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An Immunodominant Domain in Adenovirus Type 2 Early Region 1A Proteins

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Six independent rat hybridoma cell lines producing monoclonal antibodies to human subgroup C adenovirus early region 1A (E1A) proteins were isolated. Competition binding experiments revealed that each of the monoclonal antibodies was directed against the same epitope or overlapping cluster of epitopes on the E1A proteins. Viral E1A deletion mutants and deleted forms of E1A proteins expressed in *Escherichia coli* were used to localize the antibody recognition sites to sequences between amino acids 23 and 120, encoded within the first exon of the E1A gene. Similarly, polyclonal antisera raised against the *trpE*-E1A fusion protein, as well as against the native, biologically active E1A protein, were also directed primarily against this immunodominant region.

Proteins encoded in early region 1A (E1A) of the group C adenoviruses (adenovirus type 2 [Ad2] and Ad5) are of interest for a number of reasons. An E1A protein stimulates transcription of the other early viral transcription units, E1B, E2, E3, E4, and L1, during a productive infection (2, 14, 22). E1A protein can also stimulate the transcription of nonviral genes transiently introduced into cells (6, 9, 29, 31) and certain endogenous cellular genes (16, 28). In some instances E1A also appears to repress gene transcription (3, 10, 25, 26, 32). Transcription by RNA polymerase III is also stimulated by the expression of E1A proteins (1, 5, 12, 33a). Finally, E1A proteins are required for the oncogenic transformation of rodent cells by adenovirus (for a review, see reference 8).

E1A proteins expressed in transformed cells and during the early phase of a productive infection of human cells by Ad2 are encoded by two exons which are located between map units 1.3 and 4.6 on the viral genome. A primary transcript is differentially spliced into either a 12S or 13S mRNA which encodes, respectively, a 243- or 289-amino-acid protein. These proteins have identical amino and carboxy termini and differ only by an internal 46-amino-acid stretch unique to the larger protein (24).

To develop tools for studying E1A proteins directly, we isolated a number of independent anti-E1A protein monoclonal antibodies (MAbs). Fischer rats received weekly intramuscular immunizations with 100 μg of a purified *trpE*-E1A fusion protein produced in *Escherichia coli* (27). This fusion protein consists of the N-terminal 323 residues of the *E. coli trpE* protein followed by the terminal 267 amino acids of the larger E1A protein. After four immunizations, all rats showed the production of anti-E1A antibodies, assayed by using the rat sera to probe Western immunoblots of Ad2-infected HeLa cell extracts. The two animals with the strongest response were selected for separate fusions to establish hybridoma cells.

Three days before cell fusions were performed, the animals were boosted by the intrasplenic injection of 50 μg of the purified fusion protein to assure antigenic stimulation and replication of sensitized B cells in the spleen. Spleen cells were then fused with the rat Y-C-3 myeloma line by standard techniques (4). The fused cells were grown in 96-well microtiter dishes, and the supernatants from the

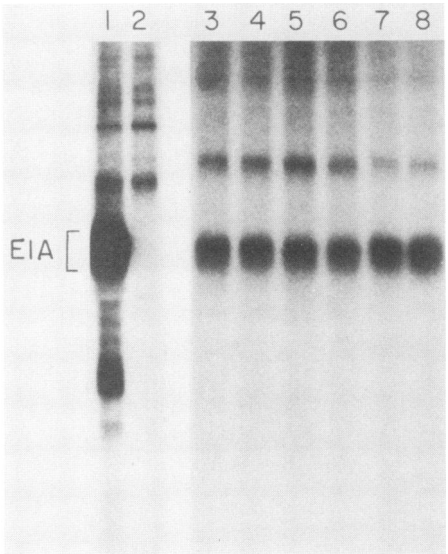

FIG. 1. Immunoprecipitation of ^{32}P-labeled E1A proteins from Ad2-infected HeLa cells. HeLa spinner cells were infected with 20 PFU of Ad2 per cell, cultured for 30 h in medium containing 20 μg of cytosine arabinoside per ml, and labeled for 2 h with $^{32}P_i$, and extracts were prepared as described previously (7). Solid-phase immunoprecipitation using rat MAbs or normal rat serum was done as described previously (30). Immunoprecipitation with rabbit polyclonal antiserum was also done as described previously (27). The antibodies used for immunoprecipitation were rabbit polyclonal anti-E1A (lane 1), normal rat serum (lane 2), and R7, R8, R12, R13, R25, and R28 (lanes 3 through 8, respectively).

