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Mapping of Cellular Protein-Binding Sites on the Products of Early-Region 1A of Human Adenovirus Type 5

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The binding sites for the 300-, 107-, and 105-kilodalton cellular proteins which associate with human adenovirus type 5 E1A products were studied with E1A deletion mutants. All appeared to bind to the amino-terminal half of E1A products in regions necessary for oncogenic transformation. These results suggest that these cellular species may be important for the biological activity of E1A products.

Early-region 1A (E1A) of human adenovirus type 5 (Ad5) encodes two major proteins of 289 and 243 residues (proteins 289R and 243R), which are identical except for the presence of an additional 46 internal amino acids in the larger species (27). Structurally, 289R can be divided into three regions: residues 1 to 139, which are common to both 289R and 243R (exon 1); residues 140 to 185, which are unique to 289R (unique); and residues 186 to 289 (exon 2), which are common to both products (Fig. 1). E1A proteins activate transcription of viral (1, 16, 17, 24) and cellular (18, 25, 31) genes, and this function has been mapped to the unique region (15, 21–23, 30). E1A products inhibit some transcriptional enhancers (2, 13, 14, 33), and this activity maps to the exon 1 region (21, 22, 30). E1A products also accomplish a number of other functions, including the induction of cell division and immortalization, participation with early-region 1B (E1B) in oncogenic transformation (see reference 3 and references therein), and stimulation of expression of an epithelial growth factor (28). These activities appear to require at least two separate regions encoded by exon 1 (21, 22, 28, 30, 34).

Little is known about the molecular mechanisms which underlie these various E1A functions. As one approach, we (6, 36) and others (11) have identified several cellular proteins that associate specifically with E1A polypeptides. The three major species observed by our group and that of Harlow are phosphoproteins of 300, 107, and 105 kilodaltons (kDa) (6, 11, 36). The identity, function, and biological importance of these cellular E1A-associated proteins are unknown. In the present report, we have investigated the binding sites of these species on E1A proteins by using a variety of E1A deletion mutants. It is difficult to define binding sites accurately without a detailed knowledge of the three-dimensional structure of E1A proteins. Nevertheless, if particular regions of the E1A protein molecule are involved in binding, either directly or indirectly, deletion of these sequences should reduce the association of one or more of the cellular species. We report that all of these species appear to bind in exon 1.

Role of the E1A amino and carboxy termini. To examine the importance of the amino and carboxy termini of E1A products in the binding of cellular proteins, human KB cells (ca. 2×10^7 in 150-mm-diameter dishes) were infected either with wild-type (wt) Ad5 or with mutant *dl*1504 or *dl*313, which lacks sequences encoding the first 14 residues at the amino terminus (5, 23) or the last 70 amino acids at the carboxy terminus (4), respectively. Infected KB and mock-infected cultures were labeled with [^{35}S]methionine (Amersham Corp.; specific activity, 1,300 Ci/mmol) from 7 to 11 h postinfection, and cell extracts prepared in either the presence (buffer B, 200 mM Tris [pH 7.0]–137 mM NaCl–1.0 mM $CaCl_2$–0.4 mM $MgCl_2$–10% [vol/vol] glycerol–1% [vol/vol] Nonidet P-40–100,000 IU of aprotinin per ml) or absence (buffer A, 50 mM Tris [pH 7.2]–150 mM NaCl–1% [vol/vol] Triton X-100–100,000 IU of aprotinin per ml) of divalent cations were immunoprecipitated with anti-peptide serum E1A-C1 or E1A-N1, which is specific for the carboxy or amino terminus, respectively, of both 289R and 243R (35, 37). In some cases, the peptides to which the sera were generated were also added. Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by using discontinuous 12% polyacrylamide gels, and migration patterns were determined by autoradiography, as described previously (29, 36). Figure 2 (lane B) shows that with extracts from wt Ad5-infected cells, E1A-C1 serum precipitated the 300-, 107-, and 105-kDa cellular proteins, in addition to E1A products. As shown previously (36), the 300-kDa species was absent when extracts were prepared in the presence of divalent cations (lane E). The three cellular proteins were not precipitated from mock-infected cells (lanes A, G, and J), and addition of E1A-C peptide blocked their precipitation (lanes C, I, and L). With E1A-C1 serum and extracts from cells infected with mutant *dl*1504, the 107- and 105-kDa species were detected, but the 300-kDa protein was not (lanes H and K). With extracts from wt- or *dl*313-infected cells precipitated with E1A-N1 serum (lanes N and Q, respectively), the 107- and 105-kDa species were present, but the 300-kDa protein was not. These results were compatible with previous data that indicated that E1A protein complexes containing the 300-kDa species were not precipitated by serum prepared against the amino terminus (36). In total, these data suggested that the amino terminus of E1A products may be involved in binding the 300-kDa protein, but that neither this region nor the last 70 residues at the carboxy terminus play a significant role for the 107- and 105-kDa species.

