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Analysis of Adenovirus Transforming Proteins from Early Regions 1A and 1B with Antisera to Inducible Fusion Antigens Produced in *Escherichia coli*

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Plasmid vectors were constructed which expressed three adenovirus tumor antigens fused to a portion of the *trpE* protein of *Escherichia coli*. Insertion of adenovirus type 2 DNA from early region 1A (E1A) into such a plasmid led to a fusion protein which contained the C-terminal 266 amino acids of the 289-amino acid protein encoded by the viral 13S mRNA. Similarly, insertion of adenovirus type 5 DNA corresponding to the E1B 55- and 21-kilodalton proteins led to production of fusion proteins containing amino acid sequences from these proteins. After induction with indoleacrylic acid, fusion proteins accumulated stably in the *E. coli* cells. By using a simple extraction of insoluble protein, 1 to 10 mg of fusion protein per liter of culture was obtained. The fusion proteins were purified on preparative polyacrylamide gels and used to immunize rabbits. Specific antisera for the E1A 289- and closely related 243-amino acid proteins and the E1B 55- and 21-kilodalton proteins were obtained. These sera were used to immunoprecipitate the tumor antigens in cells infected with wild-type and various mutants of adenovirus or to analyze them by an immunoblotting procedure. Mutant E1A proteins in which the C-terminal 70 amino acids are deleted were phosphorylated to much lower extents than the wild-type E1A proteins. This indicates that the deleted region is important for the process of phosphorylation. The E1A proteins were extracted, sedimented in glycerol gradients, analyzed by immunoprecipitation, and found to sediment primarily as monomers.

The portion of the adenovirus genome responsible for the transforming activity of the virus is subdivided into two regions, early regions 1A (E1A) and 1B (E1B) (Fig. 1). These regions are defined by two transcriptional units (2, 8) and two genetic complementation groups (20, 25). Two overlapping mRNAs transcribed at early times of infection from E1A, 13S and 12S, encode two closely related proteins of 289 and 243 amino acids (1, 45). The primary sequences of these proteins differ only by 46 internal amino acids unique to the larger protein (45). During infection two mRNAs accumulate from E1B, a 22S and a 13S mRNA, from which the two major early products of E1B, a 55-kilodalton (kd) and a 21-kd protein, are translated (4, 46, 60). Transcription and translation of these major proteins of E1A and E1B (and others which may be encoded by E1 mRNAs; see references 11, 18, 26) are important for a variety of reasons. (i) The gene products of E1A and E1B are required for cellular transformation of rodent cells in vitro (14, 16, 22, 25, 58). (ii) The E1A 289-amino acid protein induces the expression of all other early viral genes (3, 24, 42, 48). (iii) Transcription of E1A is regulated by both upstream sequences and sequences within the structural portion of the gene (43, 44; T. Osborne and A. Berk, unpublished data). (iv) E1A sequences or gene products or both may enhance transcription of nonviral genes introduced into cells by infection or transfection (R. Gaynor, D. Hillman, T. Osborne, and A. Berk, unpublished data). (v) In adenovirus-transformed cells the E1B 55-kd protein is associated with a cellular 54-kd protein; this cellular 54-kd protein is also found in association with simian virus 40 large T antigen in simian virus 40-transformed cells (51). Thus, the regulation of transcription by E1 and its role in oncogenic transformation have focused considerable interest in the proteins encoded by E1A and E1B.

The proteins of E1A are present at very low levels in both transformed and productively infected cells (21). They have been visualized on two-dimensional gels of total protein isolated from cells in which E1 mRNA and protein syntheses were increased due to prolonged inhibition of DNA synthesis (11). The E1 proteins have also been observed by immunoprecipitation of productively infected and transformed cells, using sera from animals either bearing adenovirus-induced tumors or immunized with early infected cell extracts (5, 12, 23, 30, 32, 50, 53). Monoclonal antibodies directed against adenovirus-transformed mouse cells have been used to identify the 55-kd protein of the E1B region (52). Proteins identified with these various antisera have been correlated with specific E1 messages by mRNA hybrid selection and in vitro translation studies (17–19, 33, 35, 55). Recently synthetic peptides corresponding to unique regions or carboxy termini of E1 proteins have been used to raise antisera to the E1 proteins (10, 62; K. R. Spindler and A. J. Berk, unpublished data).

We describe here the production of high-titer rabbit antisera directed against specific adenovirus E1 proteins. By using an approach similar to one used to make a foot-and-mouth disease virus vaccine (28), we have made use of DNA sequence information to produce large, in-frame fusion proteins of the *Escherichia coli trpE* gene product with adenovirus E1A and E1B proteins. Upon induction of *E. coli* containing these plasmids, large amounts of the *trpE*-adenovirus fusion proteins accumulated stably and were recovered by a relatively simple extraction of insoluble proteins. These proteins have been used to immunize rabbits, and antisera specific for the E1A, E1B 21-kd, and E1B 55-kd proteins have been obtained and used to examine the tumor antigens in wild-type and mutants of adenovirus.

MATERIALS AND METHODS

Cells and viruses. Adenovirus type 2/type 5 (Ad2/5) pm975 has a point mutation mapping at nucleotide 975 (42). Ad5

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dl312 is a deletion mutant from nucleotides 448 to 1,349 (54); Ad5 dl313 is a deletion mutant from nucleotides 1,275 to 3,625 (6, 54); and Ad5 hr1 is a single base deletion of nucleotide 1,055 (48). Wild-type Ad5 and mutant Ad2/5 pm975 were grown in HeLa cell suspension cultures, and titers were determined by plaque formation on HeLa cell monolayers. Mutants Ad5 dl312, Ad5 dl313, and Ad5 hr1 were grown and titrated on the complementing 293 cell line (15, 20).

Construction of recombinant plasmids. The construction and structure of the *trpE*-containing vectors pKRS101 and pKJB231.1 are described in Fig. 2a. Plasmids were kindly supplied as follows: pPS21 by R. Gunsalus, University of California, Los Angeles; pKJB1, pKJB231.1, and pUC8CM by K. Buckley, University of California, San Diego; and C131 by U. Petterson, University of Uppsala, Uppsala, Sweden. Enzymes were obtained from New England Biolabs or Bethesda Research Laboratories and used as recommended. *Bam*HI linkers were obtained from Collaborative Research and treated with kinase before use. Restriction fragments were purified from 7% polyacrylamide gels (40). Preparative quantities of plasmids were obtained, and ligations and transformation of *E. coli* C600 or HB101 were performed according to the methods of Maniatis et al. (37). A 20-μg/ml portion of tryptophan was added to all media used in transformations using plasmids containing *trp* operon sequences. HB101 was initially transformed with recombinant plasmids; after screening, desired plasmids were used to transform C600.