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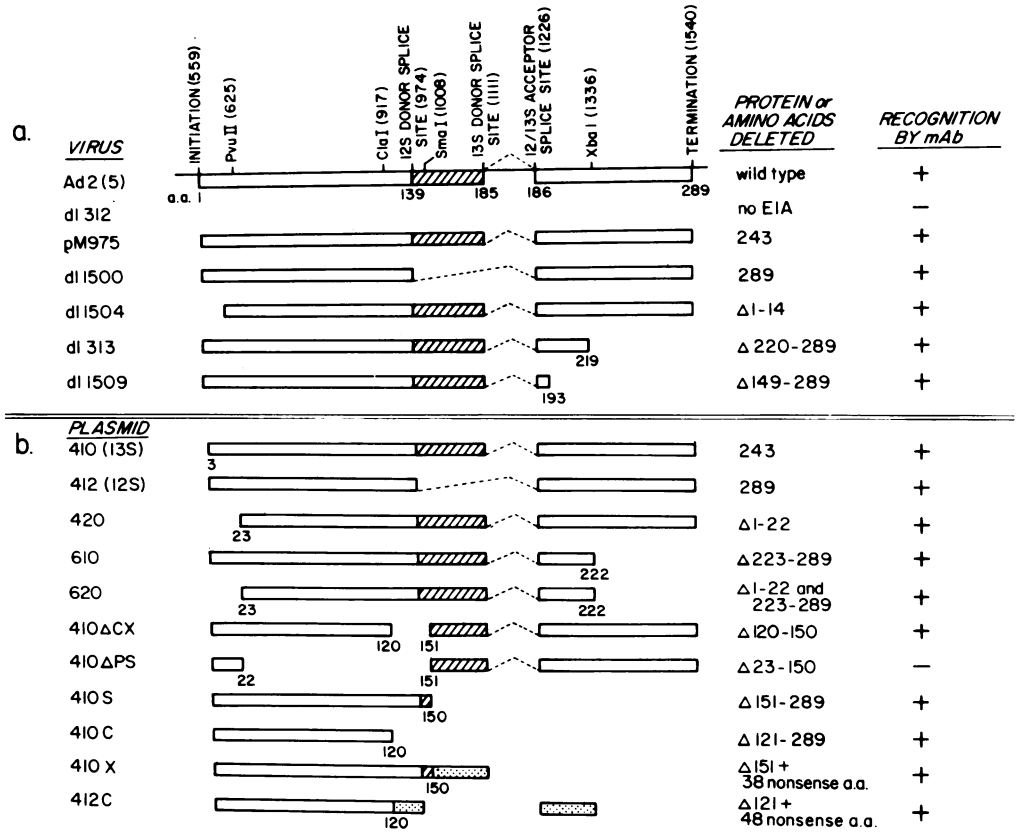

FIG. 2. Adenovirus E1A shown with the relevant restriction enzyme cleavage sites, RNA translation initiation and termination sites, and 12S and 13S 5′ and 3′ RNA splice sites indicated. The bars represent the E1A coding sequence. The hatched region is unique to the 13S mRNA. (a) Diagram of virus mutants used to map MAb recognition site (*dl*312 and *dl*313 [15], *pm*975 [20], *dl*1500 [19], *dl*1504 [23], and *dl*1509 [D. S. E. Rosser, Ph.D. Thesis, University of California, Los Angeles, 1985]). (b) Diagram of plasmids containing sequences encoding wild-type and mutant E1A proteins (16a). The number of amino acids deleted in each mutant is indicated. Recognition by MAbs as detected by Western immunoblotting is indicated as positive (+) or negative (−). All six independent MAbs analyzed showed the same pattern of recognition.

