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Human Adenovirus 2 E1B-19K and E1B-53K Tumor Antigens: Antipeptide Antibodies Targeted to the NH_2 and COOH Termini

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The human adenovirus 2 (Ad2) transforming region is located in the left 11.1% of the viral genome and encodes two early transcription units, E1A and E1B. Based on the amino acid sequence deduced from the Ad2 E1B DNA sequence (Gingeras et al., J. Biol. Chem. 257:13475–13491, 1982), we have prepared antibodies against synthetic peptides, 8 to 16 amino acids in length, encoded at the NH_2 and COOH termini of the major E1B-19K and E1B-53K tumor antigens. The antipeptide antibodies immunoprecipitated the targeted E1B-19K or E1B-53K tumor antigens from extracts of Ad2-infected cells. The specificity of the antipeptide antibody recognition of the E1B tumor antigens was confirmed by peptide competition studies. Antipeptide antibodies directed to the NH_2 and COOH termini immunoprecipitated the E1B-19K and E1B-53K tumor antigens from two Ad2-transformed rat cell lines, F17 and F4, providing evidence that identical tumor antigens are synthesized in Ad2-infected and Ad2-transformed cells. These results show that the E1B-19K and E1B-53K T antigens are not processed proteolytically at either the NH_2 or COOH terminus. Our data provide strong evidence at the protein level that the E1B-19K and E1B-53K tumor antigens partially overlap in DNA sequence, with the E1B-19K initiating translation at the first ATG at nucleotide 1711 in translation reading frame 1 and the E1B-53K tumor antigen initiating translation at the second ATG at nucleotide 2016 in reading frame 3. This confirms the results of others on the N-terminal amino acid sequence of E1B-19K and theoretical deductions based on the DNA sequence. Our findings prove that the large E1B-53K T antigen initiates translation at the second ATG at nucleotide 2016 and not at equally plausible initiation codons located farther downstream at nucleotides 2202 and 2235. Thus, the E1B-53K T antigen is another example of a protein which initiates translation at an internal ATG rather than at the 5'-proximal ATG.

During the early stages of adenovirus 2 (Ad2) productive infection, seven regions of the viral genome are transcribed, early region 1A (E1A), E1B, E2A, E2B, E3, E4, and late region 1 (L1) (see reference 45 for a recent review). E1A, located at map position 1.3 to 4.5, and E1B, located at map position 4.6 to 11.1, are of special interest because they encode the information for Ad2-induced cell transformation. Much is known about E1 of Ad2 and the closely related Ad5 (both group C). The DNA encoding the transforming genes has been sequenced (7, 16, 57). The major E1-coded mRNAs and proteins have been identified by cell-free translation and by immunoprecipitation with antisera against Ad-transformed cells (Ad tumor sera).

Cell-free translation studies with Ad2 and Ad5 E1 mRNAs have detected about eight proteins that are encoded by E1A and E1B (7, 13, 17, 23, 24, 35, 37, 38). As determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, two early proteins of apparent M_r 41,000 to 53,000 are translated from the E1A-13S mRNA, two early proteins of apparent M_r 35,000 to 47,000 from the E1A-12S mRNA, and an intermediate protein of apparent M_r 28,000 from the E1A-9S mRNA that apparently plays no role in transformation. A protein of M_r 53,000 is translated from the E1B-22S mRNA, a protein of M_r 19,000 from both the E1B-22S and E1B-13S mRNAs, and a protein of M_r 20,000 from an unidentified E1B-mRNA. Peptide map analysis has shown that the E1B-protein of M_r 20,000 (E1B-20K protein) is unrelated to the E1B-19K protein but that it shares amino acid sequences with E1B-53K (8, 17). By the use of Ad tumor

sera, Ad2- and Ad5-specific proteins have been immunoprecipitated from Ad2- and Ad5-infected cells (15, 17, 19, 27, 34, 58) and from Ad2- and Ad5-transformed cells (17, 38, 48, 51). These tumor (T) antigens possess sizes and peptide maps similar to those produced by cell-free translation.

Ad-induced cell transformation appears to be a two-step process, involving functions encoded by both E1A and E1B (45, 54). An analogous situation appears to exist with polyomavirus (52) and simian virus 40 (47). For example, a specific T antigen (middle T for polyomavirus and large T for simian virus 40) can induce most of the functions associated with cell transformation, but a second tumor antigen (small t for simian virus 40 and the NH_2 portion of large T for polyomavirus) may also play a role (47, 52). A division of function within the Ad transforming region has been defined by transforming cells with Ad5 restriction fragments containing only portions of E1. Cells transformed by the left 8% (map position 0 to 8) of the Ad5 genome, which encodes E1A plus the left half of E1B, have essentially the same phenotype as cells transformed by virions or larger fragments (56). However, cells can also be transformed partially with the left 4.5% of the viral genome, which encodes only E1A. Although cells transformed by the E1A fragment have an unlimited life span, they remain fibroblastic rather than epithelial in form, and their growth properties are similar to those of untransformed cells (25). These findings suggest that E1A encodes functions that may confer some properties associated with the transformed cell, e.g., immortality, but that E1B encodes functions which are needed for the complete expression of the transformed phenotype (25).

Studies during the past few years have provided clues to the possible functions of E1A and E1B T antigens. Studies with E1A host range and deletion mutants have shown that an E1A function regulates the expression of other early genes (4, 28). E1A encodes two major early mRNA species, 13S mRNA and 12S mRNA, which code for closely related T antigens. The gene product(s) of the E1A-13S mRNA has been recently identified as the regulatory element that modulates early gene expression (41, 46). Additional studies have suggested the possibility that the E1A gene product may function by interaction with a cellular component (30, 42). Of added importance, recent studies have provided evidence that an E1A T antigen can activate the expression of specific cellular genes, including the 70K heat shock gene (43) and the human β-globin gene introduced into cells in the form of a plasmid (21).

Much less is known about the Ad E1B T antigens. McKinnon et al. (39), using deletion mutants of Ad5 early region E1, indicated that sequences present in both E1A and the N-terminal region of E1B are necessary for DNA-mediated transformation. Recently, Chinnadurai (9) has provided direct genetic and biochemical evidence that the N-terminal portion of the Ad2 E1B-19K T antigen is an essential functional domain for the induction of cell transformation. Bernards et al. (6) have described recent studies suggesting that the E1B-coded large T antigen (E1B-53K) may play a role in the tumorigenicity of Ad-transformed cells in nude mice. More recently, van den Elsen et al. (55) have shown that E1B does not cause morphological transformation in the absence of E1A but that morphological transformation is dependent on the cooperative activities of both E1A and E1B.

