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Identification of Adenovirus Type 2 Early Region 1B Proteins That Share the Same Amino Terminus as Do the 495R and 155R Proteins

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Adenovirus type 2 early region 1B (E1B) proteins synthesized in vitro were fractionated chromatographically and characterized by peptide and sequence analysis and by reaction with peptide-specific antisera targeted to either the N or C terminus of either of two overlapping E1B reading frames (175 or 495 codons). In addition to the previously identified E1B-495R, E1B-175R, and E1B-155R species, two other E1B proteins of similar electrophoretic mobility to the 175R protein were identified. E1B-82R is an abundant product in vitro and in vivo that has the same N terminus as that of the 495R and 155R proteins but a different C terminus. The structure of 82R is predicted by the structure of the abundant 13S (1.02-kilobase) E1B mRNA. E1B-168R is a novel minor species consisting of the 24 amino-terminal residues of the 495R protein fused to the entire polypeptide IX sequence. An additional, minor 16,000-molecular-weight polypeptide was detected that may correspond to a predicted 92R E1B protein, but definitive identification was not possible. These observations establish that the leftmost portion (78 codons) of the 495-codon reading frame, which overlaps the right half of the 175-codon reading frame, is expressed as an abundant protein that does not contain other 495R sequences. This region, which may participate in the regulation of region E1A expression, may thus constitute a functional domain distinct from the rightward portion of the 495R protein.

Early region 1B (E1B) of adenovirus is usually necessary for the complete transformation of rodent cells by adenovirus (for a review, see reference 7), although it contains no transforming function of its own, and is, at least for some lines of established rodent cells, dispensable if E1A is expressed at high levels (30). E1B also provides several functions necessary for productive infection of human cells. E1B functions are expressed from two overlapping open reading frames (ORFs) of 175 and 495 codons, beginning with the first and second methionine codons, respectively, from the E1B RNA start (10, 15). Mutations in the former ORF lead to a large-plaque phenotype and increased cytopathic effect, the degradation of DNA, and host range effects (12, 28, 31, 37). Mutations in the latter lead to inefficient shutoff of host protein synthesis and to a decrease in adenovirus RNA transport (5, 6, 29). The relationship between the E1B functions identified for productive infection and for oncogenic transformation is still unclear.

Information about how the two E1B ORFs are expressed may be relevant to the question of what diverse functions are associated with each subregion of E1B. Proteins and mRNAs expressed from region E1B are diagrammed in Fig. 1. Although only one protein has been identified as the product of the 175-codon ORF (E1B-175R, sometimes referred to as 15K, 19K, or 21K) (2, 17), the expression of the 495-codon ORF is more complex. Expression of the intact ORF produces the E1B-495R protein (3), but one abundant and two nonabundant RNAs that splice out portions of the 495-codon ORF are also produced in infected cells (13, 18, 34). The E1B-155R protein has been identified as the product of one of these nonabundant mRNAs (3). An abundant protein designated E1B-18K or E1B-20K has also been described that shares homology with 495R (11, 16, 22, 23), although the exact sequence overlap has not been determined. We were initially inclined to believe that E1B-20K corresponded to E1B-155R, although the latter is not particularly abundant. However, experiments for which results are reported here revealed the existence of an abundant E1B protein related to 495R that was missed in our previous experiments because of an electrophoretic mobility equal to that of the abundant 175R protein. This species (E1B-82R) has been predicted from the structure of the abundant E1B 13S (1.02-kilobase [kb]) mRNA (10, 34) but not hitherto described. One unexpected protein related to the N terminus of 495R is also described that is presumably the translation product of a very rare mRNA.

MATERIALS AND METHODS

Cells and viruses. Human adenovirus type 2 (Ad2) was propagated on HeLa cells as previously described (2).

Preparation of E1B proteins by translation in vitro. Translation products were prepared as previously described (3) except that most of the experiments described here used late RNA prepared at 16 to 20 h postinfection (see references 13 and 34 for a comparison of E1B RNA structures present at early and late times). In some experiments, translation products were labeled with [^{3}H]proline or [^{3}H]leucine instead of [^{35}S]methionine. Contrary to our expectations based on the high proline content of most reticulocyte lysates (1), it was not necessary to remove endogenous proline by gel filtration of the lysate to obtain efficient incorporation of labeled proline.