Studies with other deletion mutants and *hr5*. Cells infected with wt Ad5, with *hr*5, or with one of a series of deletion

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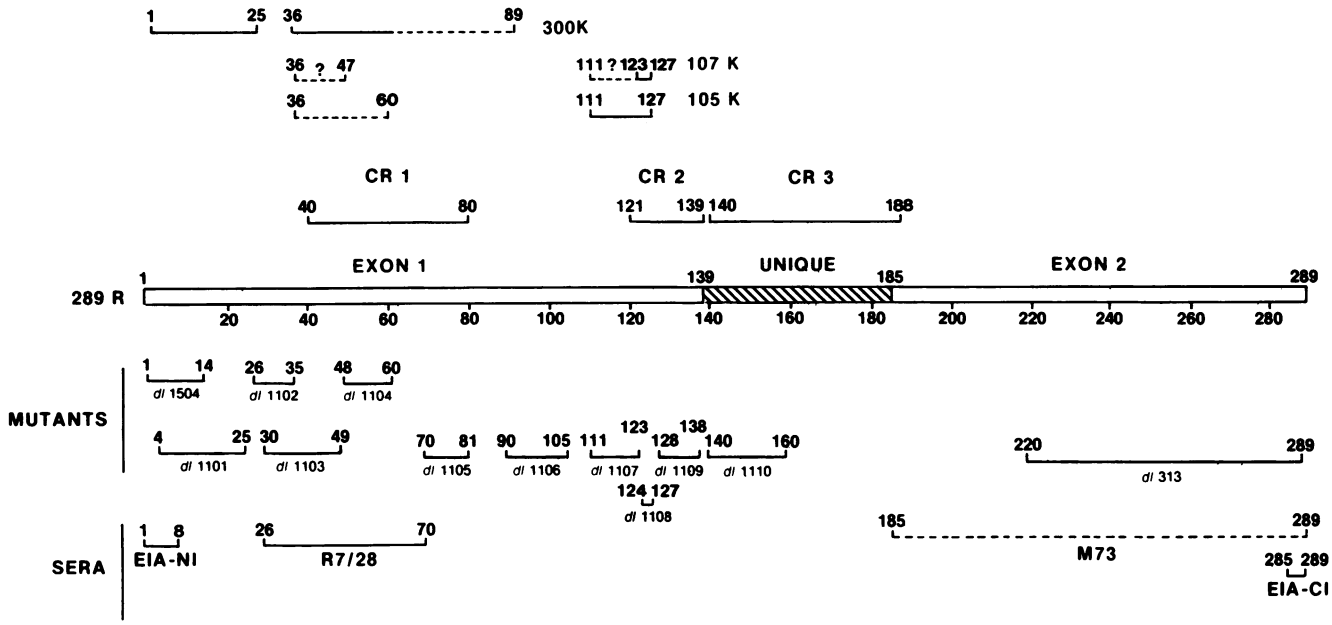