wells were tested by Western immunoblotting with peroxidase-conjugated rabbit anti-rat immunoglobulins. Of 240 wells tested, 71 were positive in this assay. The hybridoma populations, grown in liquid culture, arose from wells containing multiple fused cells. Cells from 30 of the 71 positive wells were randomly chosen, cloned by limiting dilution, and expanded. All of the hybridoma clones that survived subcloning and expansion were tested further by immunoprecipitation and Western blotting to identify expanded clones which still produced anti-E1A antibodies. Six independent rat-rat hybridoma clones, designated R7, R8, R12, R13, R25, and R28, each producing E1A-specific antibodies, were isolated. Each of these hybridomas represents independent fusion and cloning events. Clones R12 and R13 arose from a fusion in which spleen cells from one rat were used, whereas the other four clones were isolated from a separate fusion in which spleen cells from a second rat were used. Each of the MAbs specifically immunoprecipitated ^{32}P-labeled E1A proteins from Ad2-infected HeLa cells (Fig. 1). They also bound specifically to E1A proteins in Western immunoblot analyses.

To determine the regions of the E1A proteins to which these independently derived MAbs bound, we tested the MAbs for binding to a number of truncated E1A proteins expressed by adenovirus mutants (Fig. 2a) and for binding to truncated E1A proteins expressed in *E. coli* (Fig. 2b; 16a). The results for MAb R7 assayed against truncated E1A proteins expressed in *E. coli* are shown in Fig. 3. Each of the six independent MAbs recognized the same set of truncated E1A proteins. Each MAb bound to all of the truncated proteins tested, except 410ΔPS. The mutant 410ΔPS protein was present on these blots since it could be detected by an antiserum raised against a C-terminal peptide of the E1A proteins (data not shown; 33) and by rabbit polyclonal sera raised against the *trpE*-E1A fusion protein (see Fig. 5). Since all of the MAbs bound to mutant protein 410C, which deletes all of the sequence following amino acid 120, and to mutant proteins 420 and 620, which delete amino acids 1 through 22, we conclude that the epitope(s) recognized by each MAb is contained between amino acids 23 and 120. Moreover, amino acids 1 through 120 (present in mutant proteins 410C and 412C) can fold to form the epitope(s) recognized by these MAbs.

The region between amino acids 23 and 120 is relatively large (98 amino acids) and presumably contains multiple possible antigenic determinants. To determine whether the MAbs recognized overlapping epitopes or different nonoverlapping epitopes within this domain, a competition binding experiment was performed. In this experiment, Western blots were incubated with an excess of a nonlabeled

MAb and then with the ^{125}I-labeled E1A-specific MAb R7 (Fig. 4). Each of the E1A-specific MAbs inhibited labeled MAb R7 binding to E1A protein (lanes 1 through 6). A control T-cell-specific rat MAb, 53.7/313, which does not bind to E1A protein, had no effect on R7 binding (lane 7). These results indicate that each of the six independent rat MAbs recognize the same epitope or physically overlapping epitopes so that binding of one MAb sterically blocks the binding of another. In repeated experiments, MAb R28 (lane 6) was less effective in blocking R7 binding than any of the other rat MAbs. This may indicate that R28 recognizes a slightly different epitope or is of lower affinity than the other five rat MAbs analyzed.

The finding that each of the six independently isolated E1A-specific MAbs recognizes the same epitope or overlapping epitopes suggests that there is an immunodominant domain in the *trpE*-E1A antigen. To address this question, we determined whether the E1A-specific MAbs could block the binding of rabbit polyclonal E1A-specific antibodies raised against the *trpE*-E1A fusion protein. Western blots of E1A protein were incubated with excess MAb and subsequently with a rabbit polyclonal E1A-specific antiserum raised against the *trpE*-E1A fusion protein (27). The binding of rabbit antibody to E1A was determined by measuring the subsequent binding of ^{125}I-protein A, which binds to rabbit immunoglobulin G but not to the rat MAbs. Remarkably, the binding of the rabbit polyclonal antibodies to E1A was reduced to less than 10% by each of the MAbs but not by the control antibodies (Table 1). In contrast, prebinding of MAb R28 did not interfere with the binding of a polyclonal antibody raised against a C-terminal peptide of the E1A proteins (serum 44C; 33). These results show that approximately 90% of the protein A-binding antibodies in the rabbit polyclonal antiserum bind to the same epitope or overlapping epitopes that are recognized by the six rat MAbs and substantiate the conclusion that the region between amino acids 23 and 120 in E1A contains an immunodominant antigenic domain.

