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Monoclonal Antibodies Specific for Adenovirus Early Region 1A Proteins: Extensive Heterogeneity in Early Region 1A Products

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Hybridomas secreting monoclonal antibodies specific for the adenovirus early region 1A (E1A) proteins were prepared from BALB/c mice immunized with a bacterial *trpE*-E1A fusion protein. This protein is encoded by a hybrid gene that joins a portion of the *Escherichia coli trpE* gene and a cDNA copy of the E1A 13S mRNA (Spindler et al., J. Virol. 49:132–141, 1984). Eighty-three hybridomas that secrete antibodies which recognize the immunogen were isolated and single cell cloned. Twenty-nine of these antibodies are specific for the E1A portion of the fusion protein. Only 12 of the monoclonal antibodies can efficiently immunoprecipitate E1A polypeptides from detergent lysates of infected cells. E1A polypeptides were analyzed on one-dimensional, sodium dodecyl sulfate-polyacrylamide gels and two-dimensional, isoelectric focusing polyacrylamide gels. The E1A proteins that are specifically immunoprecipitated by the monoclonal antibodies are heterogeneous in size and charge and can be resolved into approximately 60 polypeptide species. This heterogeneity is due not only to synthesis from multiple E1A mRNAs, but also at least in part to post-translational modification. Several of the monoclonal antibodies divide the E1A polypeptides into immunological subclasses based on the ability of the antibodies to bind to the antigen. In particular, two of the monoclonal antibodies bind to the polypeptides synthesized from the 13S E1A mRNA, but not to other E1A proteins.

The temporal regulation of adenovirus gene expression has become one of the important systems for the study of the control of eucaryotic transcription. In recent years much of the focus of these studies has centered on the control of early gene expression by the proteins of the adenovirus early region 1A (E1A). These polypeptides are the first virus-specific proteins to be synthesized after the infection of appropriate host cells with adenovirus (31, 36). Shortly after the appearance of the E1A proteins, other early transcription units from the E1B, E2, E3, and E4 regions are activated (36, 46). Genetic studies with adenovirus mutants that either fail to express the E1A region or synthesize altered E1A polypeptides have shown that the E1A proteins specifically activate transcription from a number of the adenovirus early promoters (1, 22, 34, 38) as well as from several host promoters (24, 35, 48). The mechanism by which the E1A proteins cause these transcription units to be utilized is not known at present.

In addition to the role that the E1A proteins play in the activation of other early promoters, a number of studies have shown that expression of the E1A proteins is essential for the transformation of cells by adenovirus (8, 11, 13, 14, 23). Although full transformation by adenovirus requires expression of both the E1A and E1B regions, Houweling et al. (21) have shown that cells that express the E1A proteins alone display a phenotype that is intermediate between that of normal and transformed cells. Recently, Land et al. (28) and Ruley (41) have shown that a number of different viral or cellular genes can complement one another to transform normal primary rat cells. These cotransfection assays have suggested that the E1A proteins allow normal cells to grow indefinitely in culture. Whether transcriptional activation and cellular establishment are separate functions of E1A or whether they are the pleotropic effects of one biochemical activity is not known. This issue remains unresolved primarily because of the difficulties created by the structural relationship of the E1A protein products.

RNA transcripts from the E1A region itself are differentially spliced during the early phase of virus infection to produce predominantly two mRNAs (2, 3, 25). These RNAs are normally referred to by their sedimentation coefficients of 12S and 13S. The 12S and 13S mRNAs have the same 5′ and 3′ termini, but differ in the size of the intron that is removed during processing. The splice site for each of these mRNAs falls within the coding region; because of the structure of the splice sites, the reading frames for the second exons of both proteins are identical (37). Therefore, the primary amino acid sequences of the polypeptides synthesized from these mRNAs are identical, except for a stretch of 46 amino acid residues found only in the 13S products. The overlapping coding regions of the E1A proteins have made the genetic dissection of the functions of the E1A proteins difficult.

Advances in our understanding of the protein products encoded by the E1A mRNAs have directly paralleled the development of better techniques and reagents to identify and study these proteins. Early studies relied heavily on hybrid selection and in vitro translation of the E1A mRNAs (6, 17, 20, 38, 44, 45). These studies were complemented by analysis of the E1A proteins with antisera from animals bearing adenovirus-induced tumors (15, 29, 39, 42). Although antitumor sera often have antibodies specific for the E1A proteins, their anti-E1A titers are normally low, and the sera almost always have antibodies specific for other, more abundant or immunogenic adenovirus proteins. Recently, several groups have reported the production of rabbit antisera specific for the E1A proteins (7, 32, 40, 43, 47, 52). These sera were raised against both synthetic peptide conjugates and bacterially produced, E1A-related antigens. We have extended these immunochemical studies and have raised a series of monoclonal antibodies specific for the E1A proteins. We present here the initial characterization of

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TABLE 1. Immunization schedule

Fusion	Day	Route	Antigen	^{125}I-labeled screen	Antibodies
1	1	i.p.[a]	Powder[b]		
	14	i.p.	Powder		
	43	i.p.	Gel slice[c]		
	45	i.v.[d]	Powder		
	48	Fusion		Protein A	M1, M2, M3
2	1	i.p.	Powder		
	17	i.p.	Powder		
	38	i.p.	Powder		
	52	i.p.	Powder		
	63	i.p. and i.v.	Powder		
	67	Fusion		Rabbit anti-mouse immunoglobulin	M4, M5, M6
3	1	i.p.	Gel slice		
	197	i.p.	Gel slice		
	233	i.p.	Powder		
	270	i.p.	Powder		
	277	i.p. and i.v.	Powder		
	300	Fusion		Rabbit anti-mouse immunoglobulin	M7 through M83

[a] i.p., Intraperitoneal injection.
[b] Purified *trpE*-E1A protein was solubilized in PBS and injected intravenously or mixed with Freund adjuvant and injected intraperitoneally.
[c] *trpE*-E1A protein was purified on SDS-polyacrylamide gels, mixed with Freund adjuvant, and injected intraperitoneally.
[d] i.v., Intravenous injection.

these antibodies and an analysis of the E1A proteins that are recognized by these antibodies.

MATERIALS AND METHODS

Cells and viruses. All cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. HeLa and 293 cells were from the Cold Spring Harbor Cell Culture facility and were grown and prepared by B. Ahrens. Adenovirus 5 (Ad5), Ad5.12S, and Ad5.13S virus stocks were the kind gifts of T. Grodzicker, E. Moran, and B. Zerler. Ad5.12S and Ad5.13S are recombinant adenovirus stocks that were constructed by replacing the E1A of Ad5 with cDNA copies of the 12S or 13S mRNAs from the Ad2 E1A (E. Moran, B. Zerler, E. Ruley, M. Mathews, and T. Grodzicker, manuscript in preparation).

Purification of *trpE*-E1A and *trpE*-E1B fusion proteins. Bacteria containing the plasmids pKRS103 or pKRS107 (the kind gift of K. Spindler [47]), which direct the synthesis of fusion proteins between the *Escherichia coli trpE* protein and the adenovirus E1A (pKRS103) or E1B proteins (pKRS107), were grown to saturation in M9 minimal medium (33) supplemented with 1% Casamino Acids (Difco Laboratories) and 20 μg of tryptophan per ml. The bacteria were diluted 1/100 with minimal medium supplemented with 1% Casamino Acids, but lacking tryptophan, and grown until an optical density at 550 nm of 0.2 was reached. The *trpE* promoter was then induced by the addition of indoleacrylic acid to 10 μg/ml. The cultures were incubated for a further 2 h, and the cells were collected and lysed by the procedure of Kleid et al. (26). Under these conditions the *trpE* fusion proteins can be isolated as inclusion granules. These granules were separated from the cell debris by centrifugation. The pellets were washed once and then dissolved in sample buffer (2% sodium dodecyl sulfate [SDS], 100 mM dithiotheitol, 10% glycerol, 20 mM Tris, pH 6.8) by boiling for 10 min. These samples were run on preparative 10% polyacrylamide gels (27). The proteins were located by cutting off side strips from the preparative gels and staining these strips with 0.25% Coomassie brilliant blue in 50% trichloroacetic acid. The strips were then destained in 25% methanol–7% acetic acid and used as markers to identify the position of the fusion proteins. In later experiments samples of the purified fusion proteins were iodinated (49) and added to the sample buffer before running on the preparative gels. In this case the fusion proteins were located by autoradiography. After the bands were cut from the gels, either they were used directly or the proteins were electroeluted as described by Leppard et al. (30).