Induction of expression plasmids. C600 strains containing recombinant plasmids were grown overnight at 37°C in M9 (37) plus 1% Casamino Acids, 20 μg of tryptophan per ml, and 100 μg of ampicillin per ml. These cultures were diluted 1:100 in M9 plus Casamino Acids and ampicillin. At an optical density at 600 nm of 0.2, a 1:1,000 dilution of a 10-mg/ml stock of indoleacrylic acid (Sigma Chemical Co.) in 100% ethanol was added to the cultures to be induced (9). Growth was continued to saturation (8 to 16 h) and the cells were harvested. Total cell lysates were prepared by boiling in Laemmli gel sample buffer (29). Alternatively, cells were lysed and treated with DNase I in high salt for 1 h, and the insoluble protein pellet was recovered exactly as described previously (28). This insoluble pellet was dissolved by

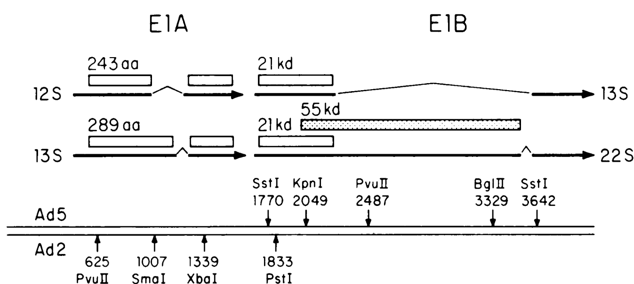

FIG. 1. E1A and E1B DNA sequences and gene products. The DNA sequences of Ad5 and Ad2 from the left end of the genome are shown at the bottom, with restriction sites used in cloning marked. Nucleotide sequence numbers are given for Ad5 and Ad2 above and below the line, respectively (57). Above this the heavy horizontal arrows joined by carets indicate the exons of mRNAs; the arrowheads indicate their 3' ends. Boxes above the exons represent the translated regions; note that the E1B 55-kd protein is translated from a different reading frame than the 21-kd protein (indicated by the shading) (4).

boiling in 1× to 2× Laemmli gel sample buffer for 5 to 30 min.

Gel electrophoresis. Proteins were electrophoresed on gels (1.5 mm by 12 cm by 18 cm) of 8, 10, or 12% polyacrylamide with a 5% stacking gel at 20 to 30 mA (constant current) until the bromophenol blue reached the bottom of the gel (29). Proteins were visualized by staining with Coomassie brilliant blue (induction experiments). Radiolabeled samples were fixed and dried ($^{32}PO_4$-labeled samples) or fixed and treated with 1 M sodium salicylate for 1 h, dried (^{35}S-labeled samples), and exposed to X-ray film, using intensifying screens. Molecular weight markers for staining were obtained from Sigma; ^{14}C-labeled protein standards were from Amersham Corp.

Immunization. Fusion proteins were prepared by electrophoresis in preparative 8% polyacrylamide gels. Protein position and concentrations were estimated by staining side marker lanes. Gel strips were excised and emulsified in complete Freund adjuvant; initial injection of 2-kg New Zealand female rabbits contained 200 to 500 μg of protein. Rabbits were boosted after 3 weeks with 100 to 200 μg of protein in incomplete Freund adjuvant, and serum was collected 7 to 10 days later. Subsequent boosts and serum collection were approximately 2 weeks apart.

Labeling, infection, and lysis of cells. A total of 2×10^7 monolayer HeLa cells were infected at the multiplicity of infection (MOI) indicated in the presence of 20 μg of cytosine arabinoside (araC) per ml (11), which was replenished every 12 h. At the indicated times (36 to 45 h after infection) the cells were washed three times with phosphate-buffered saline (for [^{35}S]methionine labeling) or 0.15 M NaCl (for $^{32}PO_4$ labeling). Dulbecco minimum essential medium minus methionine or PO_4, respectively, with 25 μCi of [^{35}S]methionine or 300 μCi of $^{32}PO_4$ per ml was added in a volume of 7 ml. After 2 h the cells were washed three times, divided into aliquots, and frozen at −20°C. Cell lysates were prepared in two ways. (i) Cells were lysed and digested with micrococcal nuclease, RNase A, and DNase I as described before (11) except that the clarified supernatant was not lyophilized but diluted directly into RIPA buffer (0.15 M NaCl, 0.01 M $NaPO_4$ [pH 7.0], 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.02% NaN_3, 100 U of Trasylol [Mobay Biochemical] per ml). (ii) Alternatively, cells were lysed by the procedure of Manley et al. (38) except that only 0.7 packed-cell volume of saturated $(NH_4)_2SO_4$ was added. After incubation for 60 min on ice, the lysates were centrifuged at 40,000 rpm for 60 min in a Beckman 50Ti rotor. The supernatant was removed and dialyzed against phosphate-buffered saline or diluted into RIPA buffer or both.

Immune precipitation and glycerol gradients. Precipitations were performed in 0.5 ml of RIPA buffer with 5 μl of antiserum at 0°C for 12 to 16 h and then incubated with 50 μl of *Staphylococcus aureus* Cowan I (27) for 30 min. The immunoprecipitates were pelleted, washed twice with 0.5 ml of RIPA and once with 1 ml of 50 mM Tris-hydrochloride (pH 6.8), and eluted by resuspending in 25 μl of Laemmli gel sample buffer (29) and boiling for 3 min. For sedimentation analysis, lysates were prepared by method (ii), dialyzed against phosphate-buffered saline, and then mixed with an equal volume of 2× RIPA buffer. Glycerol (5 to 20%) gradients (5 ml) in RIPA buffer were prepared with a 0.1-ml cushion of 55% CsCl in 20% glycerol–RIPA. Samples (0.2 ml) containing the protein extracted from 8×10^5 cells were sedimented in these gradients in a Beckman SW50.1 rotor at 4°C for 17 h at 40,000 rpm. Twenty fractions were

