-1A-FP antisera (lane 4). Immunoprecipitated ^{32}P-labeled proteins were from the following: the cytosol fraction of infected HeLa cells by the 12-1A-FP antisera (lane 6); the cytosol fraction of uninfected HeLa cells by the 12-1A-FP antisera (lane 7); the nuclei, membranes, and organelle fraction of infected HeLa cells (lane 8). [^{35}S]methionine proteins translated in vitro by RNA from 293 cells hybridization selected to a 0 to 9.4 mu restriction fragment of Ad5 DNA (lanes 5 and 9). The upper and lower arrows point to the E1A proteins synthesized in vitro (lanes 5 and 9) as previously determined by hybridization selection (35).

sion of other early viral genes (4, 24, 30). This product is the larger of the two acidic proteins encoded by Ad5 E1A (28, 34). Events leading to complete cellular transformation of rodent cells require both adenovirus E1A and E1B genes (18, 22, 44). Characterization and comparison of the proteins of Ad5 and Ad12 are important since these divergent strains are able to transcomplement one another during infection (47) and since the E1A gene product of Ad12, but not Ad5, appears to suppress class I major histocompatibility antigens in rats to at least partially account for the highly tumorigenic potential of Ad12 and the weakly tumorigenic potential of Ad5 (6, 39).

In this study, we report the production of the first Ad12 E1A monospecific antiserum. This antiserum (12-1A-FP antiserum) is specifically directed against the N-terminal half of the E1A proteins of Ad12. Moreover, the 12-1A-FP antiserum strongly cross-reacts with Ad5 E1A proteins. For both transformed and infected cells of Ad12 and Ad5, respectively, we report that the 12-1A-FP antiserum is immunospecific for E1A proteins by (i) the ability of 12-1A-FP antigen to block immunoprecipitation of E1A proteins and (ii) the comigration of immunoprecipitated E1A proteins with E1A proteins translated in vitro from hybridization-selected mRNAs. Furthermore, the 12-1A-FP antiserum immunoprecipitated phosphorylated E1A proteins from both transformed and infected cells of Ad12 and Ad5, respectively. The 12-1A-FP antiserum appeared to react strongly with E1A proteins, presumably since it is polyclonal for multiple determinants within the N-terminus.

In the Ad12-transformed hamster cell line HA12/7, the 12-1A-FP antiserum specifically immunoprecipitated two major E1A proteins of 39K and 37K, respectively. In addition, a minor E1A protein of 36K was observed. Since two open reading frames are predicted from the DNA sequence of Ad12 E1A (33, 42), it was reasonable to postulate that the minor E1A protein (36K) could be generated from a proteolytic cleavage of one or both major E1A proteins (39K and 37K). However, mRNA from HA12/7 cells that had been hybridization selected by Ad12 E1A DNA and translated in a cell-free system also synthesized two major proteins and one minor protein which comigrated with each of the respective immunopreciptated E1A proteins. Thus, there is either a susceptible proteolytic cleavage site in one or both of the major Ad12 E1A proteins which is recognized in vivo and in vitro, or, less likely, use of a different combination of reading frames or an internal start site accounts for the appearance of the third and unpredicted E1A protein of Ad12. Tryptic and N-terminal amino acid sequence analysis of these proteins will be necessary to determine the origin of the 36K protein.

It is important to note that the apparent molecular weights of the Ad12 E1A proteins reported here exceed the coding capacity of the Ad12 E1A transcripts (37). This was not surprising since the highly acidic E1A proteins of Ad5 are known to migrate aberrantly in SDS-polyacrylamide gels (34, 50). In fact, we have observed that lowering the bis/acrylamide ratio causes the Ad12 E1A proteins to migrate as proteins which are almost double in molecular weight from their predicted sizes of 29K and 26K, respectively (33, 42).

According to the DNA sequence (33, 42), the two major Ad12 E1A proteins are 266 and 235 amino acid residues in length. The 12-1A-FP antiserum recognizes the N-terminal 131 amino acids of each Ad12 E1A protein since this stretch is encoded by the bacterial expression vector pAd441, used in the production of antigen. The 12-1A-FP antiserum is also capable of immunoprecipitating Ad5 E1A proteins probably by virtue of amino sequence acid homology to Ad12 E1A, which almost exclusively resides between residues 40 and 80.

The 12-1A-FP antiserum immunoprecipitated two E1A proteins from Ad5-infected cells, when labeled with either [^{35}S]methionine or ^{32}P. In contrast, the 293 cells, which constitutively synthesize E1A mRNAs (2), appeared to contain several forms of each E1A protein when the cells were labeled with [^{35}S]methionine but fewer forms when

they were labeled with ^{32}P. As observed here and alluded to elsewhere (16 and 36), differences in the number of E1A proteins seen in Ad5-infected and transformed cells could reflect a distinction between their respective pool sizes of phosphorylated and unphosphorylated forms as well as their cellular half-lives. Rowe et al. (36) recently reported four major and four minor forms of E1A proteins from Ad5-infected cells in two-dimensional polyacrylamide gels after immunoprecipitation by an antipeptide serum directed against the carboxyl terminus. Several cautions about attempting to interpret the actual number of cellular E1A proteins by immunoprecipitation with monospecific antisera are suggested by the results of these experiments. Factors such as the time point of infection, the type of infected or transformed cell, and the type of gel matrix used in the analysis (e.g., the bis/acrylamide ratio in SDS-polyacrylamide gels) may influence the number of detectable E1A proteins which are incompletely modified or partially degraded.

The potential uses of the 12-1A-FP monospecific antisera are (i) to immunoaffinity purify cellular Ad12 and Ad5 E1A proteins, (ii) to discern whether the N termini of Ad12 and Ad5 E1A proteins perform functions which are independent of the carboxyl terminus, (iii) to determine specific biochemical features of the E1A proteins (e.g., binding to DNA), and (iv) to determine whether proteins immunoprecipitated by tumor-bearing antisera are E1A proteins (1, 17, 37, 48).

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