Production of monoclonal antibodies. BALB/c female mice were immunized with the *trpE*-E1A fusion protein by following the schedule shown in Table 1. All of the preparations for intraperitoneal injections were mixed 1:1 with Freund adjuvant. The first intraperitoneal injection contained complete Freund adjuvant, and all subsequent intraperitoneal injections were with incomplete Freund adjuvant. When the fusion protein was injected as a gel slice, the gel was first fragmented by passing it repeatedly through a 19-gauge needle. All intravenous injections were done with purified protein suspended in phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM sodium phosphate, pH 7.2). Each injection was adjusted to deliver approximately 25 to 50 μg of protein. Several days after the final injection, the mice were sacrified by cervical dislocation, the spleens were removed, and the splenocytes were washed in Dulbecco modified Eagle medium without serum. The splenocytes were fused to NS-1 myeloma cells with polyethylene glycol by standard techniques (10). These cultures were washed and suspended in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (prescreened for good cloning efficiency), 0.15 mg of oxalacetate per ml, 0.05 mg of sodium pyruvate per ml, and 0.2 U of insulin per ml and containing 1 μg of azaserine per ml and 0.1 mM hypoxanthine. The azaserine-hypoxanthine selective medium was originally suggested by Foung et al. (9) for use in preparing T-cell hybridomas, but

we have found it to be useful for the selection of B-cell hybridomas. After the fusion, the cells were plated in approximately 2,000 microtiter wells. The cultures were inspected microscopically, and when the colonies reached approximately the 200-cell stage, the tissue culture supernatants were screened for the presence of anti-fusion protein antibodies. This normally occurred 7 to 10 days after fusion.

All hybrids were single cell cloned either by limiting dilution or by microscopically picking a single cell with a drawn-out Pasteur pipette.

Radioimmunoassays. The presence of antibodies specific for the fusion proteins was determined by a solid-phase radioimmunoassay. Purified protein was bound to a nitrocellulose sheet by incubating the fusion protein (2 μg/ml) for 1 h at 20°C with a sheet of nitrocellulose (0.1 ml/cm^2). The paper was washed with 3% bovine serum albumin in PBS (blocking buffer), and then the remaining sites in the nitrocellulose sheet were blocked by incubating in blocking buffer for at least 2 h. The paper was ruled with a soft lead pencil, and 1 μl of the appropriate hybridoma tissue culture supernatant was spotted on the paper. After incubation for 30 min at 20°C the paper was washed three times in the following buffer: 150 mM NaCl–5 mM EDTA–0.25% gelatin–0.02% NaN_3–0.05% Nonidet P-40–50 mM Tris (pH 7.5). The paper was then incubated with rocking for 30 min at 20°C in the presence of ^{125}I-labeled rabbit anti-mouse immunoglobulin or ^{125}I-labeled protein A (New England Nuclear Corp., approximately 0.025 μCi per spot in approximately 1 ml of buffer per cm^2). The nitrocellulose was washed three times in buffer, and the positive reactions were detected by autoradiography.

The class and subclass of a particular monoclonal antibody were determined by a competition radioimmunoassay (18).

Immunoprecipitation and electrophoresis. Immunoprecipitations and polyacrylamide electrophoresis were performed as described previously (19). Briefly, cultures were labeled with [^{35}S]methionine for 4 h in Dulbecco modified Eagle medium without methionine. The cells were lysed in 150 mM NaCl–1% Nonidet P-40–0.5% sodium deoxycholate–0.1% SDS–50 mM Tris (pH 8.0), and the lysates were cleared by incubating with fixed *Staphylococcus aureus* Cowan I before immunoprecipitation. Samples of the cleared lysates were incubated with the appropriate monoclonal antibody for 1 h on ice. Midway through the immunoprecipitation, 1 μl of rabbit anti-mouse immunoglobulin was added. Immune complexes were collected on protein A-Sepharose (Pharmacia Fine Chemicals), washed, and suspended in 2% SDS–100 mM dithiothreitol–10% glycerol–0.1% bromphenol blue–20 mM Tris (pH 6.8). The samples were then heated to 70°C for 10 min and separated on 10% SDS–polyacrylamide gels. Two-dimensional gel electrophoresis was conducted as described by Garrels (12). Immune complexes were solubilized by the addition of isoelectric focusing sample buffer (0.3% SDS, 9.95 M urea, 4% Nonidet P-40, 2% ampholytes [pH 3.5 to 10], 100 mM dithiothreitol) and then loaded onto pH range 3.5 to 10 isofocusing gels. All second-dimension gels were 10% polyacrylamide.

Indirect immunofluorescence. Cells were grown on glass cover slips, washed twice in PBS, and fixed by incubating the cover slips in 3.7% formaldehyde in PBS for 6 min at 20°C and then treated with ice-cold acetone for 20 s. The cover slips were washed with water and allowed to dry. Samples of the appropriate hybridoma tissue culture supernatant were added, incubated at 37°C for 20 min, washed in PBS, and then rabbit anti-mouse immunoglobulin conjugated with fluorescein isothiocyanate (1/80; Cappel Laboratories) was added. The samples were incubated for a further 20 min at 37°C and washed in water. All photographs were taken at 630× on a Leitz microscope with an epifluorescent source.

RESULTS

Production of the M series monoclonal antibodies. A number of recent studies have reported the production of antisera that recognize the adenovirus E1A proteins. These antisera have been raised in rabbits immunized with E1A-specific peptides conjugated to carrier proteins (7, 32, 52) or E1A fusion proteins produced in bacteria (43, 47). We have used a bacterial fusion protein between the *E. coli trpE* gene product and the Ad2 E1A 13S cDNA product (47; kindly supplied before publication) as an immunogen, and we have produced a series of hybridoma cell lines that secrete monoclonal antibodies specific for the E1A proteins. The scheme that was used to produce these antibodies is outlined in Fig. 1. The *trpE*-E1A fusion protein that was used in these experiments joins the amino-terminal 323 residues of the *trpE* protein to the carboxy-terminal 266 amino acids of the 13S E1A protein. This protein contains approximately 60% of the coding region of the *trpE* gene and about 90% of the E1A 13S coding sequence. The fusion protein was purified in a denatured form on SDS-polyacrylamide gels. The *trpE*-E1A polypeptide was either injected together with the polyacrylamide gel slice or was purified from the gel by electroelution, dialysis, and lyophilization. Both of these preparations proved to be good immunogens, but mice that received multiple injections with the gel slices often developed severe side effects. Consequently, most of our injections used the purified protein. The sera from immunized mice were assayed periodically to determine the titer of anti-E1A antibodies, and mice that showed a good response were used for the construction of hybridomas.

After the fusion of splenocytes with NS-1 myeloma cells, the hybridoma tissue culture supernatants were screened for the presence of antibodies that bound to the *trpE*-E1A fusion protein in a solid-phase radioimmunoassay. These antibodies were detected with either ^{125}I-labeled protein A or ^{125}I-labeled rabbit anti-mouse immunoglobulins. The first assays used ^{125}I-labeled protein A and were designed to identify anti-E1A immunoglobulins with high affinity for protein A. We screened for these antibodies first because they should be useful in immunochemical assays, including immunoprecipitations, which rely on protein A binding. After several monoclonal antibodies of this type had been isolated, we broadened the screening assay in an attempt to isolate as many antibodies specific for the E1A protein as possible. After a positive tissue culture supernatant was identified, the hybridoma was single cell cloned. Eighty-three hybridomas that secrete antibodies specific for the *trpE*-E1A antigen were isolated with this approach.

Characterization of the M series monoclonal antibodies. After each hybridoma line was single cell cloned, a small volume of tissue culture supernatant was collected, and this supernatant was used for the initial characterization. Radioimmunoassays and indirect immunofluorescence assays were used to determine which portion of the fusion protein contained the antibody binding site. The antibodies were tested first for their ability to bind to a fusion protein between the amino-terminal 323 residues of the *trpE* gene and a portion of the adenovirus E1B protein. This protein has the same segment of the *trpE* protein as the *trpE*-E1A