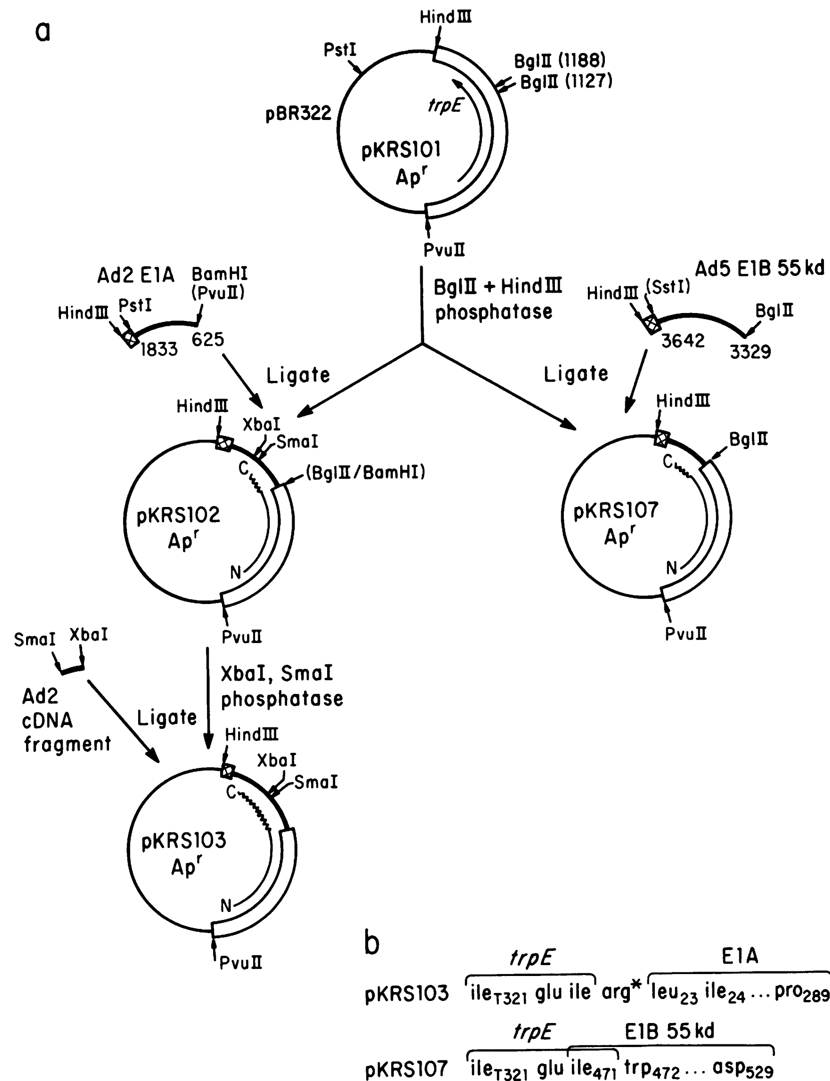

FIG. 2. Construction of *trpE*-adenovirus expression vectors for E1A and E1B 55-kd fusion proteins. (a) A *Pvu*II-*Hin*dIII fragment containing nucleotides −260 to 1,999 of the *E. coli* tryptophan operon (61) was gel purified from pPS21, a plasmid containing a portion of the *trp* operon. This fragment was ligated to the large *Pvu*II-*Hin*dIII fragment (containing the pBR322 origin) of pKJB1, a pBR322 derivative lacking the *Eco*RI site. For pKRS102 an Ad2 fragment of genomic DNA was prepared from BE5, which contains the left 3.4 kilobases of DNA (56), as follows. BE5 was digested with *Pvu*II and *Bam*HI linkers were added, followed by digestion with *Pst*I and *Bam*HI. A 1,200-base pair fragment representing nucleotides 625 to 1,833 of Ad2 DNA was cloned into *Pst*I-*Bam*HI-digested M13mp9 (41) and subsequently excised by *Bam*HI-*Hin*dIII digestion. Ligation of this fragment to *Bgl*II-*Hin*dIII-digested pKRS101 yielded pKRS102, with the loss of the *Bgl*II and *Bam*HI restriction sites. To remove the DNA corresponding to the intervening sequence, a fragment from a cDNA clone of Ad2, C131 (45), was used to replace a fragment of pKRS102. The cDNA clone C131 and pKRS102 were digested with *Sma*I and *Xba*I. An excess of gel-purified fragment from C131 was mixed and ligated with the digested pKRS102 to yield pKRS103. For pKRS107, an Ad5 fragment was first cloned into pUC8CM (a chloramphenicol-resistant derivative of pUC8; 41) as follows. An Ad5 fragment extending from the *Kpn*I site (2,049) to the *Sst*I site (3,642) was treated with T4 polymerase to remove the 3′ protruding ends (37) and cloned into the *Sma*I site of pUC8CM. A *Bgl*II-*Hin*dIII fragment was excised from this plasmid and ligated with *Bgl*II-*Hin*dIII-digested pKRS101 to yield pKRS107. Open bars represent *trp* sequences; heavy lines, adenovirus sequences; striped regions, polylinker sequences from M13mp9 or pUC8CM. Nucleotide numbers indicate adenovirus sequence numbers (see Fig. 1). Transcription and translation from *trp* sequences are indicated: N and C represent amino and carboxy termini of the proteins, respectively. Former restriction sites are indicated in parentheses. (b) Amino acid sequences at the *trpE*-adenovirus junctions of pKRS103 and pKRS107. T321 refers to residue 321 of the *trpE* sequence; the amino acid sequence numbers for the adenovirus proteins are indicated; * indicates an amino acid derived from the *Bam*HI linker.

collected from each gradient. The cushion fraction from each gradient was dialyzed against phosphate-buffered saline to remove the CsCl. Fractions were immunoprecipitated with anti-E1A fusion serum and analyzed by gel electrophoresis. Marker gradients were sedimented in parallel and contained 30 μg each of rabbit immunoglobulin and bovine serum albumin and 60 μg each of ovalbumin and lysozyme. Marker gradient fractions were analyzed by gel electrophoresis and Coomassie blue staining of the gel.