To provide tools for the further study of the Ad2 E1B T antigens, we have synthesized peptides 8 to 16 amino acid residues in length, encoded at the NH_2 and COOH termini of the Ad2 E1B-19K T antigen in translation reading frame 1 and the E1B-53K T antigen in reading frame 3, based on (i) the amino acid sequence deduced from the Ad2 DNA sequence (16) and (ii) the unproven assumption that the E1B-53K T antigen initiates translation at the second ATG codon (nucleotide 2016) in reading frame 3. The E1B-19K antigen is probably the same as the E1B-15K protein that was shown by N-terminal amino acid sequence analysis to initiate translation at the first ATG (nucleotide 1711) in reading frame 1 (1). We report in this communication that antibodies prepared in rabbits against the NH_2- and COOH-terminal peptide recognize the authentic Ad2 E1B-19K and E1B-53K T antigens in Ad2-infected and Ad2-transformed cells. These data establish at the protein level the minimum gene boundaries, translation-initiation sites, and reading frames of the Ad2 E1B-19K and E1B-53K genes, thus confirming theoretical deductions based on DNA sequence (7, 16) and peptide map analyses (7, 8). The antipeptide antibodies should prove useful for studies on the purification and functions of the Ad2 E1B-19K and E1B-53K T antigens.

MATERIALS AND METHODS

Materials. Merrifield resin (1% cross-linked divinyl benzene) having a substitution of approximately 0.7 meq per g of N^{α}-*t*-butyloxycarbonyl(tBoc)-L-*S*-methoxybenzyl cysteine was purchased from Peninsula Laboratories, Inc., Belmont, Calif. The tBoc derivatives of L-amino acids were purchased as follows: glutamine-β-nitrophenyl ester, isoleucine, leucine, proline, *O*-benzyl-serine, N^{G}-tosyl arginine, valine, phenylalanine, *O*-benzyl-threonine, and N^{in}-formyl-tryptophan from Peninsula Laboratories, Inc.; and glycine, alanine, aspartic acid-γ-benzyl ester, asparagine-*p*-nitrophenyl ester, and glutamic acid-γ-benzyl

ester from Chemical Dynamics Corp., South Plainfield, N.J.

Methylene chloride (CH_2Cl_2), anisole, and *N,N'*-dimethylformamide (DMF) were purchased from Fisher Scientific Co., Pittsburgh, Pa. Tertiary butanol was from Mallinckrodt, Inc., St. Louis, Mo. *N,N'*-Diisopropyl-ethylamine was from the Aldrich Chemical Co., Milwaukee, Wis. *N,N'*-Dicyclohexylcarbodiimide was from Sigma Chemical Co., St. Louis, Mo. Hydrogen fluoride was from Union Carbide Corp. (Linde Division), New York, N.Y.

Keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) (crystalline) were purchased from the California Foundation for Biochemical Research and from Sigma Chemical Co., respectively. *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) was purchased from the Pierce Chemical Co., Rockford, Ill.

Solid-phase peptide synthesis. Solid-phase peptide synthesis was accomplished by the method of Merrifield, i.e., the sequential addition of protected amino acid derivatives to a polymeric resin support starting with the COOH terminus (3). Merrifield resin containing esterified N^{α}-tBoc-L-*S*-methoxybenzyl cysteine was used to initiate the synthesis of all peptides described in this paper. Cysteine was used as the COOH terminus of all peptides to facilitate coupling to the carrier protein through the sulfhydryl group (see below). Each synthesis was initiated with 1.5 g of resin which contained about 1.0 mmol of cysteine. All reactions and washings of the resin were performed in a reaction vessel constructed as described by Merrifield et al. (40). The vessel was agitated at 280 cycles per min in a Burrel mechanical wrist shaker with 25 ml of solvent or reagent, unless otherwise stated.

The repeated washing, deprotection, neutralization, and amino acid coupling steps for each cycle were performed as follows with fresh solvent or reagent added at each step. The resin was washed three times for 3 min each with (i) 50% DMF in CH_2Cl_2, (ii) 95% tertiary butanol in CH_2Cl_2, and (iii) CH_2Cl_2. Deprotection, i.e., removal of the tBoc group from the α position of the cysteine esterified to the resin, was achieved by treatment for 3 min and then for 30 min with 25% trifluoroacetic acid in CH_2Cl_2. The resin was then washed three times for 3 min each with (i) CH_2Cl_2, (ii) 95% tertiary butanol in CH_2Cl_2, and (iii) CH_2Cl_2. Neutralization of the resin was then achieved by three treatments for 3 min each with 7% *N,N'*-diisopropylethylamine in CH_2Cl_2, followed by three washes for 3 min each with (i) CH_2Cl_2 and (ii) DMF. At this stage, a small sample of the resin (10 mg) was analyzed by the ninhydrin reaction (50) to ensure that deprotection was complete.

Amino acid coupling was performed as follows. A fivefold molar excess of the appropriate tBoc amino acid derivative in 10 to 15 ml of DMF was added to the reaction vessel containing the resin. After shaking for 5 min, a fivefold molar excess of *N,N'*-dicyclohexylcarbodiimide in 15 ml of CH_2Cl_2 was added. Coupling was performed by shaking for 12 h to ensure complete addition of amino acid; longer times were necessary for the addition of some tBoc amino acid derivatives to certain peptides. After the coupling reaction, the resin was washed three times for 3 min each with (i) 50% DMF in CH_2Cl_2, (ii) 95% tertiary butanol in CH_2Cl_2, and (iii) CH_2Cl_2. A small sample of resin (10 mg) was removed for analysis of the extent of coupling by the ninhydrin reaction for free amino groups (50). If coupling was not complete (>98%), the reaction was repeated for 12 to 48 h with the addition of a fivefold molar excess of fresh tBoc amino acid derivative and *N,N*-dicyclohexylcarbodiimide.

Protected tBoc amino acid derivatives were added sequentially to the growing peptide attached to the resin as described above, i.e., deprotection, neutralization, and amino acid coupling. Each deprotection and coupling reaction was monitored by the ninhydrin reaction. One-half of the peptide resin was removed after the addition of eight amino acid residues to the cysteine-resin. This permitted the isolation of peptides containing the first eight amino acid residues of the final longer peptide. Additional amino acid were then coupled to the remaining peptide resin to prepare the peptides of 13 to 16 amino acid residues.

Cleavage and deprotection was achieved by treatment with freshly distilled liquid hydrogen fluoride–5% anisole at 0°C for 1 h. This step simultaneously cleaved the peptide from the peptide-resin and removed the side chain protecting groups from the amino acids. Excess hydrogen fluoride was removed under reduced pressure, and the remaining anisole and residues from amino acid side chain protecting groups were extracted with ether. The peptide was dissolved in 10 to 20% acetic acid by stirring for 2 h, filtered, and lyophilized. The calculated overall recovery of synthetic peptide was generally 80 to 90%. A portion of each synthetic peptide was subjected to acid hydrolysis at reduced pressure (6 N HCl, 110°C, 24 h), and amino acid analysis was performed. The amino acid composition agreed well with the predicted values.