Preparation of peptide-specific antibodies. Four peptides were synthesized to represent the N and C termini of the 495R protein and the N and C termini of the 175R protein. These peptides are identified in Table 1. The peptides were synthesized by the Merrifield technique and labeled at the N terminus with [^{14}C]acetate (roughly 20,000 cpm/mg) to facilitate purification as previously described (20). The production of antiserum by using peptide 3 (p-3) conjugated to bovine serum albumin has been described (3). p-15, p-25, and p-27 were conjugated to ovalbumin by using bis-

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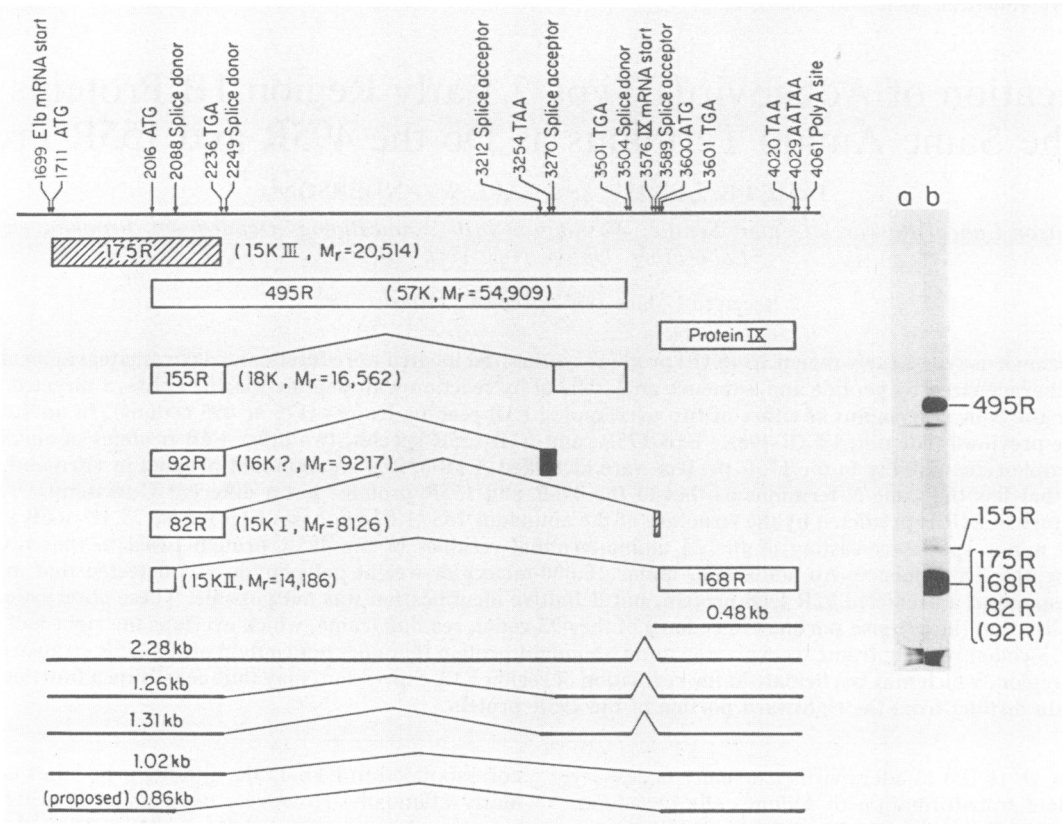

FIG. 1. Map of Ad2 early region 1B showing the protein-encoding regions and mRNA structures. The positions (nucleotide numbers) on the Ad2 DNA sequence (15) of various E1B landmarks are shown along the top, with the mRNA structures and protein-encoding locations shown below. The structures of the E1B-82R protein and the E1B-168R protein and mRNA are deduced from the peptide and sequence information presented here. The designations in parentheses refer to molecular weight in thousands estimated from electrophoretic mobility. Roman numerals I, II, and III refer to the order of elution from HPLC columns. Theoretical molecular weights are included. The three different translational reading frames that are used to encode protein segments are identified by open, hatched, and solid boxes. mRNA structures are identified by theoretical size, not including poly(A) tails, rounded to the nearest 0.01 kb. The 2.28-kb mRNA is sometimes referred to as 22S, and the 1.02-kb mRNA is sometimes referred to as 13S. The products of translation in vitro of E1B mRNA are shown on the right (lane b) adjacent to a lane without added mRNA (lane a).

diazotized benzidine, as described by Bassiri et al. (8) and Walter et al. (35). To obtain efficient conjugation with p-15, the peptide was first purified by C_{18} reverse-phase chromatography by using 4% formic acid and a gradient of methanol for elution. p-25 and p-27 were purified before conjugation by sequential gel filtration chromatography by using Sephadex G-25 and G-50 equilibrated in phosphate-buffered saline.