"Western" immunoblotting. Cells were infected at the indicated MOI in the presence of 20 μg of araC and harvested at 45 to 46 h postinfection (p.i.). A total of 2×10^5 cells were boiled in 40 μl of Laemmli gel sample buffer and loaded onto polyacrylamide gels. Proteins were electropho-

retically transferred to 0.2-μm nitrocellulose (Schleicher & Schuell) for 6 h at 200 mA (7). Remaining protein sites were blocked by soaking the nitrocellulose filter in 5% ovalbumin in 50 mM Tris (pH 7.5)–0.9% NaCl–0.02% azide (TSA) for 10 min. Filters were incubated with a 1:100 dilution of the appropriate antiserum in 2.5% ovalbumin–TSA for 5 to 15 h in a volume of 7 ml, washed for 1 h with five changes of TSA, and incubated for 2 h with 5×10^5 cpm of ^{125}I-labeled *S. aureus* protein A prepared by the method of Markwell (39). The filters were washed with seven to eight changes of TSA over 3 h and exposed to X-ray film. Anthranilate synthetase component I (the *trpE* gene product) of *Salmonella typhimurium* was included as a marker protein and internal control in the Western transfers; the purified protein was obtained from S. French, University of Virginia, Charlottesville.

RESULTS

Construction of inducible expression plasmids for E1A and E1B. An expression vector, pKRS101, was constructed (Fig. 2a) which contains the *E. coli trp* operator, promoter, *trpL* (leader), and attenuator sequences, in addition to the entire *trpE* coding sequence and the 5′ portion of the *trpD* gene cloned into a pBR322 derivative. To express fusion proteins, adenovirus DNA fragments were inserted at the *Bgl*II site at *trp* nucleotide 1,127 within the *trpE* gene.

For expression of the E1A gene, an Ad2 DNA fragment was prepared from the left end of the viral genome, its ends were modified, and it was cloned into the *Bgl*II-*Hin*dIII-digested pKRS101 (Fig. 2). Since the resulting plasmid, pKRS102, contains DNA sequences corresponding to the intron of the 13S mRNA of Ad2 E1A, translation of an mRNA transcribed from this plasmid in bacteria would result in a fusion protein terminating in the intron region. To circumvent this problem, we obtained a cDNA clone corresponding to the spliced Ad2 13S mRNA (45). Unique restriction sites *Sma*I and *Xba*I flank the 13S mRNA intron sequence (Fig. 1) and were used to generate a fragment 213 nucleotides long from the cDNA clone. This 213-base pair fragment was then substituted into *Sma*I-*Xba*I-digested pKRS102 to give pKRS103 (Fig. 2a). This plasmid contains the coding information for the C-terminal 266 amino acids of the E1A 289-amino acid protein, fused to the *trpE* sequences; the reading frame for the adenovirus E1A protein is maintained. Translation of the fusion protein gives the N-terminal two-thirds of the *trpE* protein fused to the C-terminal portion of the E1A protein (Fig. 2b).

An E1B 55-kd fusion protein expression plasmid was obtained by cloning a DNA fragment corresponding to the C-terminal portion of the Ad5 55-kd protein into pKRS101, resulting in pKRS107 (Fig. 2a). The fusion protein encoded by pKRS107 contains the N-terminal two-thirds of the *trpE* protein fused to the C-terminal 59 amino acids of the E1B 55-kd protein (Fig. 2b).

A slightly different vector was used to construct an E1B 21-kd expression plasmid. This vector, pKJB231.1, contains sequences from −260 to 1,127 of the *trpE* region (Fig. 3a). Cloning an *Eco*RI-*Bam*HI fragment into this vector destroys tetracycline resistance and allows the expression of *trpE*-adenovirus fusion protein. An Ad5 DNA fragment corresponding to the coding region for the 21-kd protein was prepared and inserted into pKJB231.1, resulting in pDR21 (Fig. 3a). The resulting fusion protein from this plasmid contains four amino acids derived from polylinker sequences and the 157 C-terminal residues of the 21-kd protein fused to the N-terminal two-thirds of the *trpE* protein.

Induction of *trpE*-adenovirus fusion proteins. Cells containing the *trpE* vector pKRS101 or pKJB231.1 or the *trp*-adenovirus plasmid pKRS103, pKRS107, or pDR21 were tested for their ability to produce the expected fusion proteins upon induction of the *trp* operon with indoleacrylic acid (9). Cells were grown in the presence or absence of 10 μg of indoleacrylic acid per ml, and the insoluble protein pellets were subjected to gel electrophoresis as described in Materials and Methods (Fig. 4). The fusion proteins are insoluble and resistant to proteolytic degradation and accumulate inside the *E. coli* cell (13). Large amounts (1 to 10 mg of fusion protein per liter of culture) of the expected size fusion proteins were produced from all of the adenovirus DNA-containing plasmids.

Figure 4, lane 4I, shows the induced protein from pKRS103, whose fused *trpE*-Ad2 E1A protein should have an estimated molecular weight of 65,000. The induced protein from these cells has an apparent molecular weight of