FIG. 1. Ad5 E1A 289R protein, mutants, antisera, conserved regions, and binding sites. The 289R protein is shown with its three structural regions: that encoded by exon 1 of the 0.9-kilobase mRNA (residues 1 to 138), the region unique to the 289R product of the 1.1-kilobase mRNA (residues 139 to 185 of the 289R protein), and the region encoded by exon 2 of both E1A mRNAs (residues 186 to 289 of the 289R protein). Shown below are the regions believed to be recognized by various E1A-specific sera and the locations of deletions in the various Ad5 mutants. Shown above are the positions of conserved regions 1, 2, and 3 (CR1, CR2, and CR3, respectively) and the locations of the regions which play a role in the binding of E1A-associated proteins (300-, 107-, and 105-kDa proteins [300 K, 107 K, and 105 K, respectively), as interpreted from data obtained in the present report. ——, Sequences essential for binding; ---, sequences having some effect on binding.

mutants were labeled with [^{35}S]methionine, and extracts were precipitated with E1A-specific monoclonal antibody M73 and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Mutant *hr5* (8, 12) contains a point mutation at nucleotide 1229, which alters Gly-139 in 243R to Asp and alters Ser-185 in 289R to Asn (7). The other mutants contain in-frame deletions and yield E1A products lacking various regions (15; see Fig. 1, Table 1, and the legend to Fig. 3 for complete details). All of these mutants produced reasonable amounts of E1A proteins, although migration rates were anomalous and increased or even decreased in a fashion unrelated to the size of the deletion (Fig. 3). An analysis (not shown) indicated that removal of acidic, and to a lesser extent, proline residues was the principal cause of increased gel mobility of mutant E1A proteins. These results suggested that high contents of these two types of amino acids cause E1A proteins to migrate much slower than would be predicted from their molecular masses.

Figure 3 shows that the 300-, 107-, and 105-kDa species coprecipitated with E1A products from cells infected with wt Ad5 and *hr5* but were reduced or absent with some of the deletion mutants. To quantify the amount of binding, appropriate exposures of the gel shown in Fig. 3 were scanned by a microdensitometer. The amounts of 300-, 107-, and 105-kDa proteins were normalized to the total amount of E1A products present, and then results were expressed relative to the values obtained with wt Ad5 (Table 1). The 300-kDa species was undetectable with *dl*1101 and *dl*1104, greatly reduced with *dl*1103, and partially reduced with *dl*1105. These results, together with that obtained with *dl*1504, showed that binding was affected by deletions within two regions of exon 1, namely, between residues 1 and 25 and between amino acids 36 and at least 60 (Fig. 1). The involvement of the first 14 to 25 residues at the amino terminus supported observations made previously (36) and in the present study (Fig. 2), which indicated that E1A-N1 anti-peptide serum failed to recognize E1A protein complexes containing the 300-kDa species. This failure may have resulted from the binding of the 300-kDa protein to this region, although it could also have been due to a conformational change induced by binding at another site. The 105-kDa species was undetectable with *dl*1107 and somewhat reduced with *dl*1103 and *dl*1104, indicating that the region between residues 111 and 123 (and perhaps the region between residues 36 and 60) plays a role in binding this protein. The increased binding with *dl*1102 was not seen in other experiments in which levels similar to wt Ad5 were observed (data not shown). With the 107-kDa species, binding was somewhat reduced with mutants *dl*1101, *dl*1103, and *dl*1107. It is unlikely that residues 1 to 25 are involved, as reduced binding was not seen consistently in other experiments with mutant *dl*1101 (data not shown) and was never seen with *dl*1504 (Fig. 2), which lacks residues 1 to 14. Less binding was seen reproducibly in several experiments with *dl*1103 and *dl*1107, thus suggesting that residues 36 to 47 and 111 to 123 may be of some importance. The region between residues 36 and 60 was also implicated in the binding of the 107- and 105-kDa proteins by results obtained with the E1A-specific rat monoclonal antibody R28. This antibody (and a similar monoclonal antibody, R7) was known to interact with an epitope mapping between residues 23 and 120 (32), and by using the present set of E1A deletion mutants, we have now shown that this epitope requires all of the region between amino acids 26 to 60 (data not shown). This serum immunoprecipitated E1A complexes containing the 300-kDa protein but failed to recognize those bound to the 107- and 105-kDa species (data not shown).