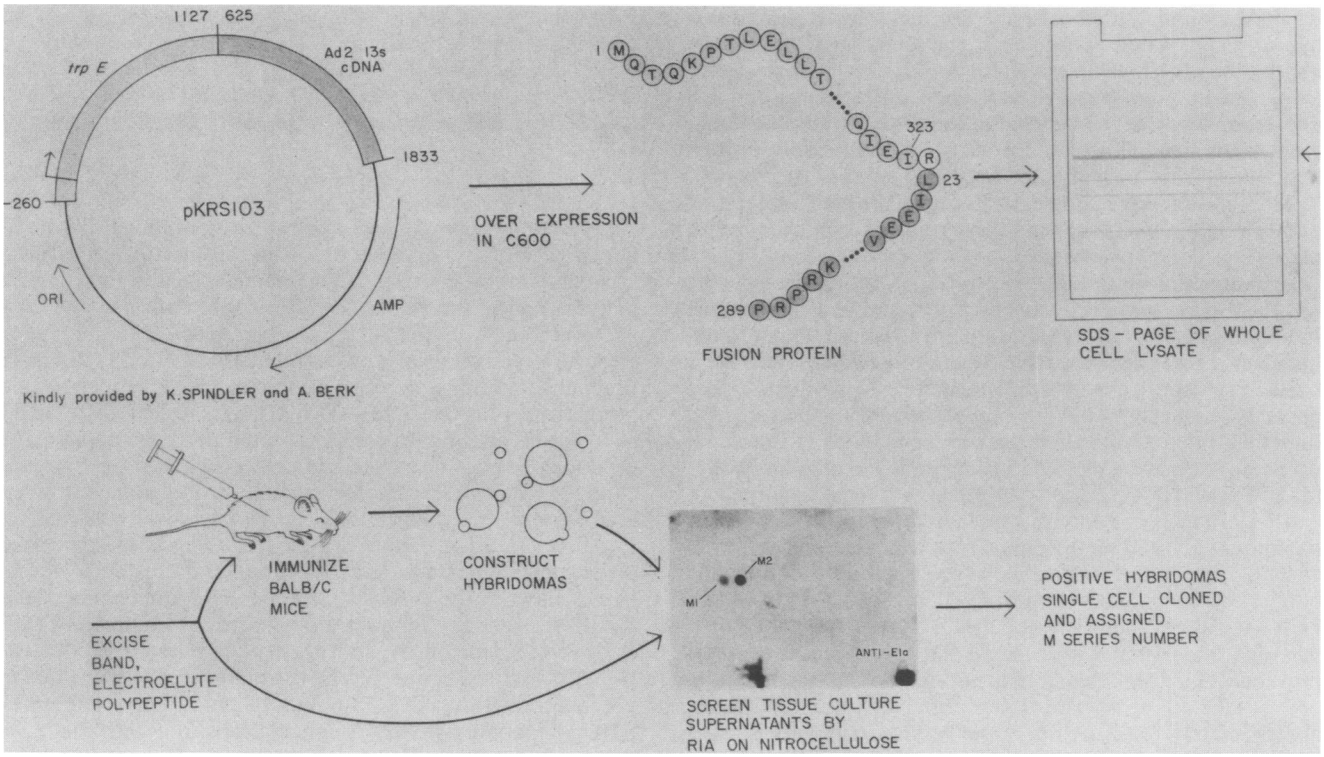

FIG. 1. Production scheme for the M series monoclonal antibodies. The *trpE*-E1A fusion protein was isolated from bacteria carrying the pKRS103 plasmid and purified on SDS-polyacrylamide gels. The protein was used to immunize BALB/c mice, and hybridomas were prepared by fusing splenocytes and NS-1 myeloma cells. Hybridoma tissue culture supernatants were tested for the presence of antibodies that could recognize the *trpE*-E1A fusion protein by a solid-phase radioimmunoassay. Positive hybridomas were single cell cloned and used for further analysis.

fusion, but the E1A region of the fusion protein has been replaced with an unrelated coding region. An example of this type of assay is presented in Fig. 2. The M series antibodies were also tested for their ability to bind to authentic E1A proteins in immunofluorescence assays of adenovirus-infected HeLa cells. Examples of the immunofluorescent staining patterns are shown in Fig. 3. Table 2 compares the results of these two assays. Three of the hybridomas, M24, M82, and M83, did not score in either of these assays. Because these hybrids produced antibodies that could bind to the *trpE*-E1A fusion protein when they were assayed after single cell cloning, but were negative in the same assay when the supernatants were collected after these cultures were expanded, we assume that the secretion of immunoglobulin from these cells was unstable. There are a number of possible explanations of why these hybrids have lost the ability to secrete the anti-E1A immunoglobulins, but this phenotype most often results from the loss during mitosis of one of the chromosomes that carry the genes for the heavy or light immunoglobulin chain.

Comparison of the results in Table 2 shows that the remaining hybrids can be grouped into four categories. Before we analyze these data, we expected the antibodies to fall into three categories, those that bound to the *trpE* protein, those that bound to the E1A proteins, and those whose binding site was found only on the immunogen. Most of the antibodies fell into one of the first two sets; they were positive for binding to either the *trpE* or the E1A segment. We found two antibodies, M25 and M54, that did not bind to any of the antigens except the original immunogen. These antibodies most likely bind to either a determinant formed by amino acids that border the fusion site or one that is not exposed on the E1A antigens used in these assays. It is more difficult to understand the pattern of binding seen with the M14, M20, M26, M58, M63, and M74 antibodies, which form the fourth category. The antibodies secreted by these hybridomas scored as positive in at least one test specific for each segment of the fusion protein. Further analysis has shown that M58 cells secrete two different subclasses of immunoglobulin G (IgG) molecules, IgG2a molecules, which bind to E1A sequences (data not shown), and IgG1 molecules, which our initial results suggest will bind to the *trpE* portion of the fusion protein. A number of previous reports

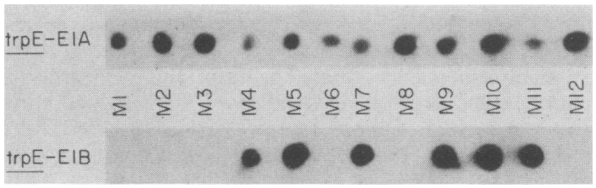

FIG. 2. Dot-blot analysis of M series monoclonal antibodies. Fusion proteins between the bacterial *trpE* protein and either the Ad2 E1A region (*trpE*-E1A) or the Ad2 E1B region (*trpE*-E1B) were purified from SDS-polyacrylamide gels by electroelution. The purified proteins were bound to nitrocellulose paper, and 1-μl samples of the appropriate hybridoma tissue culture supernatants were spotted onto the paper. After incubation, the paper was washed, and the antibodies were detected with iodinated rabbit anti-mouse immunoglobulin. The paper was washed again and prepared for autoradiography.

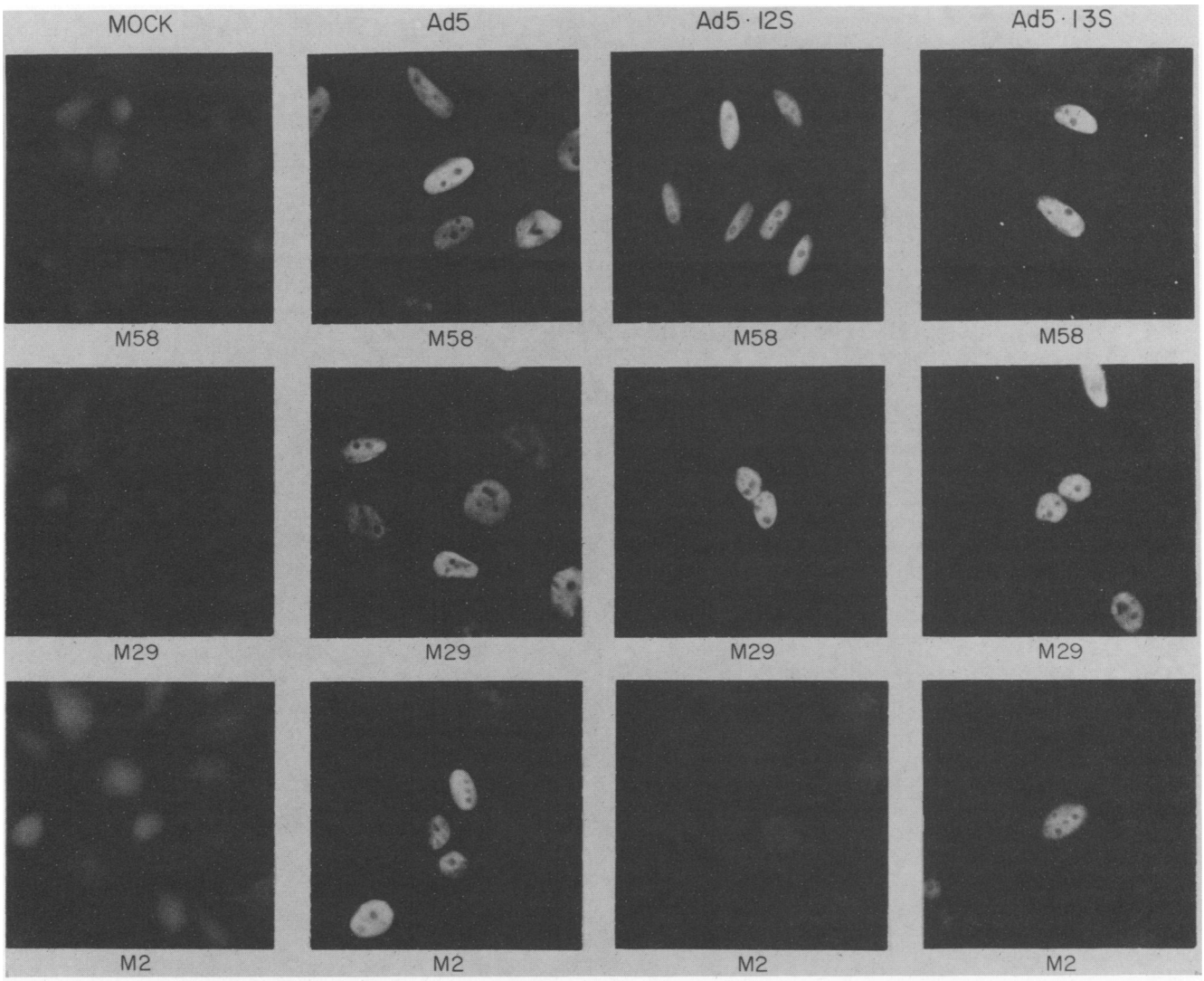

FIG. 3. Indirect immunofluorescence of infected or mock-infected HeLa cells. Subconfluent cultures of HeLa cells were infected or mock infected with wild-type Ad5 or recombinant adenoviruses containing a cDNA copy for either the 12S (Ad5.12S) or 13S (Ad5.13S) E1A mRNAs in place of the wild-type sequences. Cultures were fixed and prepared for immunofluorescence 24 h postinfection. Samples of the appropriate hybridoma culture supernatants were used as the primary antibody, and these antibodies were detected with a secondary rabbit anti-mouse immunoglobulin preparation that had been conjugated with fluorescein isothiocyanate. Magnification is 630×. Pictures of mock-infected cultures and M2-stained, Ad5.12S infected cultures were exposed at least four times longer than the other samples.

have shown that polyethylene glycol fusions can produce tribrid cell lines. We have not analyzed the other tissue culture supernatants in this group to see whether they contain multiple reactivities, but we have determined that the M29 hybridoma also secretes two different subclasses of antibodies.