Rabbits were immunized with peptide-carrier conjugates as previously described (3). Crude rabbit serum, without affinity purification, was used for immunoprecipitation.

Immunoprecipitation. Radioimmune precipitation was performed as previously described (3, 21). The specificity of the precipitation was routinely tested by control experiments (designated + in figures) in which antiserum was first incubated with an excess of cognate peptide before reaction with the sample. For analysis by two-dimensional electrophoresis, the pellet was suspended in lysis buffer (26) instead of sample buffer (19) and then analyzed as described by O'Farrell (26).

High-performance liquid chromatography (HPLC). The product of cell-free translation (usually about 250 μl) was treated with RNase and EDTA, separated from unincorporated label by Sephadex G-25 gel filtration chromatography, and clarified by filtration (pore size, 0.2 μm). In most experiments, the sample was applied to a Bio-Rad hydroxylapatite column run on a Varian LC-5000 chromatography system. Elution was at 0.8 ml/min with a linear 60-min gradient of 0.01 to 0.50 M Na_2HPO_4 (pH 6.8) in 0.01 mM $CaCl_2$–0.05% NaN_3–0.1% sodium dodecyl sulfate (SDS). Fractions (0.8 ml) were collected, the radioactivity in 25-μl portions was counted in a liquid scintillation counter, and 10 μl of each fraction that contained substantial radioactivity was analyzed by SDS-polyacrylamide gel electrophoresis. In some experiments, the sample was applied instead to a

TABLE 1. Peptides synthesized to produce antisera

Peptide	Sequence[a]	Homologous sequence in Ad2 E1B proteins
3	acMERRNPSERCK	N-terminal 9 residues of 495R and 155R proteins
15	acMEAWECLEDFSAVRY	N-terminal 14 residues of 175R protein
25	acYSDEDTD	C-terminal 6 residues of 495R and 155R proteins
27	acYPRAGLDPRE	C-terminal 9 residues of 175R protein

[a] Peptide sequences are given in the one-letter amino acid code. ac indicates an acetylated amino terminus. Underlined amino acid residues are not encoded by adenovirus but were added to facilitate conjugation to carrier protein.

Vydac wide-pore C_4 reverse-phase column. Elution was at 1.5 ml/min with a gradient of 16 to 62% acetonitrile in 0.1% trifluoroacetic acid (TFA).

Identification of peptides. Radioactively labeled peptides were subjected to amino-terminal sequence analysis with the Beckman 890C or 890M Protein Sequencer as described previously (1, 2). The amino-terminal tryptic peptide (oxidized acetyl-Met-Glu-Arg [oxidized acMER]) of proteins initiated at the E1B-495R start site was identified by thin-layer chromatography as described previously (3). Oxidized acMER eluted from our C_{18} reverse-phase column (buffered with 10 mM ammonium acetate [pH 6.0]) just after the flowthrough (fractions 8 and 9). The carboxy-terminal E1B-15KI (82R) tryptic peptide was identified by cochromatography with a synthetic standard. The peptide, NH_2-PGPSGMNVVQQPPP-CO_2H, was synthesized with a BioSearch 9500 Peptide Synthesizer. After cleavage from the support resin with hydrogen fluoride, the peptide was purified by Sephadex G-25 gel filtration chromatography in 10% acetic acid followed by reverse-phase chromatography on a Brownlee Aquapore RP-8 column in 0.1% TFA–acetonitrile. The purified peptide had the expected amino acid composition. After performic acid oxidation, the synthetic peptide and a methionine-labeled tryptic digest of E1B-15KI (82R) were fractionated by reverse-phase chromatography on an Aquapore RP-300 column equilibrated in 0.1% TFA; elution was with a linear gradient of 0.1% TFA in 80% acetonitrile–20% water. The synthetic peptide was detected by measuring A_{214}. The methionine-labeled peptide eluting at the position of the oxidized synthetic peptide (see Fig. 11) was pooled and dried. A portion was subjected to sequence analysis and identified by virtue of a methionine at position 6 (data not shown). A second portion was applied to a reverse-phase C_{18} column (RP-18e; EM Science) equilibrated with 10 mM ammonium acetate; elution was with acetonitrile. Confirmation of the identity of the peptide was based on the fact that most of the radioactivity coeluted with the synthetic peptide (data not shown).