Coupling of synthetic peptides to carrier protein. Peptides were coupled to BSA or to KLH, either through the cysteine sulfhydryl group at the COOH terminus of the peptide by using MBS as the coupling reagent (referred to as the MBS procedure) (22, 36) or through the free amino group at the NH_2 terminus by using the glutaraldehyde procedure (29). For coupling through the sulfhydryl group, 6 mg of peptide was coupled to about 5 mg of KLH activated by treatment with a 40-fold molar excess of MBS, based on an assumed M_r of 100,000 for KLH (22). To 16 mg of KLH in 1.0 ml of 10 mM phosphate buffer (pH 7.2) in a 4-ml screw-cap glass vial, 400 μl of DMF containing 2.4 mg of MBS was added dropwise with stirring. The reaction was stirred for 30 min at room temperature, and the MBS-activated KLH was purified on a Sephadex G-25 column (28 by 1.5 cm) with 50 mM phosphate buffer, pH 6.2. The visible peak of KLH in the eluate was pooled, and the absorbance at 280 nm was determined. The activated KLH was used immediately. Based on an extinction coefficient for a 0.1% solution of KLH at 280 nm of 2.02, 5 mg of activated KLH was removed and added to 6.0 mg of peptide (50- to 100-fold molar excess of peptide) in 1 to 2 ml of about 50 mM phosphate buffer at pH 7 to 7.5. The reaction mixture was incubated at room temperature for 3 h with stirring and then was placed at −20°C (without removal of free peptide) for inoculation into rabbits.

For coupling through the NH_2 group, reagent grade glutaraldehyde (1 ml, 20 mM) was added dropwise to 2 ml of 100 mM sodium phosphate buffer, pH 7.5, containing 26 mg of BSA or KLH and a 30- to 50-fold molar excess of peptide. The reaction was allowed to

continue for 1 h at room temperature with occasional stirring. The peptide-protein conjugate was dialyzed against phosphate-buffered saline (PBS) for 48 h with four changes of buffer.

The extent of coupling of peptide to carrier protein was measured in several experiments. From 10 to 40% of the peptide was bound to carrier protein.

Immunizations. Two-month-old New Zealand white rabbits were inoculated subcutaneously in the neck at three sites with peptide-protein conjugate emulsified in complete Freund adjuvant. For peptide-protein conjugates prepared by the MBS procedure, the equivalent of 400 μg of total peptide was inoculated in the initial immunization and in the first two boosts. For subsequent boosts, 200 μg of peptide was used. For peptide-protein conjugates prepared by the glutaraldehyde procedure, 1 mg of total protein plus peptide was used for the initial inoculation, and 500 μg was used for boosts. Rabbits inoculated with MBS-coupled peptides received boosts of peptide-protein conjugate emulsified with Freund incomplete adjuvant by the subcutaneous route after 2 weeks. After an additional week, a boost was given by intraperitoneal inoculation with peptide-protein conjugates prepared in an aluminum hydroxide gel (10 mg/ml in 0.15 M NaCl). Subsequent boosts were given every 3 weeks intraperitoneally. Rabbits inoculated with glutaraldehyde-coupled peptides received boosts of peptide-protein conjugate emulsified in Freund incomplete adjuvant by subcutaneous inoculation at 3-week intervals after the initial inoculation.

Blood was drawn 7 to 14 days after each immunization, starting with the third inoculation. The samples were allowed to coagulate at room temperature for several hours were then, rimmed, and placed at 4°C overnight. Sera decanted and clarified by centrifugation at 1,600 × *g* for 15 min. Sera were stored at −70°C until analyzed by enzyme-linked immunosorbent assay (ELISA) or immunoprecipitation.

ELISA analysis. The ELISA was used to determine peptide antibody titers. It was performed in 96-well polystyrene plates (Falcon Plastics, Oxnard, Calif.). Five picomoles of peptide per well in 25 μl of 0.1% BSA in PBS were allowed to adsorb overnight uncovered at 37°C. For peptides conjugated to BSA, ovalbumin must be substituted for BSA in all the ELISA solutions. Adsorbed peptide was then fixed for 5 min in 100% methanol and air dried. Plates could be stored sealed at 4°C for several months. Before use, 50 μl of 3% BSA–PBS was added to each well, and the plate was covered in a moist chamber and incubated at 37°C for 4 h. The solution was shaken off, and twofold serial dilutions of rabbit serum in 1% BSA–PBS were added to the wells (25 μl per well). The first dilution was 1:20 for the initial test of each serum, but for initially high-titer antisera, greater dilutions were subsequently used. After overnight incubation in a damp chamber, excess serum was shaken off, and the plates were washed as before. Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Sigma Chemical Co.) appropriately diluted in 1% BSA–PBS was added (25 μl per well), and the plates were incubated for 2 h in a damp chamber at 37°C. After washing 10 times in tap water, 50 μl per well of freshly prepared phosphatase substrate (Sigma Chemical Co.) was added to each well. The substrate was prepared by mixing 0.2% *p*-nitrophenyl phosphate-disodium in distilled water with an equal volume of alkaline buffer solution (100 mM glycine, 1 mM $MgCl_2$, adjusted to pH 10.5 with 1 N NaOH). After incubation for 1 h at room temperature, the enzyme action was stopped by adding 150 μl of 1 N NaOH, and the absorbance at 405 nM was determined. Controls were performed by applying the test antiserum on an unrelated absorbed peptide as well as by using normal rabbit serum and antiserum to an unrelated peptide on wells containing samples of the immunizing peptide. In addition, a chicken albumin–anti-chicken albumin system was run on each plate for comparison of different assays. The antipeptide titer is the last dilution which has an absorbance greater than twice that of controls.

Preparation of [^{35}S]methionine-labeled whole cell extracts of Ad2-early-infected cells and Ad2-transformed cell lines. Ad2 (strain 38-2) was grown in suspension cultures of KB cells in Eagle minimal essential medium (EMEM) containing 5% horse serum (18). Infected KB cells were labeled with [^{35}S]methionine essentially as described by Gaynor et al. (14). Two hundred milliliters of KB cells (4 × 10^5 cells per ml) were centrifuged and infected with 200 PFU of Ad2 per cell in 1/20 the initial volume of EMEM. After 1 h at 37°C, the cells were diluted to one-half the initial volume, and 20 μg of 1-β-D-arabinofuranosylcytosine (AraC) per ml was added. Additional AraC (20 μg/ml) was added at 15 and 26 h postinfection. At 30 h postinfection, the cells were centrifuged, washed twice with warm methionine-free EMEM containing AraC, and suspended in 50 ml of methionine-free EMEM containing 20 μg of AraC per ml, 2% dialyzed horse serum, and 1 mCi of [^{35}S]methionine (1,100 Ci/mmol). After labeling at 37°C for 8 to 10 h, the cells were centrifuged, washed twice with cold PBS containing 1 mM phenylmethylsulfonyl fluoride and 0.1% Trasylol (Aprotinin) (Mobay Chemical Corp., New York, N.Y.; 10,000 Kallikrein inactivator units per ml). The pellet was solubilized by sonication in 5.0 ml of whole cell sonication buffer containing 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, and 0.8 M urea (10) and was centrifuged at 40,000 rpm for 1 h in a Beckman Ti50 rotor (100,000 × *g*). The supernatant fluid, containing about 2 × 10^8 trichloroacetic acid-insoluble cpm/ml, was used for immunoprecipitation analysis as described below.