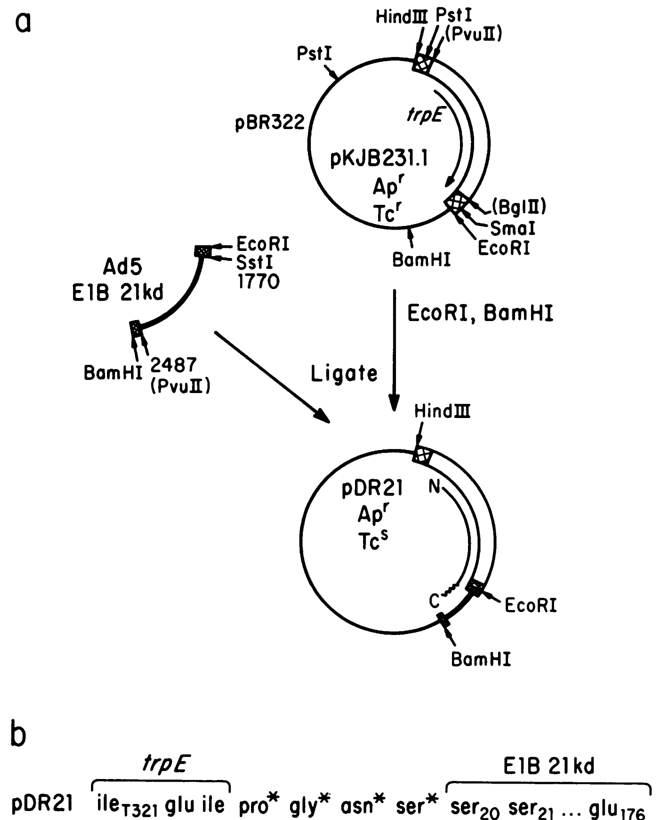

FIG. 3. Construction of *trpE*-adenovirus expression vector for E1B 21-kd fusion protein. (a) pKJB231.1 contains *E. coli trp* operon sequences from −260 to 1,127 (corresponding to the *Bgl*II site at 1,127 diagrammed in pKRS101, Fig. 1) flanked by polylinker sequences from M13mp9. An Ad5 DNA fragment extending from the *Sst*I site at 1,770 to the *Pvu*II site at 2,487 was cloned into M13mp11 (41) and excised by digestion with *Eco*RI and *Bam*HI. This 730-base pair fragment was ligated with *Eco*RI-*Bam*HI-digested pKJB231.1 to yield pDR21. Symbols are as in the legend to Fig. 2; dotted region indicates the polylinker sequence from M13mp11. (b) Amino acid sequence at the *trpE*-adenovirus fusion of pDR21. T321 designates the amino acid at position 321 of the *trpE* sequence; amino acid sequence numbers for the adenovirus protein are indicated; * indicates an amino acid derived from polylinker sequences.

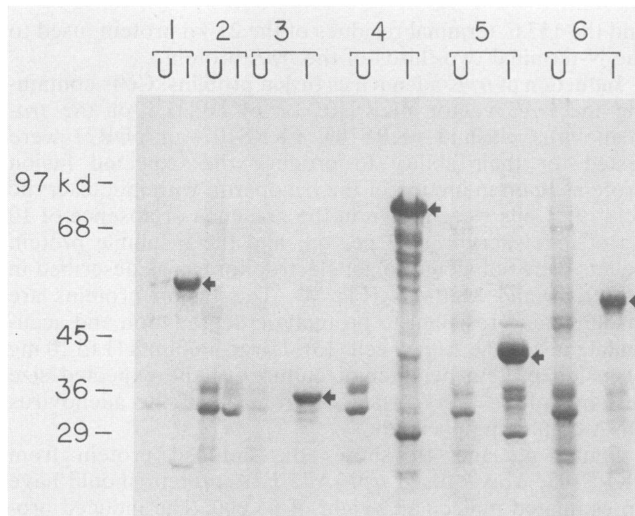

FIG. 4. Induction of fusion proteins from plasmid-bearing cells. Insoluble protein pellets (lanes 1, 2, 4 to 6) or total cell lysates (lane 3) of C600 cells containing (1) pKRS101, (2, 3) pKJB231.1, (4) pKRS103, (5) pKRS107, or (6) pDR21 uninduced (U) or induced (I) with indoleacrylic acid were analyzed on a 10% polyacrylamide gel and visualized by Coomassie blue staining. Positions of marker proteins are indicated. Arrows indicate the induced proteins of interest.

72,000. The E1A proteins from adenovirus-infected cells have been shown to migrate anomalously in sodium dodecyl sulfate-polyacrylamide gels at a molecular weight of 45,000 to 50,000, although their sequence predicts a molecular weight of 27,000 to 32,000 (55). Thus, the E1A sequence in the fusion protein may contribute to the apparently high molecular weight of the fusion protein.

The fusion protein from pKRS107 migrates close to the predicted molecular weight of approximately 42,000 (Fig. 4, lane 5I). To be certain that this protein was not simply a termination at the end of the *trpE* coding region in pKRS107, the protein produced by pKJB231.1 was examined. pKJB231.1 should produce such a truncated *trpE* protein with an expected molecular weight of approximately 35,000. As expected, this protein can be seen to migrate faster than the protein induced in pKRS107 (Fig. 4, cf. lanes 3I and 5I). In fact, the protein from pKJB231.1 was not observed in the "insoluble pellet" method of preparation (lanes 2U and I) and could only be observed in whole-cell lysates (lanes 3U and I). After induction of pDR21, a protein whose mobility is in good agreement with the expected fusion protein molecular weight of 53,000 can be seen in Fig. 4, lane 6I.

Immunoprecipitation of tumor antigens with antisera directed against fusion proteins. Preparative quantities of the fusion proteins were used to immunize rabbits, as described in Materials and Methods. Four weeks after immunization sera were obtained and used to immunoprecipitate lysates from infected cells (Fig. 5). [^{35}S]methionine-labeled extracts of HeLa cells mock infected or infected with Ad5 or dl313 were tested with antisera directed against the E1A, E1B 55-kd, or E1B 21-kd fusion protein. dl313 eliminates 70 amino acids from the C terminus of the E1A protein and lacks the E1B 21- and 55-kd proteins. The anti-E1A antibody precipitates several proteins with apparent molecular weights ranging from 45,000 to 54,000 from Ad5-infected cells and slightly lower-molecular-weight proteins from dl313-infected cells (Fig. 5, lanes 5 and 6).

Antiserum directed against the E1B 21-kd protein specifically precipitates a 21,000-molecular-weight protein from wild-type- but not dl313-infected cells (Fig. 5, lanes 12 and 13). The anti-E1B antiserum directed against the *trpE*-E1B 55-kd fusion protein recognizes a protein of approximately 55,000 from wild-type- but not dl313-infected cells (Fig. 5, lanes 16 and 17). Labeled material remaining at the top of the gel in lane 16 may be aggregated or incompletely solubilized E1B 55-kd protein. This material has been seen only in immunoprecipitations of wild-type Ad5 (and not E1B mutants) with the anti-55-kd serum. Both E1B protein antisera precipitate a protein which comigrates with the adenovirus hexon protein in the marker lane and probably represents nonspecific coprecipitation of this protein. Much lower levels of hexon protein are induced in dl313-infected cells under these conditions.

Several experiments were performed to confirm the specificity of these antisera. The anti-E1A serum precipitated proteins from wild-type-infected cells which comigrated with proteins precipitated by an antiserum directed against a C-terminal synthetic peptide (62) of the E1A proteins coupled to keyhole limpet hemocyanin (Spindler and Berk, unpublished data), on both one-dimensional (Fig. 6) and two-dimensional gels (data not shown). The E1A proteins precipitated by the antifusion E1A serum also correspond to those observed in two-dimensional electrophoresis of whole-cell lysates (11) from adenovirus-infected cells (data not shown). The 21-kd protein immunoprecipitated with the corresponding antifusion serum comigrated on two-dimensional gels with the 21-kd protein identified by Gaynor et al. (11) in whole-cell lysates (data not shown).