After determining which of the antibodies bound to the E1A segment of the immunogen, we compared the ability of these monoclonal antibodies to immunoprecipitate E1A proteins from infected cells. HeLa cells were infected with Ad5 and labeled for 4 h beginning at 14 h postinfection. Samples of detergent lysates from these cultures were immunoprecipitated with the 29 antibodies that gave good nuclear fluorescence in the previous assays. We also included in these assays several of the monoclonal antibodies that gave unusual immunofluorescent staining patterns on other adenovirus-infected and transformed cells (data not shown). Only 12 of the monoclonal antibodies that we tested precipitated detectable amounts of the E1A proteins (Fig. 4). Because different subclasses of IgG molecules have different affinities for the immunoadsorbent, protein A-Sepharose, rabbit anti-mouse immunoglobulin was used as a second antibody in all of the immunoprecipitations. This insured that the monoclonal antibodies were quantitatively removed before SDS-polyacrylamide gel electrophoresis. Of the monoclonal antibodies used in these immunoprecipitations, 29 bound to the E1A proteins in immunofluorescence assays, but only 12 efficiently precipitated the E1A antigens. We assume that this frequency of positive reactions indicates that the structure of the E1A proteins in the detergent lysate is different than the structure of the E1A polypeptides prepared for immunofluorescence. These data also suggest that the conditions used for the immunofluorescence alter the structure of the E1A proteins to produce a molecule that more closely resembles the structure of the *trpE*-E1A gel-purified immunogen. Subsets of epitopes between denatured

TABLE 2. Radioimmunoassays and immunofluorescence assays of M series monoclonal antibodies

Antibody	Radioimmunoassay[a]		Immunofluorescence assay[b]		
	trpE-E1A	*trpE*-E1B	Ad5	Ad5.13S	Ad5.12S
M1	+	−	+	+	+
M2	+	−	+	+	−
M3	+	−	+	+	−
M4	+	+	−	−	−
M5	+	+	−	−	−
M6	+	−	+	−	−
M7	+	+	−	−	−
M8	+	−	+	+	+
M9	+	+	−	−	−
M10	+	+	−	−	−
M11	+	+	−	−	−
M12	+	−	+	+	+
M13	+	+	−	−	−
M14	+	+	+	+	+
M15	+	+	−	−	−
M16	+	+	−	−	−
M17	+	−	+	−	−
M18	+	−	+	+	+
M19	+	+	−	−	−
M20	+	+	−	+	−
M21	+	+	−	−	−
M22	+	+	−	−	−
M23	+	−	+	+	−
M24	−	−	−	−	−
M25	+	−	−	−	−
M26	+	+	+	−	−
M27	+	+	−	−	−
M28	+	−	+	+	+
M29	+	−	+	+	+
M30	+	+	−	−	−
M31	+	+	−	−	−
M32	+	+	−	−	−
M33	+	+	−	−	−
M34	+	+	−	−	−
M35	+	−	+	−	−
M36	+	−	+	−	−
M37	+	−	+	+	+
M38	+	+	−	−	−
M39	+	+	−	−	−
M40	+	+	−	−	−
M41	+	−	+	+	−
M42	+	−	+	+	+
M43	+	−	+	−	−
M44	+	−	+	−	−
M45	+	+	−	−	−
M46	+	+	−	−	−
M47	+	−	+	+	+
M48	+	+	−	−	−
M49	+	+	−	−	−
M50	+	−	+	+	−
M51	+	−	+	+	−
M52	+	−	+	+	+
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M55	+	+	−	−	−
M56	+	+	−	−	−
M57	+	+	−	−	−
M58	+	+	+	+	+
M59	+	+	−	−	−
M60	+	+	−	−	−
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M62	+	−	+	+	+
M63	+	+	+	−	−
M64	+	+	−	−	−
M65	+	+	−	−	−
M66	+	+	−	−	−
M67	+	−	+	+	+
M68	+	+	−	−	−
M69	+	+	−	−	−
M70	+	+	−	−	−
M71	+	+	−	−	−
M72	+	+	−	−	−
M73	+	−	+	+	+
M74	+	+	−	+	−
M75	+	+	−	−	−
M76	+	+	−	−	−
M77	+	+	−	−	−
M78	+	+	−	−	−
M79	+	+	−	−	−
M80	+	+	−	−	−
M81	+	+	−	−	−
M82	−	−	−	−	−
M83	−	−	−	−	−

[a] Monoclonal antibodies were tested for binding to purified *trpE*-E1A or *trpE*-E1B fusion proteins in a solid substrate dot blot. Antibodies were detected with iodinated rabbit anti-mouse immunoglobulin.

[b] HeLa cells were infected with Ad5, Ad5.12S, or Ad5.13S at a multiplicity of infection of 20 PFU per HeLa cell and assayed by immunofluorescence for the presence of E1A proteins at 24 h postinfection.

and more native proteins are often seen when a denatured antigen is used to raise antibodies.

Analysis of the E1A polypeptides. Previous work has shown that the E1A proteins can be separated into a number of polypeptide species. At least three polypeptide products have been shown to be synthesized from each of the 12S and 13S mRNAs, and one is synthesized from the 9S mRNA (see below). As expected from these previous observations, the anti-E1A monoclonal antibodies specifically immunoprecipitated a series of polypeptides from infected cells with relative molecular weights of 30,000 (30K) to 50K (Fig. 4 and 5A). However, the pattern of the bands did vary among several of the monoclonal antibodies. To understand the relationship between these bands and to gain better resolution of the E1A polypeptides, we analyzed the proteins precipitated by several of the M series monoclonal antibodies on two-dimensional, isoelectric focusing polyacrylamide gels. To help in the comparison of our results with previously published data, the immunoprecipitated samples were divided before electrophoresis, and identical fractions were run on both one- and two-dimensional gels. Although other researchers have shown that the E1A products undergo post-translational modification, we were surprised by the extensive heterogeneity of the proteins immunoprecipitated by the monoclonal antibodies (Fig. 5B and C). Comparisons of both short and long exposures of these gels have suggested that the E1A polypeptides from these lysates can be resolved into approximately 60 different spots. Several results suggest that the heterogeneity is not caused by proteolytic cleavage of the E1A proteins after the cells have been lysed. The patterns do not change when the proteases in the samples are inactivated by boiling the samples in 2% SDS before immunoprecipitation (Fig. 6). Second, there is no apparent degradation of any of the other proteins that are non-specifically precipitated in this or other experiments. In addition, including any of the commonly used protease inhibitors in the lysis buffer does not change the patterns