The Ad2-transformed rat cell lines F17 and F4 were grown as described previously (20). Monolayer cultures (75 cm^2) when 70 to 80% confluent were washed twice with methionine-free Dulbecco modified EMEM containing 2% fetal bovine serum (labeling medium) and were preincubated for 1 h with 10 ml of labeling medium. The medium was removed, and the cells were incubated for 6 h at 37°C in 2 ml of labeling medium containing 410 μCi of [^{35}S]methionine. The monolayer was washed twice with PBS containing 1 mM phenylmethylsulfonyl fluoride, and 0.1% TRASYLOL. Five milliliters of whole cell sonication buffer was added to the monolayer, effectively removing the cells by solubilization. The cell extract was sonicated and clarified as described above. From 5 × 10^7 to 5 × 10^8 trichloroacetic acid-insoluble cpm was obtained per monolayer.

Immunoprecipitation analysis of [^{35}S]methionine-labeled cell extracts. Antipeptide sera were clari-

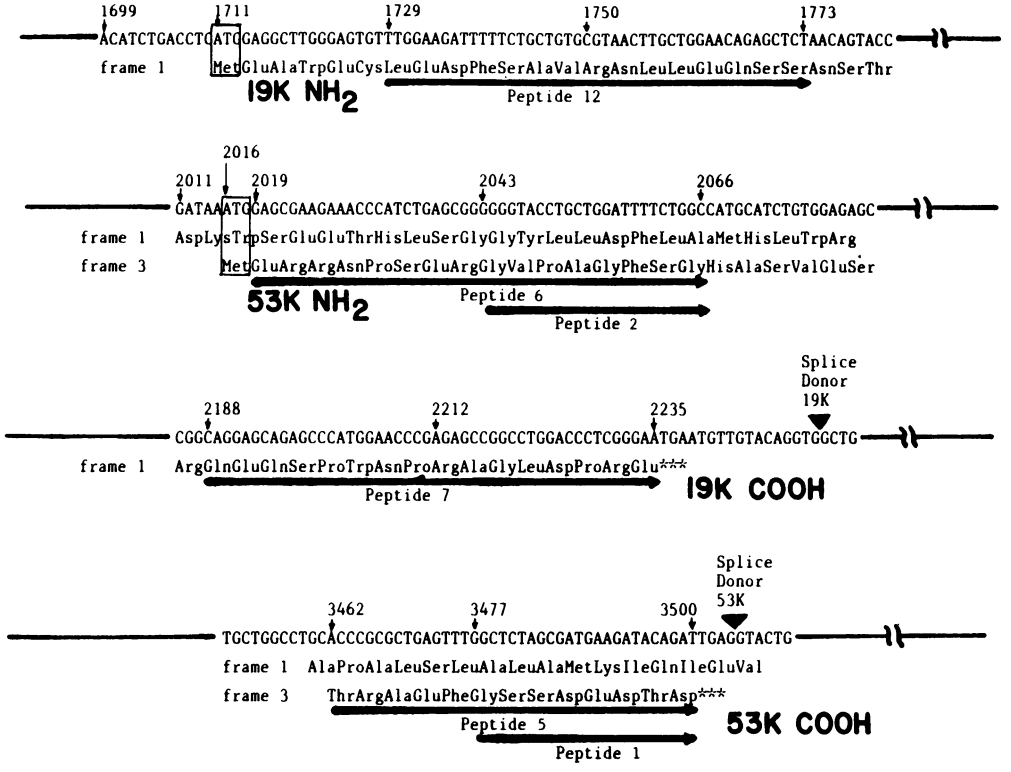

FIG. 1. Genomic location, DNA sequence, and amino acid sequence of the coding regions of the Ad2 E1B-19K and E1B-53K T antigens to which synthetic peptides are directed. The DNA sequence and amino acid sequence are taken from Gingeras et al. (16). Peptide 12 contains a 15-amino-acid (15aa) sequence close to the NH_2 terminus, and peptide 7 contains the 16-amino-acid sequence at the COOH terminus of the E1B-19K T antigen. Peptide 6 contains the 16-amino-acid sequence at the NH_2 terminus of the E1B-53K T antigen; peptide 2 contains the 8-amino-acid sequence at the COOH end of peptide 6. Peptide 5 contains the 13-amino-acid sequence at the COOH terminus of the E1B-53K T antigen: peptide 1 contains the 8-amino-acid sequence at the COOH end of peptide 5. See the text for a discussion of viral gene location and regulatory signals.

fied before use by centrifugation at 100,000 × *g* for 5 min in a Beckman airfuge. [^{35}S]methionine-labeled whole cell extracts of Ad-infected or Ad-transformed cells (5×10^6 cpm per immunoprecipitation reaction), prepared as described above, were immunoprecipitated with 5 or 10 μl of antipeptide sera, and the immunoprecipitates were analyzed by SDS-acrylamide gel electrophoresis as described previously (19). For peptide competition experiments, the peptide used to generate the antipeptide antibody (homologous peptide) was included in the immunoprecipitation reaction of a concentration of 0.1 to 5.0 μg/ml. In some experiments, an unrelated peptide (heterologous peptide; peptide 19, Glu-His-Phe-Leu-Pro-Leu-Arg-Asn-Ile-Cys) was included in the reaction at a concentration of 5 μg/ml.

RESULTS

Organization of the Ad2 E1B-transforming region and preparation of antipeptide antibodies directed to the NH_2 and COOH termini of the E1B-19K and E1B-53K T antigens. As illustrated in Fig. 1, we have synthesized six peptides containing 8 to 16 amino acid residues (peptides 1, 2, 5, 6, 7, and 12) encoded at the NH_2 and COOH termini of the Ad2 E1B-19K or E1B-53K T antigens. The peptides were coupled to carrier protein and were used to generate antibodies in rabbits. In this report, we describe immunoprecipitation analysis of Ad2-infected and Ad2-transformed cells by using antipeptide antibodies targeted to peptides 1, 2, 5, 7, and 12; studies with peptide 6 will be reported separately (see below).