The preceeding results identify an immunodominant epitope in a *trpE*-E1A fusion protein that contains at its carboxyl terminus amino acids 23 to 289 of the E1A 13S mRNA product fused to 323 amino-terminal amino acids of the *trpE* protein. To assess whether full-length nonfused

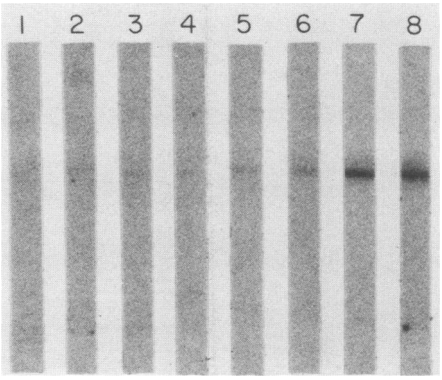

FIG. 4. MAb competition experiment. Western immunoblot strips of Ad2-infected HeLa cell extracts were preincubated overnight at 4°C with 100 μg of the E1A-specific MAbs R7, R8, R12, R13, R25, and R28 (lanes 1 through 6, respectively), a control rat MAb, 53.7 (lane 7), and no antibody (lane 8). The filters were washed, incubated with ^{125}I-R7 MAb for 3 h at 25°C, washed, and exposed to X-ray film. Similar results were observed when 10 μg of the MAbs was used in the preincubation.

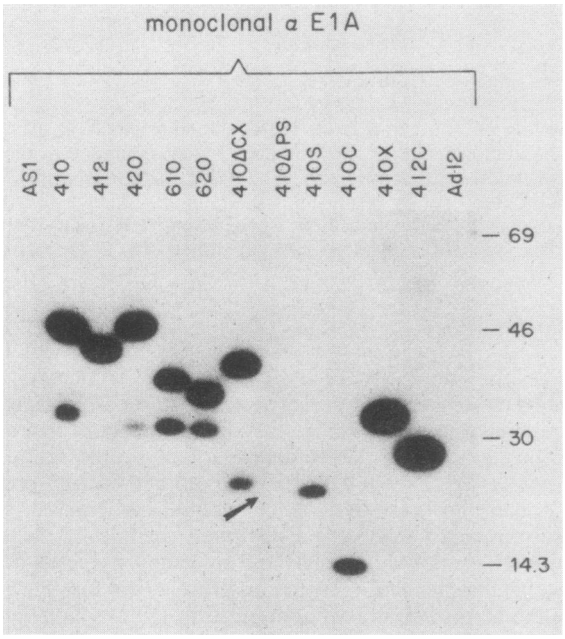

FIG. 3. Immunoblot analysis of truncated E1A proteins with an E1A-specific MAb. The structures of full-length and deleted forms of E1A protein encoded by the designated plasmids are shown schematically in Fig. 2b. Lane Ad12 contained an extract of the *E. coli*-expressed human Ad12 E1A 13S mRNA product (N. Jones, B. Ferguson, and M. Rosenberg, manuscript in preparation). *E. coli* AR120 cells containing the designated plasmids were induced with nalidixic acid (21), and at 6 h postinduction a 0.01-ml sample of the culture (except for 410ΔPS, for which a 0.3-ml sample was used) was analyzed by SDS-PAGE and immunoblotting by using the E1A-specific MAb R7. After electrophoretic transfer, the nitrocellulose-bound proteins were incubated sequentially with 3% bovine serum albumin, excess rat MAb R7, and excess rabbit antiserum specific for rat immunoglobulin and ^{125}I-protein A. The numbers on the right indicate the positions of molecular size markers (kilodaltons).