Both of the proteins encoded by the E1A region and the E1B 55-kd protein have been shown to be phosphorylated

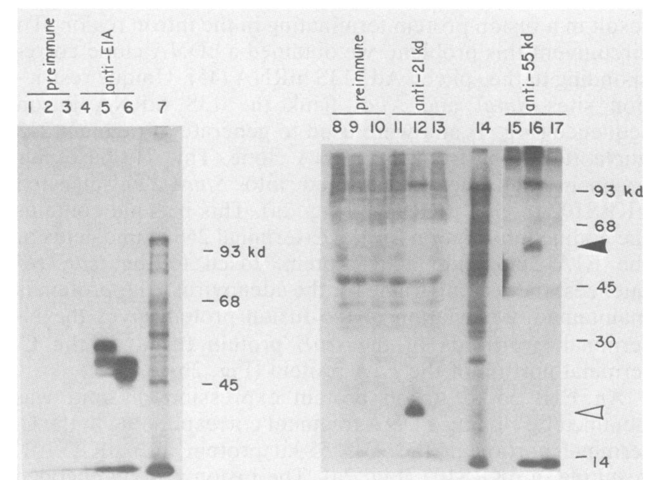

FIG. 5. Immunoprecipitation of [^{35}S]methionine-labeled, adenovirus-infected cell extracts. HeLa cells were mock infected (lanes 1, 4, 8, 11, 15) or infected in the presence of araC at an MOI of 25 with Ad5 (lanes 2, 5, 9, 12, 16) or dl313 (lanes 3, 6, 10, 13, 17) and labeled with [^{35}S]methionine at 36 h p.i. Extracts were prepared by method i (see text). The extract from 10^6 cells was immunoprecipitated with 5 μl of the indicated antiserum and analyzed on 8 or 12% polyacrylamide gels (lanes 1 to 7 and 8 to 17, respectively) by autoradiography. Lanes 7 and 14 are an extract of adenovirus-infected cells labeled with [^{35}S]methionine at 24 h p.i. in the absence of araC (late lytic marker). The positions of ^{14}C-labeled marker proteins are indicated by their molecular weights. The filled arrow indicates the E1B 55-kd protein; the open arrow indicates the E1B 21-kd protein. Preimmune indicates normal rabbit serum.

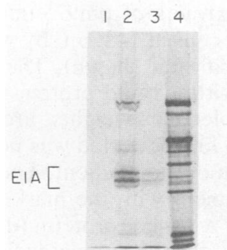

FIG. 6. Immunoprecipitation by E1A antifusion protein and antipeptide sera. Cells infected at an MOI of 150 in the presence of araC were labeled with [^{35}S]methionine at 40 h p.i. and lysed by method ii (see text), and the extract from 2×10^5 cells was precipitated with 5 μl of (1) normal rabbit serum, (2) E1A antifusion protein serum, or (3) E1A antipeptide serum and analyzed on an 8% polyacrylamide gel. Lane 4 is a late lytic marker. The position of the E1A proteins is indicated.

(11, 31, 36). Immunoprecipitation of cells infected with wild-type and various adenovirus mutants labeled with $^{32}PO_4$ are shown in Fig. 7. HeLa cells were infected with Ad5, dl313, or dl312 and labeled at 36 h p.i. with [^{35}S]methionine or $^{32}PO_4$. Extracts were immunoprecipitated with normal, anti-E1A, or anti-55-kd serum and analyzed by gel electrophoresis. The phosphorylated E1A proteins of Ad5 and dl313 can be seen in Fig. 7, lanes 6 and 7. The E1A proteins of dl313 appear to be much less phosphorylated than those of Ad5; as a control, the amount of [^{35}S]methionine-labeled E1A proteins in these two virus infections can be seen in lanes 2 and 3. Total E1A proteins in these infections can also be seen in an immunoblotting experiment (Fig. 8A, lanes 2 and 6). The total amount of E1A proteins present in dl313 is less than in the wild type, but the decrease in phosphorylation of E1A proteins in dl313 is much greater than that in the amount of protein. Since the dl313 deletion removes the C-terminal 70 amino acids of the two E1A proteins, this C-terminal region must be important for the phosphorylation of these proteins.

The E1B 55-kd protein is phosphorylated (Fig. 7, lane 16). As expected, no material is seen in the corresponding position in an immunoprecipitation of dl313-infected cells (lane 17). The 55-kd protein is not visible in the dl312 infection (lane 18). Such a result is expected, since at the low MOI of dl312 used induction of the E1B transcription unit is greatly reduced (24).

The antisera produced against the fusion proteins were also used in Western immunoblotting experiments. Cell extracts from wild-type, pm975, dl312, dl313, or hr1 infections were separated on polyacrylamide gels and transferred to nitrocellulose as described in Materials and Methods. Proteins separated on an 8% gel were probed with anti-E1A antibody (Fig. 8A); proteins separated on a 12% gel were probed with anti-21-kd antibody (Fig. 8B). The results of these experiments reiterate the specificity of the antibodies produced by immunization with the bacterial fusion proteins and indicate that the antibodies are able to recognize denatured proteins. The mutant Ad2/5 pm975 eliminates the E1A 12S mRNA (42) and the 243-amino acid protein (11). Ad5 hr1 has a single base deletion causing an out-of-frame termination of the E1A 289-amino acid protein (48). Ad5 dl312 has a large deletion which eliminates production of the E1A proteins (54). Three predominant bands of E1A proteins can be seen in Ad5-infected cells (Fig. 8A), whereas only two can be seen in the Ad2/5 pm975 and hr1 infections, and their proportions vary relative to the corresponding bands in the wild type. Multiple forms of the E1A proteins have been described (5, 48, 55, 62) and may represent post-translational modifications of the proteins. As expected, no E1A bands are detected in dl312-infected cells, and as mentioned above, there are decreased amounts of E1A in dl313-infected cells relative to wild-type-infected cells.