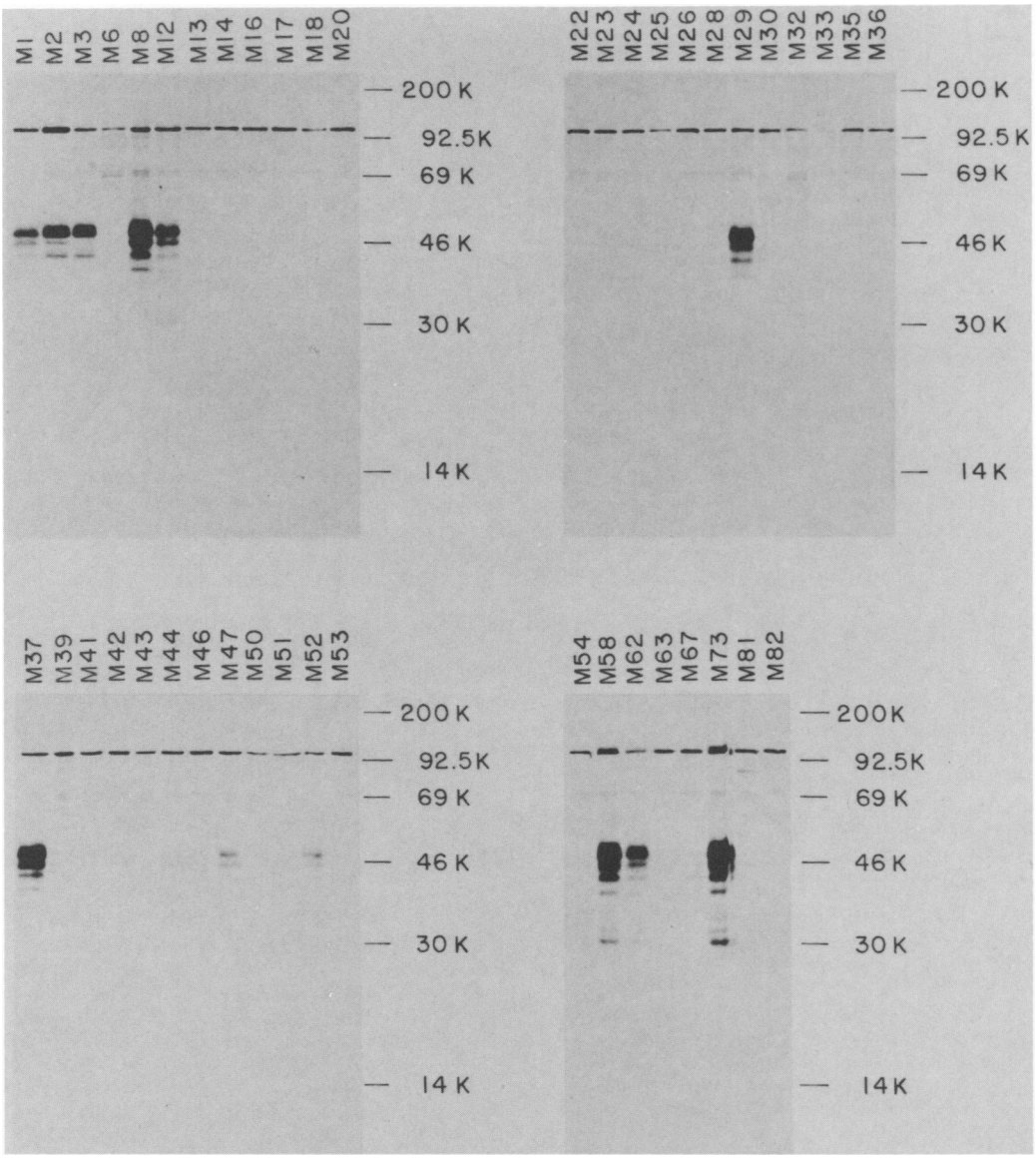
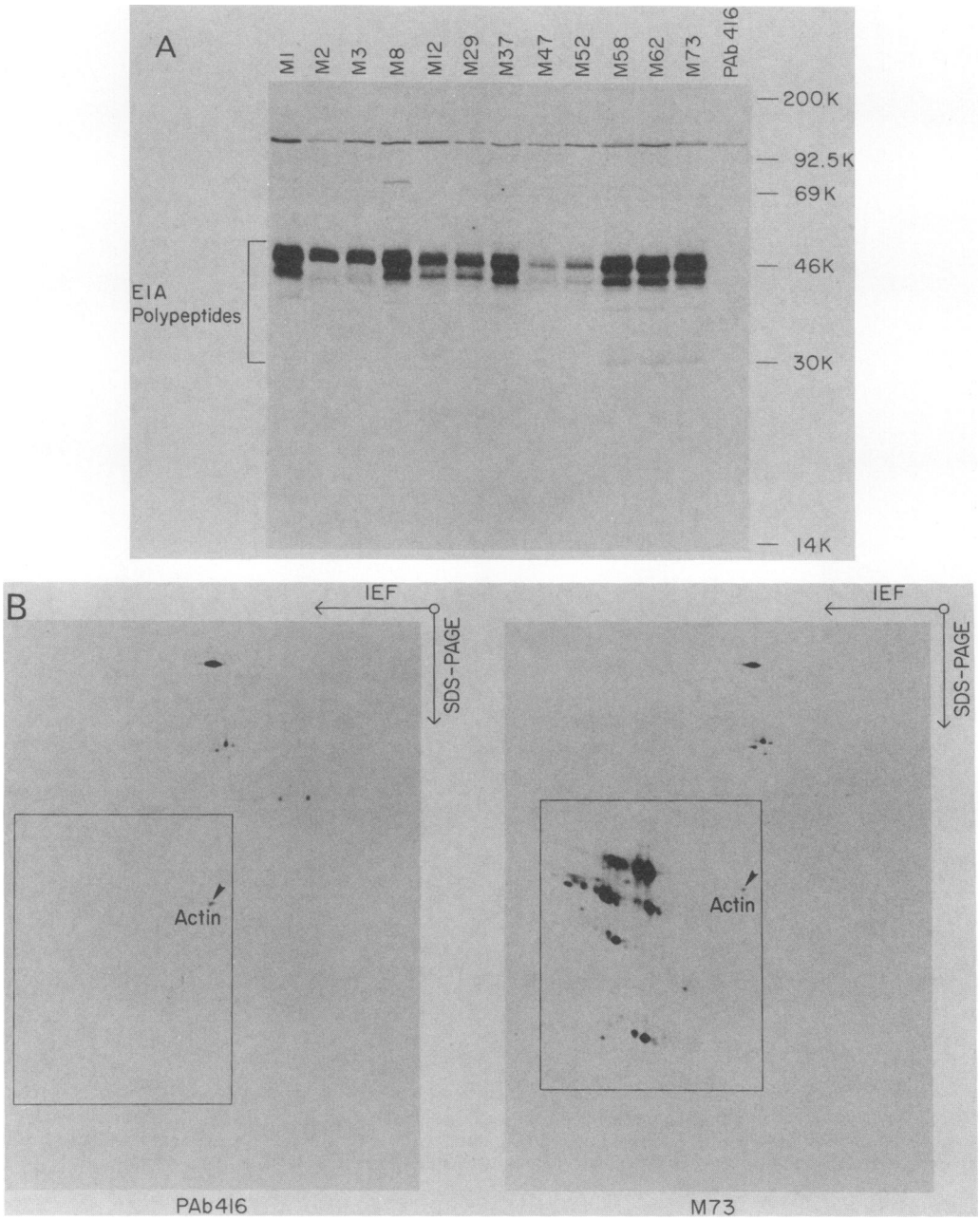


FIG. 4. Immunoprecipitation screen of the M series monoclonal antibodies. Ad5-infected HeLa cells were labeled with [^{35}S]methionine 14 to 18 h postinfection. Lysates from these cultures were immunoprecipitated with tissue culture supernatants from the M series hybridomas. The immune complexes were collected on protein A-Sepharose, washed, and separated on 10% polyacrylamide gels.

(data not shown). Although these results do not rule out the possibility that the patterns on these two-dimensional gels are generated by proteolytic cleavage, they do suggest that if the E1A proteins are modified by proteolytic clipping it most likely occurs within the intact cell (see below). Because the E1A species are recognized directly by an anti-E1A monoclonal antibody (Fig. 6), the polypeptides are probably E1A-related products and not host proteins that are bound to E1A or other proteins that are non-specifically trapped in the immune complexes. In addition to the E1A polypeptides in these immunoprecipitations, a number of other proteins are non-specifically precipitated. These include a doublet at approximately 70K that we have identified as the 70K heat shock proteins. Other proteins that are non-specifically precipitated are actin, which serves as a useful landmark on two-dimensional gels, and myosin.

Because the patterns of the E1A polypeptides on the two-dimensional gels were complicated, we also tested the same set of monoclonal antibodies for immunoprecipitation of E1A proteins synthesized by either Ad5.12S or Ad5.13S (Fig. 7). The autoradiograms in Fig. 7 have not been exposed for as long as the ones for Fig. 5, and therefore several of the more minor polypeptides cannot be seen. Comparison of the patterns on two-dimensional gels of the Ad5, Ad5.12S, and Ad5.13S immunoprecipitated proteins have shown that the array of spots seen in the Ad5-infected HeLa cell lysates can be separated into the groups depicted in Fig. 8. The 12S and 13S specific polypeptides are resolved in these gel systems; although they have somewhat different mobilities in both dimensions, there is a remarkable similarity in patterns between these groups. This is not altogether surprising, since these proteins have identical amino and carboxy sequences and vary only in the presence or absence of an internal 46-amino-acid stretch generated by alternative splicing. The two-dimensional gels have also resolved a series of E1A polypeptides from Ad5-infected cells with relative



molecular weights of approximately 30K that are specifically immunoprecipitated by some, but not all, of the antibodies we have tested. Although there remains some controversy over the protein products generated by the 9S mRNA (see below), the 30K polypeptides must be at least partially encoded by the E1A region. We are currently attempting to construct and sequence a cDNA clone that will direct the synthesis of these proteins. This will allow us to determine the coding region for these 30K polypeptides. A fourth series of spots (Fig. 8) can also be seen in immunoprecipitations from the Ad5-infected cells. These species are less prominent than the other E1A polypeptides, and it has not been possible to locate the coding region for these polypeptides with data from these experiments.