TABLE 1. Inhibition of binding of rabbit polyclonal anti-E1A fusion protein antiserum to E1A proteins by E1A-specific MAbs[a]

Preincubation antibody	% of ^{125}I counts bound
$\alpha E1A_{fp}$	100
53.7/313	86
NRtS	69
R7	9
R8	2
R12	8
R13	2
R25	2
R28	2
None	0
R7	0

[a] E1A protein on Western immunoblot strips was preincubated with 100 μg of the indicated antibody. $\alpha E1A_{fp}$, E1A-specific rabbit polyclonal serum raised against the *trpE*-E1A fusion protein; 53.7/313, T-cell-specific rat MAb; NRtS, normal rat serum; R7, R8, R12, R13, R25, and R28, E1A-specific rat MAbs. After being washed, the filter strips were incubated with the rabbit polyclonal anitserum $\alpha E1A_{fp}$ (except the strips preincubated with $\alpha E1A_{fp}$ and the last two entries of the table). The filters were washed and then probed with ^{125}I-protein A to detect the amount of rabbit *trpE*-E1A antiserum bound; 100% was 27,000 cpm.

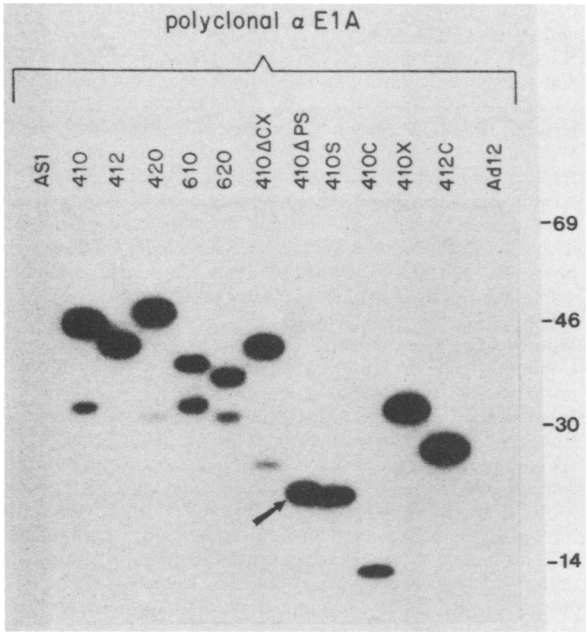

FIG. 5. Immunoblot analysis of truncated E1A native proteins with rabbit polyclonal antibody (33-1) raised against the native 289-amino-acid E1A protein. *E. coli* cells containing the designated plasmids (Fig. 2b) were analyzed exactly as described in the legend to Fig. 3 except that rabbit polyclonal E1A-specific antibody (33-1) (17) was used instead of rat MAb R7 and bound immunoglobulin was detected with ^{125}I-protein A. Lane 410ΔPS contained 30-fold more induced *E. coli* cell lysate than any of the other lanes.

E1A proteins also contain this immunodominant antigenic determinant, we analyzed rabbit antiserum raised against the full-length, nonfused Ad2 E1A 13S mRNA product produced in *E. coli* (polyclonal antibody 33-1; 17). The binding of polyclonal antiserum 33-1 to various *E. coli*-expressed deleted forms of E1A is shown in Fig. 5. Antiserum 33-1 bound with similar efficiency to each of the deleted forms of E1A except mutant 410ΔPS, which was recognized poorly by this polyclonal antibody. It was necessary to load 30-fold more of the E1A-derived 410ΔPS product on the sodium dodecyl sulfate-polyacrylamide gel to obtain a level of antibody binding (as measured by ^{125}I-protein A binding and autoradiography) similar to that observed for each of the other deleted forms of E1A protein (Fig. 5). The relative amounts of E1A-derived proteins were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining and by immunoblot analysis with an antibody specific for the E1A carboxy-terminal peptide (3). Thus, the polyclonal antibodies raised against the E1A 13S mRNA product bind efficiently to a region between amino acid residues 23 and 120 but do not bind efficiently to a deleted form of E1A lacking these amino acids.