Peptides were conjugated to BSA (peptide 12) or to KLH (peptide 7) by the glutaraldehyde procedure or to KLH by the MBS procedure (peptides 1, 2, and 5), as described above. Rabbits were immunized with the protein-conjugated peptide, and sera were analyzed by ELISA and by immunoprecipitation of [^{35}S]methionine-labeled extracts of Ad2-infected cells starting 2 months after the first inoculation. Rabbits inoculated with peptides 1, 2, 5, and 12 raised sera with positive ELISA titers of 1:320 to 1:1,280 and positive immunoprecipitation of the targeted T antigen in 2 to 3 months after the

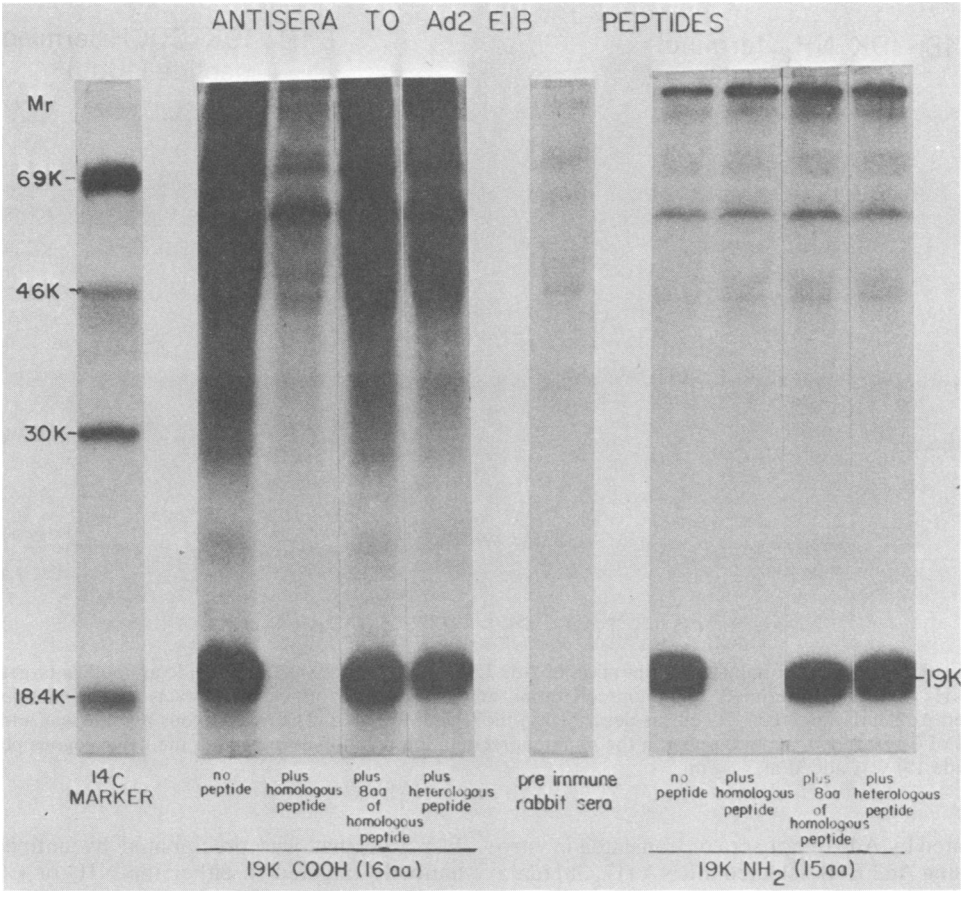

FIG. 2. Immunoprecipitation of extracts of [^{35}S]methionine-labeled Ad2-early-infected KB cells by antibody targeted to peptide 7 (COOH terminus) and peptide 12 (close to the NH_2 terminus) of the Ad2 E1B-19K T antigen (see Fig. 1). Immunoprecipitation analysis and SDS-acrylamide gel electrophoresis were performed as described in the text. Peptide competition was performed by including 5 μg of peptide per ml in the immunoprecipitation reaction. Homologous peptide refers to the peptide used to generate the antibody. Peptides homologous to the 8-amino-acid sequence at the COOH end of peptides 7 and 12 were also studied. The heterologous peptide was peptide 19 (see text). The lane at the left end contains molecular weight markers of *methyl*-^{14}C-labeled BSA (69K), ovalbumin (46K), carbonic anhydrase (30K), and lactoglobulin A (18.4K).

initial inoculation. Peptide 7 was not positive until 5 months.

The results of our studies, which are described below, are consistent with the genomic location and expression of the E1B-19K and E1B-53K T antigens that can be predicted from the Ad2 DNA sequence (16) (see Fig. 1). We have synthesized and prepared antibodies against peptide 12 (15 amino acids), coded close to the amino end of the 19K gene at nucleotide 1729, and against peptide 7 (16 amino acids), coded at the COOH terminus of the 19K gene at nucleotide 2235 (Fig. 1). In addition, we have synthesized two peptides encoded at the NH_2 terminus of the 53K at nucleotide 2019, peptide 6 (16 amino acids), and at nucleotide 2043, peptide 2 (8 amino acids) (Fig. 1), and two peptides encoded at the COOH terminus of the 53K gene at nucleotide 3500, peptide 1 (8 amino acids) and peptide 5 (13 amino acids).

Immunoprecipitation of the Ad2 E1B-19K T antigen from extracts of [^{35}S]methionine-labeled Ad2-early-infected KB cells by antipeptide antibodies and competition by homologous peptides. As shown in Fig. 2, antibody directed against peptide 7 (19K, COOH terminus, Gln-Glu-Gln-Ser-Pro-Trp-Asn-Pro-Arg-Ala-Gly-Leu-Asp-Pro-Arg-Glu) and peptide 12 (19K, NH_2 terminus, Leu-Glu-Asp-Phe-Ser-Ala-Val-Arg-Asn-Leu-Leu-Glu-Gln-Ser-Ser) (see Fig. 1) immunoprecipitated a 19K protein from extracts of Ad2-early-infected cells. The peptide map of the immunoprecipitated 19K protein was identical to that of authentic E1B-19K that was immunopre-