When we compared the pattern of polypeptides immunoprecipitated from cells infected with wild-type Ad5 and Ad5.12S or Ad5.13S, we found that there were several new polypeptide species found in the immunoprecipitations from cells infected with Ad5.12S or Ad5.13S. These new spots are not seen in the infections with the wild-type virus and are found in addition to the authentic 12S or 13S products. One possible origin for these new polypeptide species is that they are synthesized from novel mRNAs that are formed by cryptic splicing events. The use of cryptic splice sites is common when cDNA copies of genes that normally have spliced mRNAs are expressed by recombinant DNA techniques in eucaryotic cells. Because Ad5.13S still contains the 12S splice donor, one possible origin for these RNAs would be the splicing of RNA from this site to other splice donors downstream. Although we have not determined the origin of these new proteins, infections with recombinant adenoviruses similar to the ones used here produce new

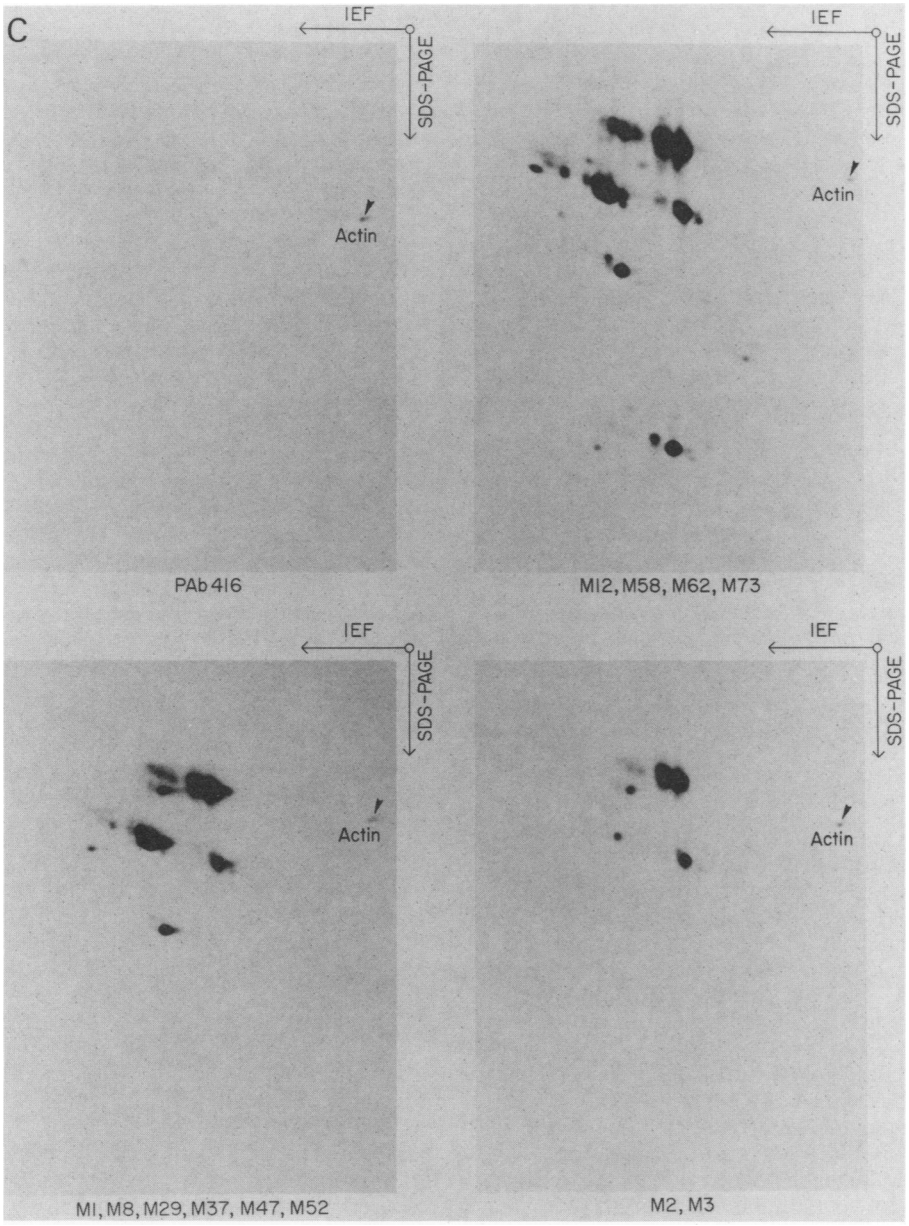


FIG. 5. One- and two-dimensional analysis of the E1A proteins in Ad5-infected HeLa cells. Ad5-infected HeLa cells were labeled with [^{35}S]methionine 14 to 18 h postinfection. Lysates of these cultures were immunoprecipitated with M series monoclonal antibodies or PAb416, a monoclonal antibody specific for the simian virus 40 large T antigen (18). The immune complexes were collected on protein A-Sepharose and washed. The immunoprecipitates were split after the last wash; 10% of the sample was run on a one-dimensional SDS-polyacrylamide gel (A), and the remaining 90% was processed for two-dimensional analysis. Panel B shows representative autoradiograms of the entire two-dimensional gel, and panel C shows enlargements of the area in which the E1A proteins migrate. Antibodies are grouped by the pattern of the E1A polypeptides (not including the 35 to 40K polypeptides) that were specifically immunoprecipitated. PAGE, polyacrylamide gel electrophoresis.

mRNA species that are formed by splicing events from the 12S donor (51; J. Logan, personal communication). We do not know the origin of the novel E1A-related polypeptides from the Ad5.12S-infected cells.

The patterns of E1A polypeptides that are immunoprecipitated by different members of the M series monoclonal antibodies varies widely. Figures 5 and 7 show the polypeptides precipitated by the antibodies which have proven to be useful in immunoprecipitation. Interestingly, two of the monoclonal antibodies, M2 and M3, will immunoprecipitate the 13S polypeptides, but not the 12S polypeptides. Preliminary evidence suggests that these antibodies both bind to the 13S unique region. Similarly, a number of the antibodies will bind to the 12S and 13S products, but do not precipitate the 30K proteins. Table 3 summarizes the precipitation patterns of these antibodies.

DISCUSSION

A number of studies have shown that the adenovirus E1A codes for a series of related polypeptides. These proteins

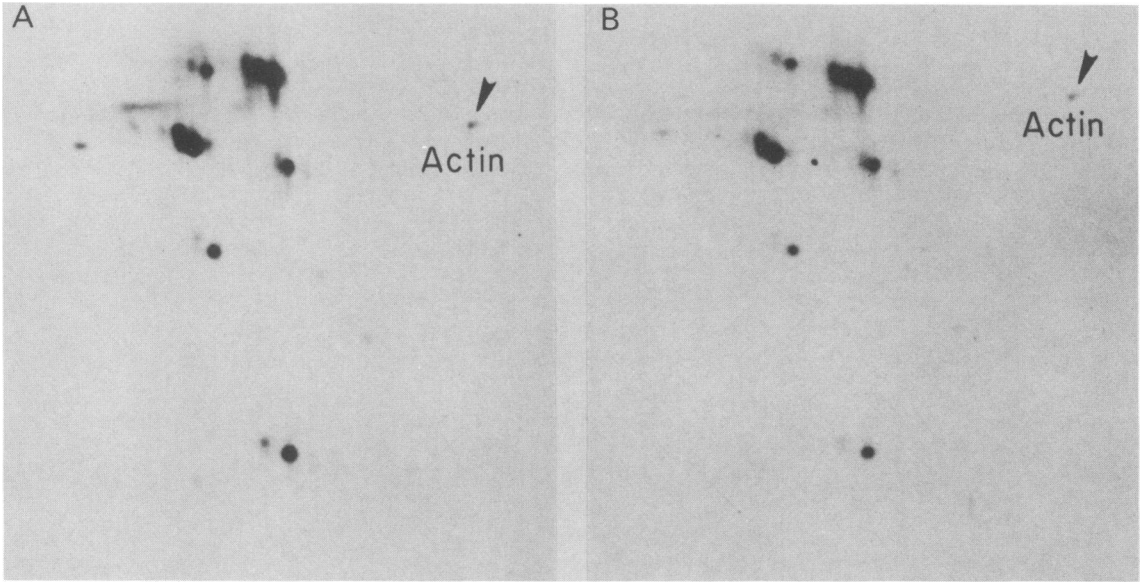


FIG. 6. Two-dimensional analysis of native (A) or denatured (B) E1A polypeptides. Ad5-infected HeLa cells were labeled with [^{35}S]methionine 14 to 18 h postinfection. The cells were lysed as quickly as possible. One half of the lysate was adjusted to 2% SDS and boiled for 10 min, and the other half was incubated on ice. Both samples were diluted 20-fold with 150 mM NaCl–5 mM EDTA–0.25% gelatin–0.02% NaN_3–0.05% Nonidet P-40–50 mM Tris (pH 7.5), and the DNA was fragmented by sonication. The lysates were precleared with *S. aureus* and then immunoprecipitated with M73 monoclonal antibody tissue culture supernatant. The immune complexes were collected on protein A-Sepharose, washed, and run on two-dimensional gels as described in the text. The region of the gel in which the E1A polypeptides migrate is shown.