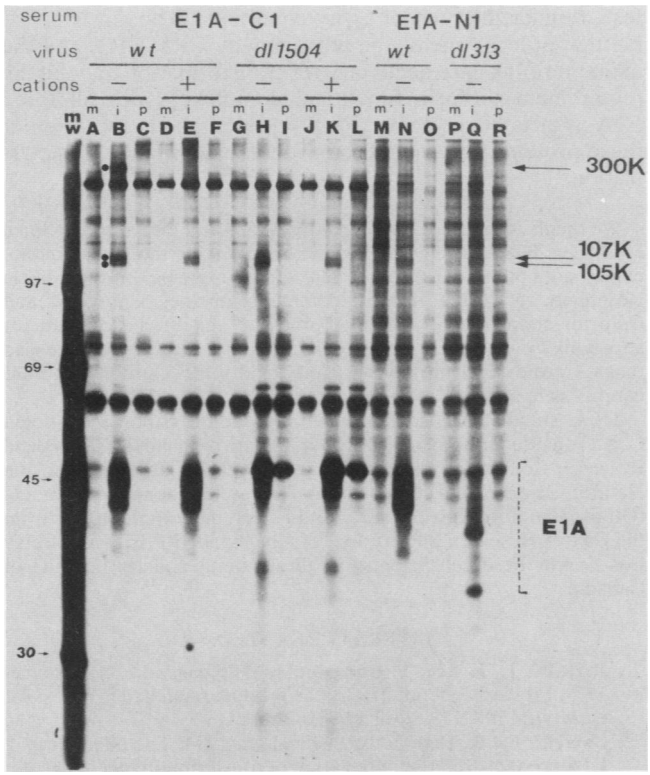

FIG. 2. E1A-associated proteins in cells infected with the mutants *dl*1504 and *dl*313. Human KB cells infected with wt Ad5 or with mutant *dl*1504 or *dl*313 were labeled with [^{35}S]methionine, and cell extracts prepared either in the presence or absence of divalent cations were immunoprecipitated by using either E1A-C1 or E1A-N1 anti-peptide serum, in some cases in the presence of the appropriate synthetic peptides. Lanes: mw, ^{14}C-labeled molecular weight markers (Du Pont NEN Research Products); m, mock-infected cells; i, Ad5-infected cells; p, precipitations carried out in the presence of 10 μg of appropriate peptide. Extracts were prepared in the presence (+) or absence (−) of divalent cations. To the right of the gel are shown the positions of the 300-, 107-, and 105-kDa proteins (300K, 107K, and 105K, respectively); to the left are shown the molecular masses of the ^{14}C-labeled markers.