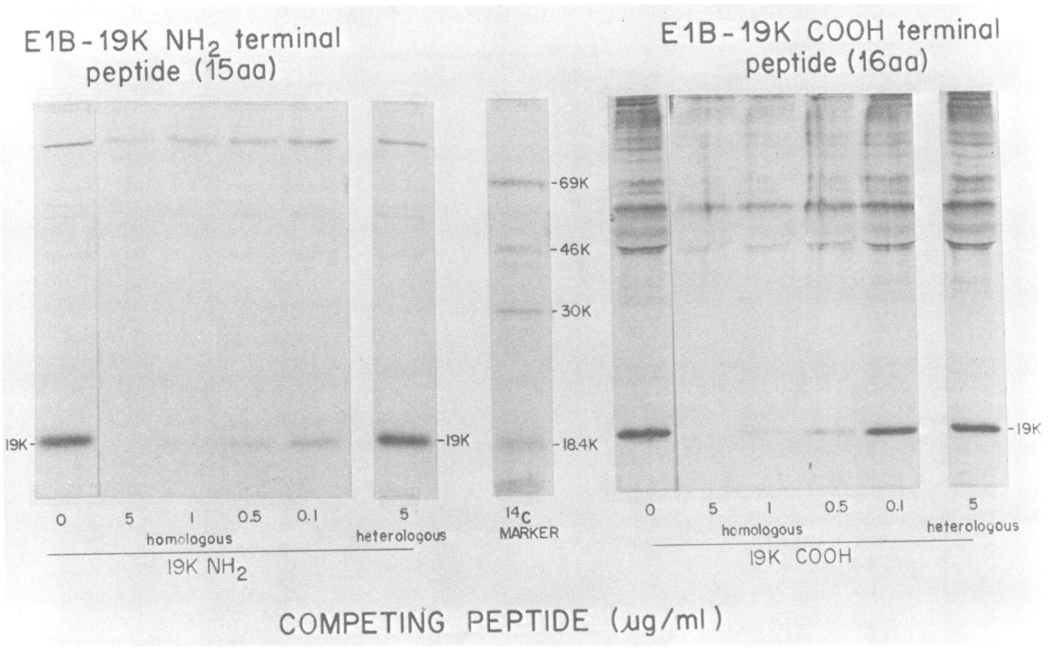

FIG. 3. Specificity of immunoprecipitation of Ad2 E1B-19K T antigen by antipeptide antibodies targeted to the NH_2 and COOH termini. Immunoprecipitation analysis was performed on extracts of [^{35}S]methionine-labeled Ad2-early-infected KB cells as described in the legend to Fig. 2. The homologous peptide was added at levels of 0.1, 0.5, 1.0, and 5.0 μg/ml in the immunoprecipitation reaction as indicated; the heterologous peptide (peptide 19) was added at 5 μg/ml.

cipitated by Ad2 tumor sera or translated in vitro by using Ad2 E1B-selected mRNA (17, 38) (data not shown).

Immunoprecipitation of the E1B-19K T antigen by antibody against peptide 7 (COOH terminus) was blocked completely by inclusion of 5 μg of peptide 7 per ml in the immunoprecipitation reaction (Fig. 2). Immunoprecipitation was not inhibited by a heterologous peptide. It is interesting that the peptide of 8 amino acids, Arg-Ala-Gly-Leu-Asp-Pro-Arg-Glu, encoded at the COOH end of the 16-amino-acid peptide 7, did not inhibit the reaction between antipeptide 7 antibody and E1B-19K (Fig. 2).

Peptide 12, encoded at the NH_2 terminus E1B-19K, completely blocked the immunoprecipitation of E1B-19K by antipeptide 12 antibody (Fig. 2). The eight-amino acid peptide, Arg-Asn-Leu-Leu-Glu-Gln-Ser-Ser, encoded at the COOH end of peptide 12 (Fig. 1), did not compete with the immunoprecipitation of E1B-19K by antipeptide 12 antibody. These data indicate that antibodies generated against peptide 12 contain predominant recognition sites located in the NH_2-terminal half of the peptide.

The abilities of different concentrations of peptide to inhibit the immunoprecipitation of the Ad E1B-19K T antigen by antipeptide antibody were tested. As shown in Fig. 3, the amount of E1B-19K that was precipitated by antipeptide antibody targeted to either the NH_2 or COOH terminus was significantly reduced by the presence of 0.5 μg of the homologous peptide per ml.

Immunoprecipitation of the Ad2 E1B-53K T antigen from extracts of [^{35}S]methionine-labeled Ad2-early-infected KB cells by antipeptide antibodies and competition by homologous peptides. As shown in Fig. 4, antibody directed to peptide 5, which contains the 13-amino-acid sequence at the COOH terminus of the E1B-53K protein (Thr-Arg-Ala-Glu-Phe-Gly-Ser-Ser-Asp-Glu-Asp-Thr-Asp) (see Fig. 1), immunoprecipitated a 53K protein from extracts of Ad2-early-infected cells. The identity of the 53K protein with the E1B-53K T antigen that is immunoprecipitated by F17 tumor sera (17) was confirmed by peptide map analysis (data not shown). The specificity of the antipeptide antibody was demonstrated by the complete inhibition of the immunoprecipitation of E1B-53K by 5 μg of peptide 5 per ml; a heterologous peptide did not block the reaction (Fig. 4).

Antibody directed against peptide 2, which contains the eight-amino-acid sequence at the NH_2 terminus of the E1B-53K gene (Gly-Val-Pro-Ala-Gly-Phe-Ser-Gly) (see Fig. 1), immunoprecipitated the E1B-53K T antigen (Fig. 5). As little as 0.5 μg of peptide 2 per ml reduced the

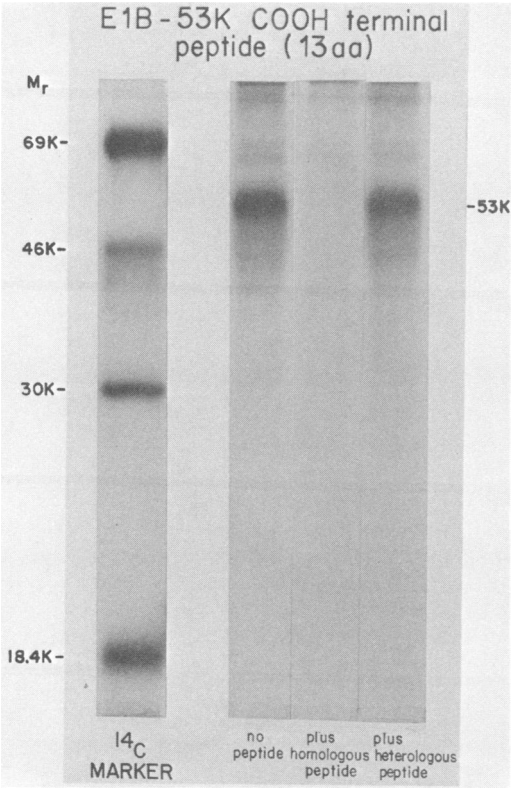

FIG. 4. Immunoprecipitation of extracts of [^{35}S]methionine-labeled Ad2-early-infected KB cells by antibody targeted to peptide 5 containing the 13-amino-acid sequence at the COOH terminus of the Ad2 E1B-53K T antigen. Immunoprecipitation analysis was performed as described in the legend to Fig. 2. Peptide competition was performed with 5.0 μg of homologous peptide (peptide 5) or heterologous peptide (peptide 19) per ml.

amount of the E1B-53K T antigen that was immunoprecipitated (Fig. 5). A heterologous peptide at 5 μg/ml did not significantly reduce the amount of immunoprecipitated E1B-53K.