The E1B-21-kd protein is visualized in the immunoblotting experiment shown in Fig. 8B. Whereas the Ad5 and Ad2/5 pm975 levels of the 21-kd protein are approximately equal, the amount of 21-kd protein in the hr1 infection is decreased, despite the high levels of E1A-specific proteins present (Fig. 8A). Note that the cells in the hr1 infection were infected at a higher MOI. As was seen in the immunoprecipitation of [^{35}S]methionine-labeled extracts (Fig. 5), no 21-kd protein is observed in an infection by dl313. Ad5 dl312 does not produce detectable 21-kd protein under these conditions of

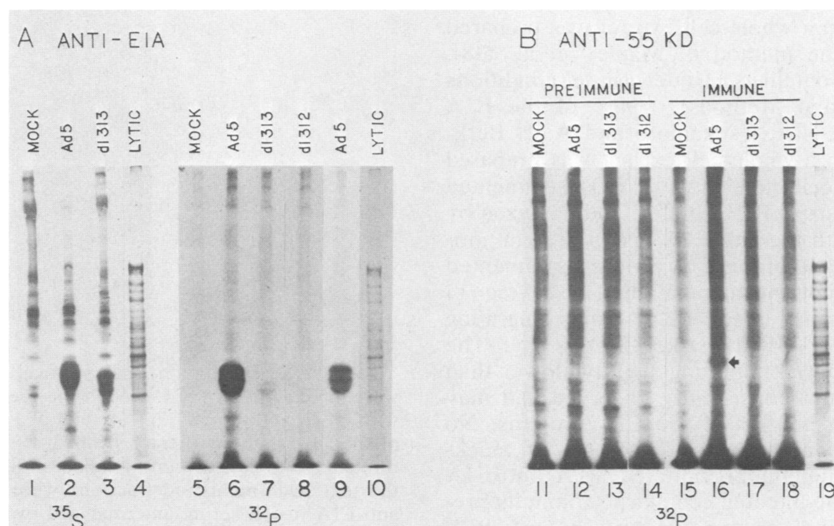

FIG. 7. Immunoprecipitation of phosphorylated E1 proteins. HeLa cells were infected at an MOI of 20 in the presence of araC and labeled with [^{35}S]methionine (lanes 1 to 3) or $^{32}PO_4$ (lanes 5 to 9, 11 to 18) at 36 h p.i. Lysates were prepared by method i from cells mock infected or infected with Ad5, dl313, or dl312 as indicated. The extract from 10^6 cells was immunoprecipitated with the indicated sera and analyzed on 8% polyacrylamide gels. (Lanes 4, 10, 19) Late lytic marker. Lane 9 is a shorter exposure of lane 6, exposed for half as long.

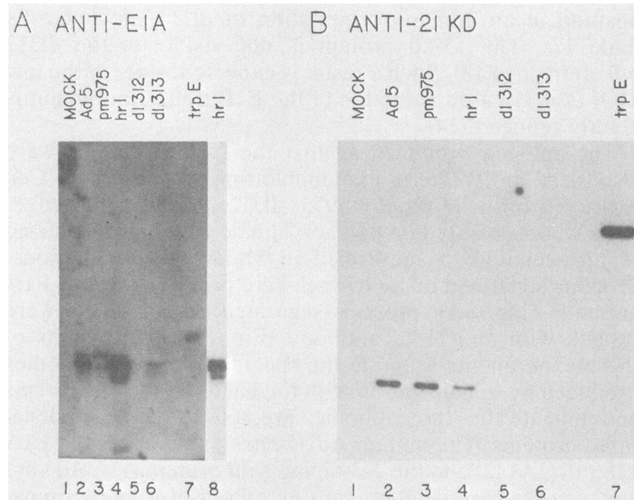

FIG. 8. Immunoblotting analysis of adenovirus mutants. HeLa cells were infected with Ad5, pm975, dl312, dl313 (MOI = 20), or hr1 (MOI = 70) in the presence of araC, harvested at 45 h p.i., and lysed in Laemmli gel sample buffer as described in the text. The lysate from 10^5 cells was loaded on each lane of an 8 or 12% polyacrylamide gel (A and B, respectively). Proteins were transferred to nitrocellulose filters which were incubated with anti-E1A (A) or anti-E1B 21-kd (B) fusion serum. Lanes: 1, Mock; 2, Ad5; 3, pm975; 4, hr1; 5, dl312; 6, dl313. Lane 7 contained approximately 1 μg of purified *Salmonella typhimurium trpE* protein. Lane 8 is a shorter exposure of lane 4, exposed for one-fifth as long.

low MOI (lane 5), as expected, since the induction of E1B in Ad5 dl312 is defective (24).

Sedimentation analysis of E1A proteins. Feldman and Nevins reported that the E1A 289-amino acid protein sediments very rapidly in a glycerol gradient prepared in RIPA buffer (10) when the protein is extracted in low salt from nuclear or cytoplasmic fractions of infected cells. These authors analyzed gradient fractions by immunoblotting, using antisera specific for the 289-amino acid protein (10). In agreement with these results, we found that after a similar low-salt extraction the E1A proteins sedimented rapidly (data not shown). However, when a whole-cell extract was prepared by a modification of the method of Manley et al. (38), different results were obtained. Under these conditions (method ii; Materials and Methods), >90% of the E1A proteins were solubilized (A. Tsukamoto and A. J. Berk, unpublished data). Such a whole-cell extract was prepared from wild-type-infected cells labeled with $^{32}PO_4$, sedimented through glycerol gradients in RIPA buffer, and analyzed by immunoprecipitation with the anti-E1A fusion protein antiserum (Fig. 9). Virtually all of the E1A proteins sedimented near the top of the gradient, at the position of lysozyme (14 kd). The E1A proteins are observed as bands migrating slightly slower than the 45 kd marker in this gel. The immunoprecipitated material migrating slightly slower than the 30-kd marker was not consistently observed and may represent a degradation product of the E1A proteins. No specific bands were seen in control gradients of mock-infected cell extracts immunoprecipitated with anti-E1A fusion serum or wild-type-infected cell extracts immunoprecipitated with preimmune serum (data not shown). In a longer exposure of the autoradiogram shown in Fig. 9, several additional bands could be seen, but all of these were also present in the control gradients. Identical results were obtained with ^{32}S-labeled extracts of wild-type-infected cells, ^{32}P-labeled extracts of pm975-infected cells, and extracts isolated from cells at 14 h p.i. by wild-type virus in the absence of araC (data not shown). The cosedimentation of the E1A proteins with a 14-kd protein was surprising since their predicted molecular weights are 27,000 to 32,000. Sedimentation for a longer period was performed to "spread out" the top portion of the gradient. The E1A proteins were again found to sediment with the marker 14-kd protein and more slowly than a 20-kd protein (data not shown). A possible explanation for the anomalously low sedimentation coefficient of the E1A proteins as extracted here is discussed below.