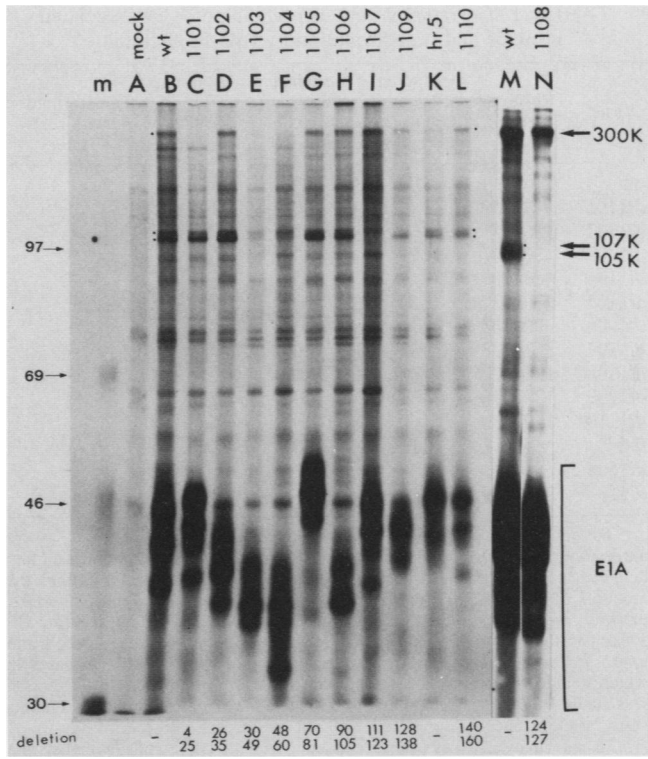

FIG. 3. E1A-associated proteins in cells infected with various deletion mutants and *hr5*. Cells were infected with wt or mutant Ad5 or they were mock-infected. The Ad5 deletion mutants *dl*1101 to *dl*1107 (1101 to 1107), *dl*1109 (1109), *dl*1110 (1110) (15), and *dl*1108 (1108) all possess in-frame deletions (see Fig. 1 and Table 1 for details of the deleted sequences), and their production has been described previously (15). Rescue of mutations into virus was by standard methods (Jelsma et al., in preparation). These mutants were all propagated in cell line 293 (9). Cells were labeled with [^{35}S]methionine, and extracts prepared in buffer A were immunoprecipitated by using the E1A-specific mouse monoclonal antibody M73, which reacts with an epitope in the exon 2 region (10). Lanes: m, molecular weight markers; A, mock-infected cells; B, wt Ad5-infected cells; C through L, mutant-infected cells (as indicated); M and N, wt Ad5 (M) and *dl*1108 (N) from a separate experiment. The residues missing in the deletion mutants are indicated at the bottom of the figure. Positions of the 300-, 107-, and 105-kDa proteins (300K, 107K, and 105K, respectively) are shown to the right of the gel; to the left are shown the molecular masses of the ^{14}C-labeled markers.

Because none of the previous mutants completely eliminated binding of the 107-kDa species, we constructed mutant *dl*1108, in which residues 124 to 127 between those removed in *dl*1107 and *dl*1109 were deleted. With this mutant, binding of the 300-kDa species was similar to that seen with wt Ad5 (Fig. 3, lane M), but both the 107- and 105-kDa proteins were absent (Fig. 3, lane N; Table 1). These data showed that the region necessary for binding of the 105-kDa protein extends to residue 127 and that amino acids 124 to 127 are required for the 107-kDa species.

It is unlikely that the unique region of E1A products plays a significant role in binding of the 300-, 107-, and 105-kDa proteins. Previous results have shown that the 243R protein is as efficient in binding these species as is 289R (6, 36), and binding of all three proteins to E1A products harboring deletions in the unique region, such as *dl*1110 (Fig. 3, lane L) and others (data not shown), was as with wt E1A. None of exon 2 appeared to be necessary for binding of the 300-kDa species, as preliminary studies with mutant E1A proteins produced in and purified from *Escherichia coli* containing the plasmid 410X, which encodes a protein lacking residues 150 to 289 (19), indicated that binding of the 300-kDa species was as efficient as with full-length E1A protein (C. Egan, B. Ferguson, M. Rosenberg, and P. E. Branton, unpublished results). Although the role in binding of the 107- and 105-kDa species of residues 186 to 220 in exon 2 has not been examined directly, results with *dl*313 suggested that residues 221 to 289 are of little importance (Fig. 2).