have been detected by a variety of techniques, most recently by immunoprecipitation and two-dimensional isoelectric focusing polyacrylamide gel electrophoresis. Early reports identified either two or four polypeptides that were coded for by mRNAs from the E1A (6, 17, 38). These results were based on in vitro translations of RNA that had been hybrid selected on E1A DNA. By separating the mRNAs on methyl mercury gels before translation, Esche et al. (6) were able to detect three E1A mRNA species. This was in good agreement with previous data from S1 and R loop analyses that also had described three E1A mRNA species (2, 3, 25). These mRNAs are commonly referred to by their sedimentation coefficients of 13S, 12S, and 9S. Using two-dimensional isoelectric focusing polyacrylamide gel electrophoresis, Harter and Lewis (20) and Smart et al. (44) were able to identify six E1A products from the 12S and 13S mRNAs. Three of these polypeptides were shown to be encoded by the 12S mRNA, and three were encoded by the 13S mRNA. Recently several groups (7, 40, 43, 47, 52) have obtained similar results in studies in which they have examined the polypeptides immunoprecipitated with antisera specific for the E1A proteins. These and other studies have shown that the six polypeptides identified as products of the 12S and 13S E1A mRNAs are closely related. These polypeptides have identical amino-terminal sequences (5), have similar chromatographic profiles of radiolabeled tryptic and chymotryptic peptides (5, 16, 44), and can be immunoprecipitated with an antiserum specific for the carboxy-terminal six amino acids (40, 52). Analysis of cDNA clones prepared from the 12S and 13S mRNAs (37) have shown that the 12S and 13S polypeptides have identical amino- and carboxy-terminal sequences, but differ by an internal stretch of 46 amino acids found only in the 13S products. The RNA that codes for the 46 amino acid residues is removed by differential splicing during the production of the 12S mRNA. In addition, several studies have shown that a third mRNA from the E1A region with a sedimentation coefficient of 9S directs the synthesis of a 28K polypeptide (6, 45).

TABLE 3. Summary table

Antibody	Subclass	12S	13S	30K	35 to 40K
M1	G2b	+	+	−	+
M2	G2a	−	+	−	+
M3	G2a	−	+	−	+
M8	G1	+	+	−	−
M12	G1	+	+	+	+
M29	G1[a]	+	+	−	−
M37	G2b	+	+	−	−
M47	G2b	+	+	−	−
M52	G1	+	+	−	+
M58	G2a[b]	+	+	+	+
M62	G1	+	+	+	+
M73	G2a	+	+	+	+

[a] The M29 hybridoma also secretes G2a antibodies specific for an unknown antigen.

[b] The M58 hybridoma also secretes G1 antibodies specific for the *E. coli trpE* protein.

In the studies presented here, the E1A proteins have been resolved into approximately 60 polypeptide species. There are a number of possible explanations why we have been able to identify a larger number of E1A products than previous workers, but there are three potentially significant differences between earlier studies and the ones reported here. The first difference is the length of time that cells were labeled before immunoprecipitation. In many of the early experiments, infected cultures were labeled for 2 h. Comparisons between different labeling periods have indicated that some of the E1A polypeptides can be more easily detected with longer labeling times (our published observa-

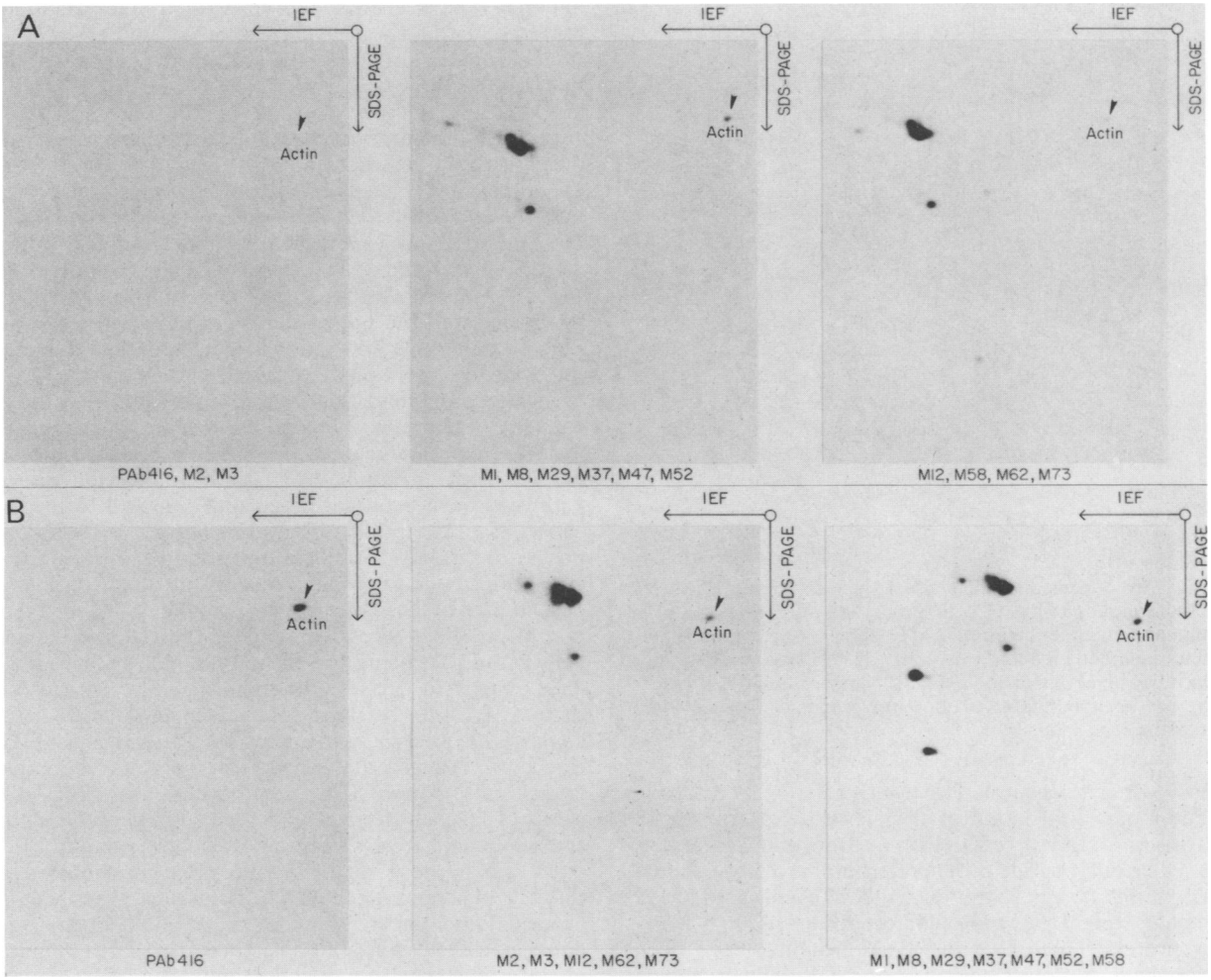


FIG. 7. Two-dimensional analysis of the E1A proteins in Ad5.12S- or Ad5.13S-infected HeLa cells. Ad5.12S- or Ad5.13S-infected HeLa cells were labeled with [^{35}S]methionine 14 to 18 h postinfection. Lysates of these cultures were immunoprecipitated with M series monoclonal antibodies or PAb416, a monoclonal antibody specific for the simian virus 40 large T antigen (18). The immune complexes were collected on protein A-Sepharose, washed, and processed for two-dimensional analysis. The region of the gel in which the E1A polypeptides migrate is shown. Shown are representative autoradiograms of immunoprecipitations from Ad5.12S-infected HeLa cells (A) and from Ad5.13S-infected cells (B).

tions); consequently, we have used 4-h pulses to radiolabel the E1A polypeptides. Second, the nonspecific background in immunoprecipitations that use monoclonal antibodies is often lower than in similar immunoprecipitations that use polyclonal sera. This lower background allows longer exposure times and the detection of some of the minor E1A polypeptides. The third and probably most important difference is the use of the Quest two-dimensional gel system (12). Use of this system has allowed us to identify polypeptide species that we could not separate by other methodology.