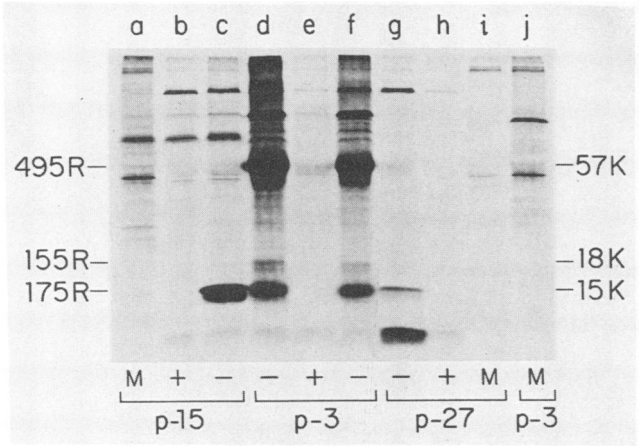

FIG. 2. Immune precipitation of E1B proteins from extracts of Ad2-infected cells by using antisera to the N terminus of the 495-codon reading frame (p-3) and to the N terminus (p-15) and C terminus (p-27) of the 175-codon reading frame. Extracts were prepared 18 h after infection or mock infection (M) of cells treated with cytosine arabinoside from 1 h after infection. Each precipitation used approximately 15 (Ad2) or 32 (M) × 10^6 cpm, and the extract was first cleared of material that precipitated nonspecifically by reaction with 10 μl of preimmune serum. Immune precipitation used 10 μl of crude antiserum. Lanes +, Antiserum was first blocked by reaction with 20 μg of cognate peptide. Fluorography was for 1 week.

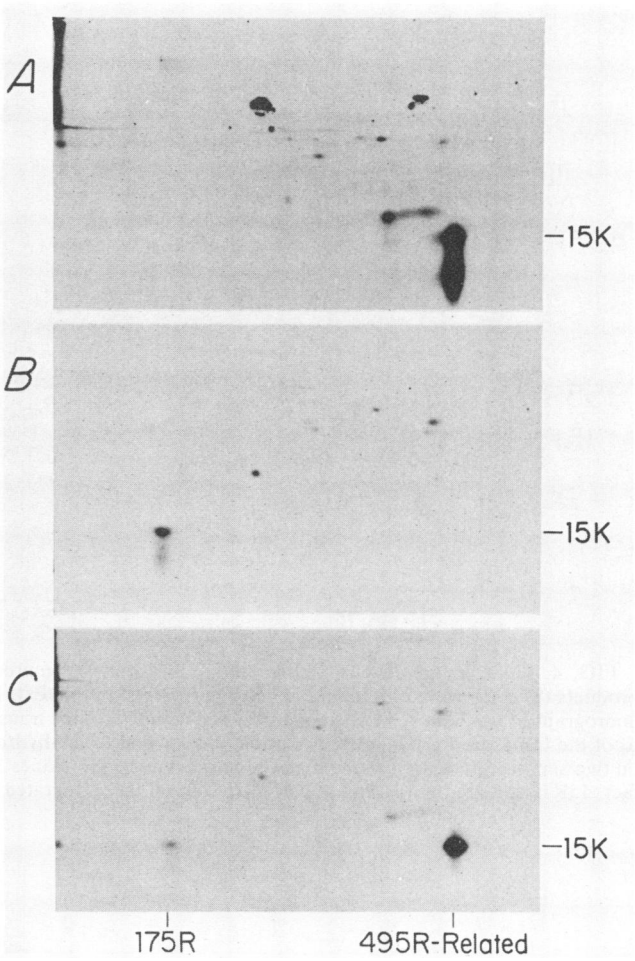

FIG. 3. Two-dimensional electrophoresis of E1B proteins precipitated by using antiserum to p-3 or p-15. An extract was prepared from cells 18 h after infection in the presence of cytosine arabinoside as in Fig. 2 and in separate experiments precipitated with antiserum to p-3 or p-15. (A) Two-thirds of the p-3 precipitate was analyzed; (B) two-thirds of the p-15 precipitate was analyzed; (C) a mixture of one-third of each precipitate was analyzed. Isoelectric focusing was in the horizontal direction, with the pH decreasing toward the right. SDS-polyacrylamide gel electrophoresis was in the vertical direction. Fluorography was for 6 weeks.