While conclusions from experiments such as these must be regarded with caution, the data suggest that exon 1 is the only portion of the E1A protein molecule involved in the binding of these cellular polypeptides, and furthermore, that regions in exon 1 in which deletions eliminate binding completely could represent the actual binding sites. For the 300-kDa species, two such regions exist which are located within residues 1 to 25 and 36 to 49. It is not clear if these two regions are combined by tertiary structure to form a single binding site or if one of these regions is necessary only for the formation of an appropriate protein structure at the other site. For each of the 107- and 105-kDa proteins, only a single

TABLE 1. Correlation between binding of E1A-associated proteins and transformation by Ad5 E1A mutants

Virus	Residues deleted	Binding (% wt) of 300-kDa protein[a]	Binding (% wt) of 107-kDa protein[a]	Binding (% wt) of 105-kDa protein[a]	Transformation with *ras*[b]
wt	0	100	100	100	+
*dl*1101	4–25	0	60	120	−
*dl*1102	26–35	160	150	380	+
*dl*1103	30–49	10	40	50	−
*dl*1104	48–60	0	90	50	−
*dl*1105	70–81	50	90	120	+
*dl*1106	90–105	130	120	170	+
*dl*1107	111–123	140	50	0	−
*dl*1108	124–127	100[c]	0[c]	0[c]	−
*dl*1109	128–138	130	80	130	−
*dl*1110	140–160	160	120	100	+
hr5[d]		110	120	100	+
*dl*1504	1–14	−	+	+	+[e]
*dl*313	220–289	+	+	+	+

[a] To quantify binding, the polyacrylamide gels shown in Fig. 3 were autoradiographed with preflashed film (20). From exposures in which all film densities fell on the linear part of the characteristic curve of the film, the amounts of the 300-, 107-, and 105-kDa cellular proteins and all of the E1A products were measured by microdensitometry with a Hoefer Scientific GS 300 apparatus. Values were normalized to the amount of total E1A protein and expressed as a percentage of the values obtained with wt Ad5. In cases in which deletions eliminated a methionine residue, an appropriate correction was made. Binding with the mutants *dl*504 and *dl*313 was not quantified in this study; the presence (+) or absence (−) of binding is indicated.

[b] Transformation assays were carried out with BRK cells with plasmid pLE2 containing mutated E1A regions and an activated *ras* gene, as described previously (21, 30). The specific transformation data will be published separately (Jelsma et al., in preparation). +, Transformation; −, no transformation.

[c] Values were obtained in a separate experiment.

[d] *hr5* contains a point mutation that alters residue 139 in the 289R product and amino acid 185 in 243R.

[e] Data of Osborne et al. (26) by a different transformation assay involving virions.

putative binding site was apparent, between residues 124 and 127 and residues 111 to 127, respectively. Other regions of the E1A protein which, when deleted, resulted in only partial reduction in binding may play a role in the tertiary structure of the primary binding site.

The fact that the E1A-binding proteins appear to interact with exon 1 of E1A proteins is of great interest, as this region has been linked to oncogenic transformation, cellular immortalization, enhancer repression, and other activities (15, 21, 22, 28, 30, 34). For transformation in association with an activated *ras* gene, two highly conserved regions encoded by exon 1, termed CR1 and CR2 (Fig. 1), are required (21, 22, 30, 33, 34). Recently, the deletion mutants described in the present study were used in plasmid form to map the regions in exon 1 necessary for transformation in cooperation with *ras*. The results showed that in addition to CR1 and CR2, a region near the amino terminus was also essential (Jelsma, et al., manuscript in preparation). Others had previously demonstrated the importance of the amino terminus, but they did not show that this region was functionally separable from CR1 (34). There was an excellent correlation between the failure to bind one or more of the E1A-associated proteins and loss of transforming activity (Table 1). The only exceptions were *dl*1109 and *dl*1504. The former transformed poorly, even though the mutant E1A product appeared to bind the E1A-associated species at levels comparable with that of wt Ad5. The latter, which fails to bind the 300-kDa species, has been shown by others, using whole virus, to be capable of transformation (26), although it has not been tested under the present assay conditions. Thus, it is possible that at least one of the reasons why CR1, CR2, and the amino terminus are necessary for transformation may be the requirement to form functional complexes with all three E1A-associated proteins. Further clues about the roles of these proteins must await their identification and characterization.

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