To better understand the origin of the different E1A polypeptides, we have analyzed the proteins produced by recombinant adenoviruses that have been engineered to synthesize only the 12S or 13S mRNAs. By comparing the pattern of these polypeptides on two-dimensional gels with similar patterns of the wild-type proteins, the E1A polypeptides synthesized from the 12S and 13S mRNAs can be identified. We have found that approximately 40 of the 60 polypeptide species are specifically encoded by these two mRNAs. In addition to these polypeptides, the anti-E1A monoclonal antibodies also immunoprecipitate two other sets of protein species. One of these consists of a series of polypeptides with molecular weights of approximately 35 to 40K. These proteins are minor E1A products when compared with the proteins of the other three groups, and we do not know their genetic origins. The E1A polypeptides from the fourth group have relative molecular weights of approximately 30K and have several of the properties expected for products of the E1A 9S mRNA. As discussed above, in vitro translation of the 9S E1A mRNA yield a 28K polypeptide. Like the 28K polypeptide, the 30K proteins are more easily detected during the late stage of viral infection. These data suggest that the 30K polypeptides may be the 28K polypeptide described previously. However, work by two groups has suggested that the 9S mRNA should code for a 6K polypeptide. Both Virtanen and Pettersson (50) working with Ad2 and Dijkema et al. (4) working with Ad7 have sequenced cDNA copies of 9S E1A mRNA. Their data suggest that the 9S mRNA should code for a polypeptide with the same amino terminus as the 12S and 13S products, but a different carboxy terminus. This is achieved by joining a donor splice site upstream from the 12S splice donor to the common

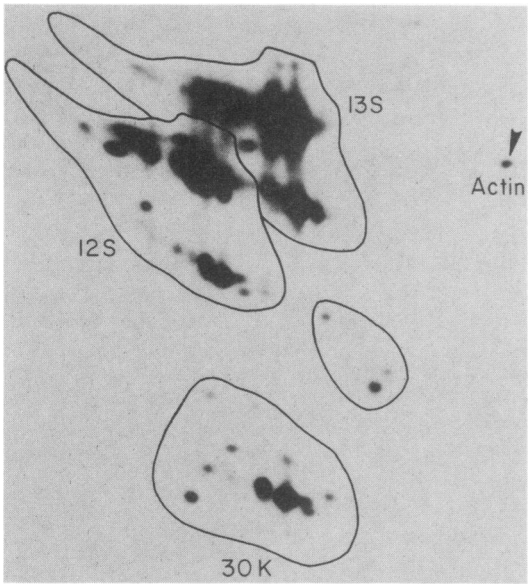


FIG. 8. Two-dimensional analysis of the adenovirus E1A proteins. The adenovirus E1A proteins from Ad5-infected HeLa cells were immunoprecipitated with M73 hybridoma tissue culture supernatant and separated on two-dimensional gels. The autoradiogram is from the same experiment as in Fig. 5, but a film from a longer exposure has been annotated to show the groups of the individual polypeptide species.

12S-13S-9S splice acceptor. However, the alignment of the splice sites puts translation of the second exon of the 9S mRNA into a different reading frame from the 12S or 13S mRNAs. The polypeptide produced from this mRNA should terminate 29 amino acids after the splice site and should have a molecular weight of approximately 6K. Although many nuclear proteins, including the E1A proteins, have been shown to have a slower mobility on SDS-polyacrylamide gels than would be expected based on their actual molecular weights, it seems unlikely that the 30K polypeptides are directly related to the 6K products. The immunochemical properties of the 30K proteins also suggest that they are not related to the 6K protein. Although several of the M series monoclonal antibodies will specifically bind to the 30K polypeptides, the fusion protein that was used as an immunogen in these studies should only have four amino acids in common with the 6K polypeptide. In other experiments we have shown that the antibodies that can immunoprecipitate the 30K polypeptides recognize at least two distinct antigenic determinants. Therefore, it seems unlikely that both groups of the monclonal antibodies would bind to the putative 6K polypeptide. Taken together, these data suggest that there may be two 9S E1A mRNAs, one which codes for the 30K polypeptides and one which codes for an unidentified protein with a molecular weight of approximately 6K (4, 50).

There are a number of mechanisms that could generate a large number of polypeptide species from a single translation product. We have checked for evidence of proteolytic breakdown of the E1A proteins and have found no evidence that the patterns are generated by nonspecific cleavage of the E1A polypeptides after they are extracted from the infected cells. The patterns of two-dimensional gels of the polypeptides synthesized from 12S and 13S mRNA are identical no matter which of the anti-E1A monoclonal antibodies is used as an immunoadsorbent. Several of these antibodies have been shown to bind to idependent sites in both the first and second E1A exons (unpublished observations). In addition, Yee et al. (52) have previously shown that the carboxy-terminal region of the E1A proteins is available for binding to an anti-peptide serum. These data suggest that the primary amino acid structure remains intact during extraction and immunoprecipitation. In pulse-chase experiments Stephens and Harlow (manuscript in preparation) have shown that the primary translation product of both the 12S and 13S mRNA runs as a single species that migrates faster and at a more basic isoelectric point than the 12S or 13S polypeptides shown here. This polypeptide can be chased into the larger and more acidic forms shown in Fig. 8. Other workers have speculated that the multiple forms of E1A previously reported were generated by post-translational modification, and it seem likely that the heterogeneity of the E1A polypeptides specifically recognized by the M series monoclonal antibodies are also the result of modification. A number of well-characterized enzymatic pathways have been described that can lead to the modification of polypeptides after translation. Most often these post-translational modifications involve the addition of chemical groups to the amino acid side chain. Probably the best studied of these mechanisms are the N-linked glycosylation of proteins during transport to the cell surface and the addition of phosphate to serine, tyrosine, or threonine residues by protein kinases. Other workers have already shown that the E1A polypeptides are phosphorylated (47, 52), so some of the differences in migration of the species of E1A on isoelectric focusing polyacrylamide gels may be explained by phosphorylation. However, because there are so many forms of E1A on these gels, it seems unlikely that all of the species result from differential phosphorylation. Further work will be necessary to identify the individual residues that are phosphorylated and to determine how these modifications alter the migration of E1A polypeptides on two-dimensional gels. Additional studies are also required to identify other possible modifications of the E1A molecules.

Proteins that undergo post-translational modifications are often processed in an ordered, sequential fashion. It is not clear whether the patterns of the E1A polypeptides synthesized from one mRNA depict a sequence of modifications to the same polypeptide chain or whether the patterns are composites of several modification pathways that act on different E1A molecules. In either case, the E1A polypeptides can be divided into subclasses based on their degree or state of modification. Other workers have previously reported evidence for different subclasses of the E1A proteins based on other criteria. Feldman and Nevins (7) have shown that the E1A polypeptides can be separated into subclasses based on the solubility of the E1A proteins in different extraction conditions. They have suggested that the species of E1A that are the most difficult to release from the nucleus may be molecules that are bound to the nuclear matrix. It will be interesting to compare the isoelectric focusing polyacrylamide gel electrophoretic patterns of E1A polypeptides prepared by these methods.

The E1A polypeptides can also be separated into different subclasses based on their immunochemical properties. Several of the monoclonal antibodies described here will bind to some, but not all, of the E1A polypeptides. The inability of these anti-E1A monoclonal antibodies to bind to certain of the E1A molecules can be explained in the following three ways: (i) the amino acids that form the antibody recognition site may not be found on the antigen, (ii) the epitope may be present, but inaccessible to the antibody because of steric

hinderance, or (iii) the antigen may fold in such a way as to distort the structure of the epitope and lower the affinity of the antibody for the binding site. In the first case, the amino acids that form the epitope would be present in some of the E1A polypeptide species, but not on others. This would be expected for proteins like the E1A 12S and 13S polypeptides, which have overlapping coding regions. The M2 and M3 monoclonal antibodies appear to fall in this first class. These antibodies recognize the 13S polypeptides, but fail to bind to the 12S proteins, and our preliminary evidence suggests that these antibodies bind to an epitope within the 46 amino acids that are unique to the 13S polypeptides. The second and third explanations are difficult to distinguish experimentally unless one has a detailed knowledge of the structure of the E1A molecules. However, we have been able to show that a number of the anti-E1A monoclonal antibodies will only recognize the E1A proteins after they have been treated with strong denaturants. The inability of some of the antibodies to bind to more native E1A molecules suggest that these epitopes are altered or masked on these proteins or the epitopes are formed by secondary or tertiary folding. In addition, there are a number of the M series antibodies that divide the E1A proteins into immunological subclasses for reasons that we do not understand at present. For example, the M8, M29, M37, and M47 antibodies are specific for the 12S and 13S polypeptides and do not bind to the 30K or 35 to 40K polypeptides. Similarly, the M1 and M52 antibodies will bind to the 12S, 13S, and 35 to 40K polypeptides, but not to the 30K proteins. Although these immunological subclasses may be due to the absence of E1A epitopes on either the 30K or 35 to 40K polypeptides it is difficult to explain the structural basis for these reactivities at present because we do not know the primary amino acid sequence of the 30K and 35 to 40K polypeptides.

We have presented here the production and initial characterization of 83 monoclonal antibodies specific for a bacterial fusion protein between the *E. coli trpE* protein and the adenovirus E1A proteins. Twelve of these antibodies appear to have properties that will make them useful reagents in the study of the E1A polypeptides. Analysis of the polypeptides specifically immunoprecipitated with these monoclonal antibodies has shown that the E1A proteins can be separated into approximately 60 polypeptide species. Although the reason for this heterogeneity is not clear at present, these antibodies will facilitate more detailed study of the E1A proteins.

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