A competition binding experiment was performed to directly determine the proportion of polyclonal antibodies in antiserum 33-1 which bind to the first exon-encoded region of E1A. In this experiment, antiserum 33-1 was incubated with the E1A-derived protein 410S or 410ΔPS (Fig. 2b) before incubation with nitrocellulose-bound E1A 13S and 12S mRNA products. Prebinding to mutant 410S reduced the subsequent binding of the antibodies in antiserum 33-1 to E1A proteins by 17-fold relative to the binding observed after preabsorption to the 410ΔPS mutant protein (Fig. 6). This result was also obtained when the full-length, native, biologically active E1A proteins (17) were spotted directly onto the nitrocellulose rather than being subjected to SDS-PAGE and electrophoretic transfer to nitrocellulose (data not shown). These results show that about 95% of the polyclonal antibodies in antiserum 33-1 bound to epitopes contained within the first exon-encoded region of E1A. Moreover, the immunodominant first exon-encoded region of E1A we observed is in fact a feature of the native E1A proteins. This was also shown by the ability of the rat MAbs to immunoprecipitate native E1A proteins from extracts of Ad2-infected HeLa cells (Fig. 1).

Recently, Harlow et al. (11) also reported the isolation of E1A-specific MAbs. Although they immunized mice with the same *trpE*-E1A fusion protein, they obtained murine MAbs with a number of distinct antigenic specificities. Our isolation of six rat MAbs with similar or overlapping specificities may have resulted from hyperimmunization of the animals. While our immunization protocol resulted in a high percentage of hybridomas producing anti-E1A MAbs, it apparently favored the expansion of B-cell clones directed against the dominant epitope we defined in these studies. The differences between our results and those of Harlow et al. (11) may also have resulted from the different animals used (rats versus mice); however, the rabbit sera we analyzed (also from animals which were immunized repeatedly) were also directed primarily against the same immunodominant epitope.

The immunodominant epitope defined here maps to the same first exon-encoded region of E1A that is sufficient to

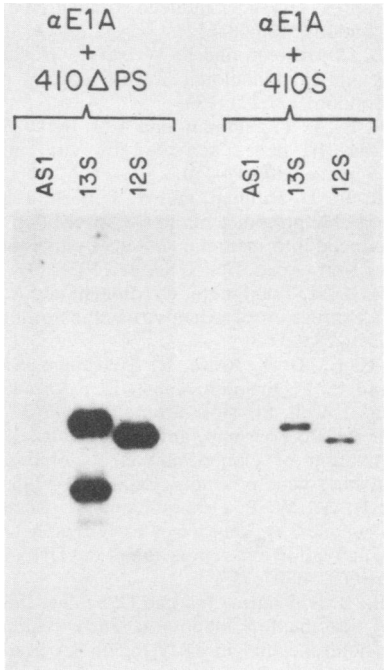

FIG. 6. Prebinding of rabbit polyclonal E1A-specific antibody (raised against the native *E. coli*-expressed E1A 13S mRNA product) to two deleted forms of E1A protein. *E. coli* cells containing pAS1 (AS1), pAS1-E1A410 (13S), and pAS1-E1A412 (12S) (Fig. 2b; 17) were induced and analyzed exactly as described in the legend to Fig. 5 except that the polyclonal E1A-specific antiserum was preincubated with the purified products of pAS1-E1A410ΔPS or pAS1-E1A410S (Fig. 2b) before use. The E1A-specific rabbit antiserum (10 μl) was preincubated with approximately 20 μg of E1A-derived protein in 1 ml of RIPA buffer (27) for 15 min at 25°C.

induce adenovirus gene transcription (albeit at levels reduced 10- to 20-fold relative to that induced by the full-length 289-amino-acid E1A protein; 16a). Thus, the MAbs bind to E1A within a region of the protein that appears to form an essential part of the active site for E1A function. Hydrophilicity plots of the E1A proteins, generated by the Kyte and Doolittle (18) and Hopp and Woods (13) programs, reveal two hydrophilic stretches in the primary sequence between amino acids 35 and 57 and between amino acids 80 and 102. In view of the preceeding findings, it is likely that one of these two regions forms surface determinants recognized by the MAbs described here.

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