Immunoprecipitation of E1B-53K and E1B-19K T antigens from extracts of [^{35}S]methionine-labeled Ad2-transformed cells by antipeptide antibodies. The Ad2-transformed rat cell line F4 contains multiple copies of the right 5% of the viral genome fused to the left 68% of the genome; F4 cells synthesize 24S and 19S hybrid mRNAs that contain both E4 and E1A sequences, as well as the normal E1A and E1B mRNAs (20, 49). As shown in Fig. 6, antibodies directed against peptide 2 and peptide 1, encoded at the NH_2 and COOH termini, respectively (see Fig. 1), of E1B-53K immunoprecipitated the E1B-53K T antigen from extracts of [^{35}S]methionine-labeled cells. In addition, antibodies directed against peptide 12 and peptide 7, encoded at the NH_2 and COOH termini, respectively, of E1B-19K immunoprecipitated the E1B-19K T antigen (Fig. 6).

The Ad2-transformed rat cell line F17 contains a major uninterrupted copy of the left 17% of the Ad2 genome and synthesizes the expected E1A and E1B mRNAs (20, 49). The presence of the E1B-53K and E1B-19K T antigens was readily demonstrated by immunoprecipitation analysis with antibodies directed against peptides 1, 2, 7, and 12, encoded at the NH_2 and COOH termini of the E1B-19K and E1B-53K T antigens (Fig. 7). In a previous study, E1B-53K was not detected in F17 cells by antitumor sera (38).

DISCUSSION

We have synthesized six peptides, ranging from 8 to 16 amino acid residues, targeted to the NH_2 and COOH termini of the Ad2 E1B-19K and E1B-53K T antigens, based on the amino acid sequence deduced from the Ad2 DNA sequence (16). Studies with five of these peptides are reported here. Peptide 12 (15-amino-acid sequence close to the E1B-19K NH_2 terminus), peptide 7 (16-amino-acid sequence at the E1B-19K COOH terminus), peptide 2 (8-amino-acid sequence close to the E1B-53K NH_2 termi-

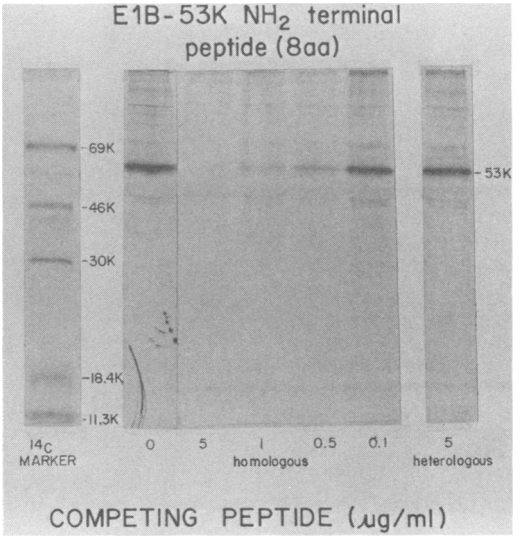

FIG. 5. Immunoprecipitation of extracts of [^{35}S]methionine-labeled Ad2-early-infected KB cells by antipeptide antibody targeted to peptide 12 containing the eight-amino-acid sequence at the NH_2 terminus of the Ad2 E1B-53K T antigen. Immunoprecipitation analysis was performed as described in the legend to Fig. 2. Peptide 2 was added to the immunoprecipitation reaction at levels of 0.1, 0.5, 1.0, and 5.0 μg/ml, as indicated. The heterologous peptide (peptide 19) was added at 5 μg/ml.

nus), peptide 5 (13-amino-acid sequence at the E1B-53K COOH terminus), and peptide 1 (8-amino-acid sequence at the COOH end of peptide 5) were coupled to carrier protein and used to generate antipeptide antibodies in rabbits. Each antipeptide antibody recognized the targeted native protein, as measured both by ELISA and by immunoprecipitation analysis with extracts of [^{35}S]methionine-labeled Ad2-early-infected cells. Peptides which contain hydrophilic amino acids, as well as peptide 2, which contains only hydrophobic amino acids, were capable of inducing specific antipeptide antibodies. The immunoprecipitation reaction was blocked by the addition of the homologous peptide, thus confirming the specificity of the immunoprecipitation reaction with antipeptide antibody. Of additional interest, peptides comprising the 8-amino-acid sequence at the COOH end of the antibody-inducing 15- or 16-amino acid-residue peptides for E1B-19K did not block the immunoprecipitation reaction. These findings suggest that antipeptide antibody recognition sites include the amino-terminal half of the peptide that was used to raise the antibody.

The organization of the Ad2 E1B-19K and E1B-53K T antigen genes has several novel features. The E1B-19K T antigen can be translated in vitro on both the E1B 22S and 13S mRNA molecules (13). E1B-19K utilizes the first ATG at nucleotide 1711 to initiate translation, as shown by N-terminal amino acid analy-

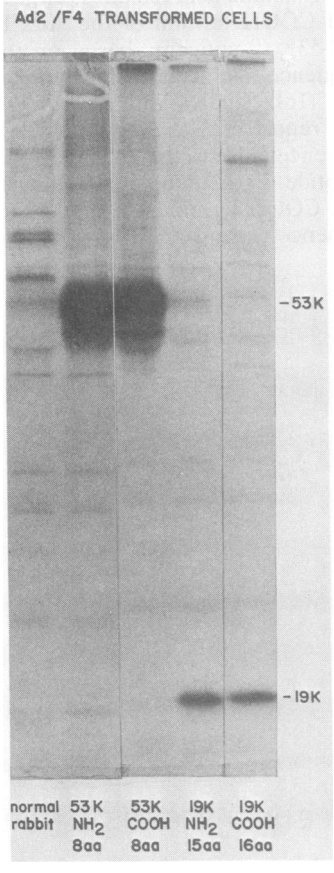

FIG. 6. Immunoprecipitation of Ad2 E1B-53K and E1B-19K T antigens from extracts of [^{35}S]methionine-labeled Ad2-transformed F4 cells by antipeptide antibody targeted to the NH_2 (peptide 2, eight-amino-acid sequence) and COOH (peptide 1, eight-amino-acid sequence) termini of E1B-53K and the NH_2 (peptide 12) and COOH (peptide 7) termini of E1B-19K. Immunoprecipitation analysis was performed as described in the legend to Fig. 2.