DISCUSSION

The proteins from E1, the transforming region of adenovirus, have been difficult to study since they are present in small amounts in infected or transformed cells. Serological methods have been used to identify these proteins. Previously, other laboratories have prepared antisera from adenovirus-induced tumor-bearing animals, which recognize an array of proteins from infected cells (12, 23, 30, 32, 50, 53). A monoclonal antibody to the E1B 55-kd protein has been prepared (52); antibodies to synthetic peptides of the E1A- and E1B-encoded proteins have also been prepared (10, 62; Spindler and Berk, unpublished data). In addition, using conventional purification procedures, Persson et al. (47) were able to purify the Ad2 E1B 15-kd protein (analogous to the 21-kd protein here) and use it to raise specific antisera. As described here, we have used fusion proteins produced in bacterial cells to raise specific antibodies to the tumor antigens of E1A and E1B.

Three specific antisera against adenovirus tumor antigens were obtained by using expression plasmids. pKRS103 was constructed to produce a *trpE*-Ad2 E1A fusion of the 289-amino acid protein. The protein induced in cells carrying this plasmid elicited specific antibodies in rabbits in 1 month.

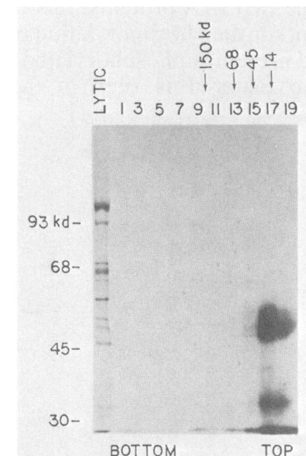

FIG. 9. Sedimentation analysis of E1A proteins. HeLa cells were infected at an MOI of 50 in the presence of araC and labeled with $^{32}PO_4$ at 36 to 38 h p.i. A total of 5×10^6 cells were lysed by method ii, and the extract from 8×10^5 cells was sedimented through a 5 to 20% glycerol gradient in RIPA buffer as described in the text. Odd-numbered fractions were immunoprecipitated with anti-E1A fusion serum and analyzed by gel electrophoresis. Fraction 1 contained the CsCl cushion and was dialyzed before immunoprecipitation. Arrows and molecular weights at the top indicate sedimentation of markers in a parallel gradient. Gel electrophoresis markers are indicated by the numbers at the side. Lytic indicates the late lytic marker.

These antibodies have been used to examine proteins produced in adenovirus-infected cells and have been shown to recognize the predicted tumor antigens of E1A (Fig. 5 to 8). Plasmids were also constructed which produce fusion proteins of the *trpE* gene with either the E1B 55-kd or the 21-kd protein. These fusion proteins also elicited a rapid antibody response, specific for the appropriate E1B tumor antigens.

The immunoprecipitation of E1A proteins from dl313, a deletion mutant lacking 70 C-terminal amino acids of the E1A proteins, has revealed that in addition to being shorter the proteins are less abundant and less phosphorylated than those of wild-type virus. We are currently investigating the decreased phosphorylation in dl313 and other E1 mutants. We are also further analyzing the multiple forms of the E1A proteins seen in infected cells, using the specific E1A antisera. The reduced E1B 21-kd protein level in hr1-infected cells (defective in the E1A 289-amino acid protein), but not in pm975-infected cells (defective in the E1A 243-amino acid protein), is consistent with a specific role for the 289-amino acid protein in induction of early viral transcription (42, 48).

When infected cell extracts were prepared by the modified method of Manley et al. (38) described here, the E1A proteins were found to sediment in RIPA buffer as monomers, without any observable association with other cellular or viral proteins. The procedure extracts >90% of the E1A proteins, as determined by comparing high-speed supernatant and pellet fractions by Western immunoblot analysis or autoradiography of two-dimensional gels of labeled extracts (Tsukamoto and Berk, unpublished data). This contrasts with the low-salt extraction procedure used by Feldman and Nevins which did not extract a large fraction of the E1A proteins (10). The E1A 289-amino acid protein extracted by their procedure rapidly pelleted through glycerol gradients prepared in RIPA buffer (10). The discrepancy in sedimentation properties of the E1A proteins in these two studies might be explained by the incomplete dissociation of ionic interactions between the highly charged E1A proteins (pI 4.5 to 5.0; 11, 48) and cellular components in the low-salt buffer. Feldman and Nevins also reported that the E1A 289-amino acid protein was not solubilized from nuclear fractions by extraction with 2M NaCl and therefore raised the possibility that the protein is tightly associated with the nuclear matrix (10). We have also observed that the E1A proteins are poorly extracted by very high-salt buffers (Tsukamoto and Berk, unpublished data). However, the ability to extract the E1A proteins in soluble form with buffers of intermediate salt concentrations indicates that the proteins are not bound to nuclear structures through salt-resistant interactions.

It was surprising to observe that the E1A proteins which have predicted molecular weights of 27,000 to 32,000 cosedimented with lysozyme (14 kd) in RIPA buffer (Fig. 9). One possible explanation which can be tested is that the proteins have a very extended conformation in this buffer.

The method of antibody production reported here is rapid and inexpensive; we have been able to obtain high-titer anti-E1A antiserum throughout 5 months of immunization. In addition, immunoprecipitates of mammalian cells, using these antibodies, have a low background since the antigens for immunization are produced in bacterial cells. Antisera produced against the E1A bacterial fusion protein are higher in titer than those directed against an E1A synthetic peptide and are directed against specific gene products. These antibodies are being used to study proteins of various adenovirus mutants and also will be useful in the purification and study of the adenovirus tumor antigens.

The antisera to the E1B 55-kd fusion protein appear to be of lower titer than those to the E1A and E1B 21-kd protein. This may be because the fusion protein used for immunization contains only 60 amino acids of the adenovirus sequence. Attempts to induce *trpE*-adenovirus fusion proteins from plasmids encoding 485 amino acids of the 55-kd protein have been unsuccessful. This is probably because the resulting fusion protein would be approximately 90 kd; *trpE* fusion proteins of >76 kd have not been obtained (Spindler and Berk, unpublished data; S. Watanabe, J. Konopka, and C. Dieckmann, personal communication). The construction of plasmid derivatives of pKRS101 with a polylinker region from pUC13 in all three reading frames has extended the usefulness of this expression vector system (A. Tzagoloff and T. J. Koerner, personal communication). Such vectors allow the rapid construction of plasmids which can express *trpE* fusion proteins for biochemical and serological uses.

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