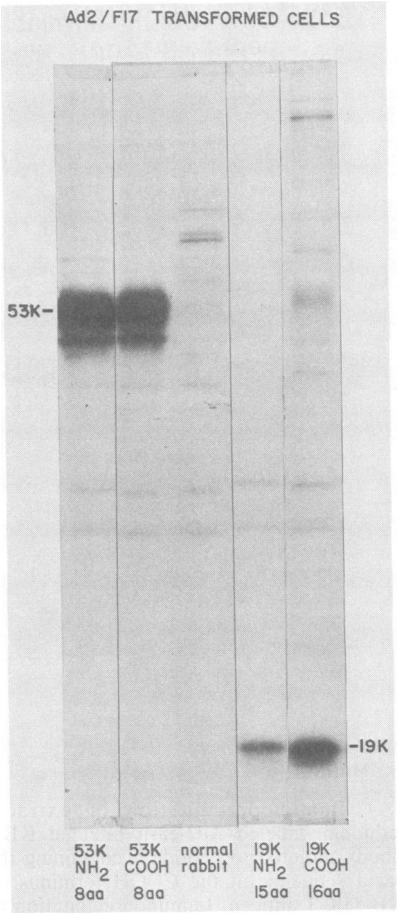

FIG. 7. Immunoprecipitation of Ad2 E1B-53K and E1B-19K T antigens from extracts of [^{35}S]methionine-labeled Ad2-transformed F17 cells by antipeptide antibody targeted to the NH_2 (peptide 2, eight-amino-acid sequence) and COOH (peptide 1, eight-amino-acid sequence) termini of E1B-53K and the NH_2 (peptide 12) and COOH (peptide 7) termini of E1B-19K. Immunoprecipitation analysis was performed as described in the legend to Fig. 2.

sis of an in vitro-translated Ad2 E1B 15K protein (1), which undoubtedly is the same protein as the E1B-19K T antigen. Brackmann et al. (8) reported that the Ad2 E1B-53K T antigen does not share peptides with Ad2 E1B-19K T antigen; this suggests that they do not initiate at the same ATG codon and cannot be translated in the same reading frame (17, 38). Similar findings were reported for the Ad5 E1B T antigens (7). The lack of relationship between the small and large E1B-encoded T antigens was difficult to interpret initially since both the 22S and 13S mRNAs appeared to have a common 5′ terminus (2, 5, 7, 11) and only one open reading frame was predicted from the DNA sequence initially reported for Ad5 E1B (57) and Ad12 E1B (53). Subsequent revisions of the original DNA sequence for Ad5 E1B (7) and for Ad12 E1B (7, 31) revealed the presence of two open reading frames which could encode small (M_r, 19,000 to 21,000) and large (M_r, 54,000 to 55,000) T antigens. Based on the corrected DNA sequences and S1 nuclease analysis, which failed to detect an E1B mRNA with a 5′ terminus in between the first and second ATG codons, Bos et al. (7) proposed that the large T antigen initiates at the second ATG in a different reading frame from E1B-19K. However, the DNA sequence data cannot exclude the possibility that initiation occurs at an ATG codon farther downstream (see below), as Bos et al. acknowledged (7). Thus, although the DNA sequence is of great value in defining the coding regions used for the synthesis of a viral protein, it is important to demonstrate at the protein level that the amino acid sequence deduced from the DNA sequence exists.

The results of our studies with antipeptide antibodies are consistent with the potential coding regions deduced from the Ad2 DNA sequence (16). The Ad2 E1B DNA sequence predicts that the E1B-19K T antigen initiates translation at the first ATG at nucleotide 1711 in translation reading frame 1 (16). Our antipeptide antibody data confirm the reading frame and the location of the NH_2 and COOH termini of the E1B-19K T antigen. The first ATG codon on the E1B-22S mRNA, which encodes the large E1B-53K T antigen, is at nucleotide 1711 in reading frame 1, whereas only reading frame 3 is large enough to encode the large E1B T antigen, E1B-53K. Translation of E1B-53K could initiate in reading frame 3 at the second, third, or fourth ATG codon at nucleotides 2016, 2202, or 2235, respectively. Initiation at the second ATG would yield a protein of M_r 54,900, whereas initiation at the third or fourth ATG would yield proteins of M_r ca. 49,000. The apparent M_r that we observed by SDS-acrylamide gel electrophoresis was 53,000. Considering the limited accuracy of protein M_r values estimated from mobilities on SDS-acrylamide gels, one cannot decide which ATG codon in frame 3 is used for initiation. The data presented here, derived by using antipeptide antibodies against peptides coded in frame 1 and frame 3, prove that the E1B-53K T antigen initiates translation at the second ATG at nucleotide 2016 in frame 3. Thus, the E1B-53K T antigen is another example of a protein that initiates translation at an internal ATG and not at the 5′-proximal ATG (32, 33). Considering the fuzzy nature of the 53K band usually observed on gels, it is conceivable that a low-level initiation occurs at the third and fourth ATG codon.

Many membrane and secretory proteins contain an N-terminal signal sequence that is removed cotranslationally. Our data demonstrate that, despite a report that the Ad E1B-19K T antigen is a membrane protein (44), it is probably not processed proteolytically at its N terminus. Indeed, our data exclude the possibility that the E1B-19K and E1B-53K T antigens are processed proteolytically at either the NH_2 or COOH terminus.

Antipeptide antibodies specific for the NH_2 and COOH termini of the E1B-19K and the E1B-53K T antigens immunoprecipitated the appropriately sized 19K and 53K proteins from the Ad2-transformed cell lines F17 and F4. This suggests that the E1B small and large T antigens that are synthesized in Ad2-transformed cells are full-length protein molecules. Cells transformed by simian virus 40 or polyomavirus often synthesize truncated T antigens or super T antigens (12, 47).

Very recently, Yee et al. (59) have prepared antibody against a peptide containing the six-amino-acid sequence at the COOH terminus of the Ad5 large T antigen. They showed that antipeptide antibody precipitated the expected protein from infected cell extracts. Immunofluorescent antibody staining detected the large T antigen in both the nucleus and cytoplasm of infected cells (59), confirming immunoprecipitation data reported previously with biochemically fractionated cells (17).

We have shown previously that an E1B-20K T antigen is a major species that is synthesized in Ad2-infected and Ad2-transformed cells (17, 38). The mRNA that codes for the E1B-20K T antigen has not been identified. Since E1B-20K is closely related to E1B-53K, we expected that antipeptide antibodies directed to some domains of the E1B-53K T antigen would recognize E1B-20K. We have found recently that antibody directed to a 16-amino-acid peptide at the NH_2 terminus of the E1B-53K protein (peptide 6 in Fig. 1) will immunoprecipitate the E1B-20K T antigen from infected and transformed cells

(Lucher, Brackmann, Symington, and Green, Virology, in press).

The Ad2 E1B-19K and E1B-53K T antigens appear to possess several interesting biological properties. The E1B-19K, specifically the NH_2-terminal domain, is essential for cell transformation, as shown recently by genetic and biochemical studies on Ad2 large-plaque mutants that are defective in cell transformation (9). In addition, recent studies suggest that the E1B large T antigen may play a role in the tumor-inducing ability of the transformed cell (6, 26). The availability of antibody specific to different domains of E1B-19K and E1B-53K should prove valuable for the purification and functional analysis of these Ad2-encoded tumor antigens.

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