RESULTS

Our previous description of the E1B-155R protein (3) included some indications that additional E1B proteins might exist. First, antibodies directed against the N terminus of the E1B-495R protein not only precipitate the 495R and 155R proteins but also another protein with electrophoretic mobility greater than that of 155R and similar to that of the unrelated E1B-175R. At that time we had not determined whether this species was indeed 175R, precipitated via complex formation with 495R or 155R, or another E1B protein. Second, we also chemically characterized a minor 16,000-molecular-weight species (16K species) with electrophoretic mobility intermediate between the 155R and 175R proteins as having the same N terminus as 495R but an unidentified C terminus. These considerations plus the sub-

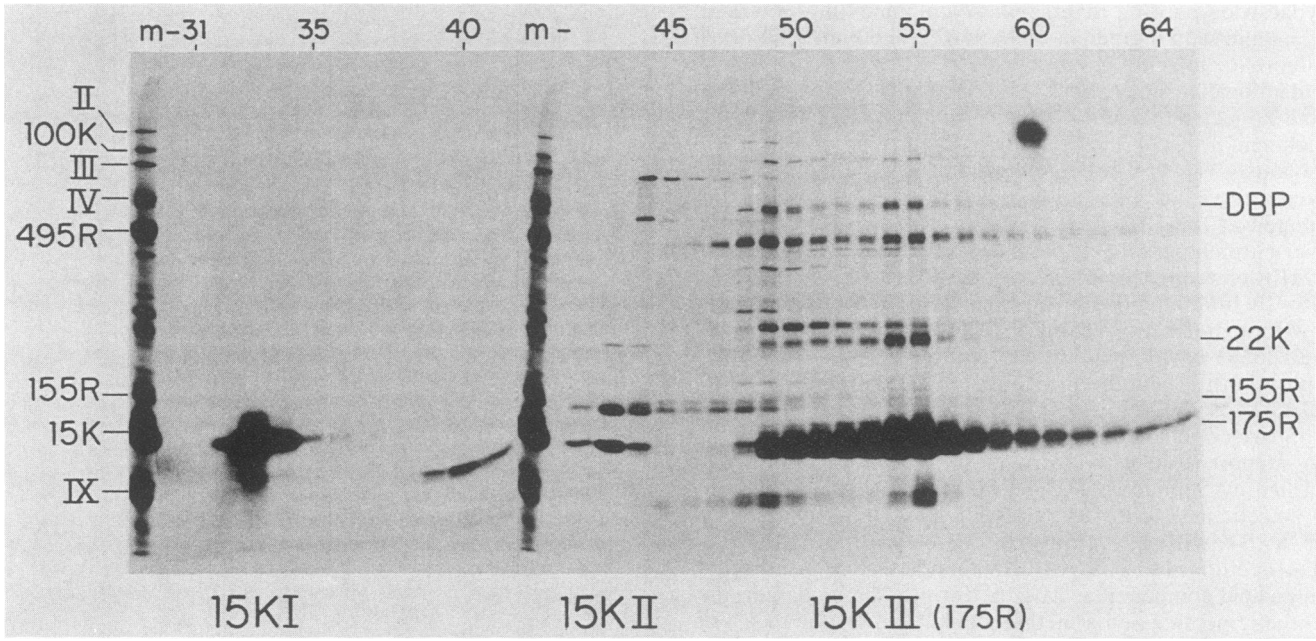

FIG. 4. Hydroxylapatite chromatography of E1B translation products. Late Ad2 E1B RNA was used as a template for translation, and the products (87.5 μl) were chromatographed as described in the text. Fractions 31 to 64 were analyzed by using a 15% polyacrylamide gel and fluorographed for 3 days. Lanes m were loaded with 5 μl of the translation product after dilution by gel filtration chromatography; about 1,400 μl of the translation product after dilution was applied to the hydroxylapatite column. Material in lanes 31 to 41 and 42 to 64 was analyzed on two separate gels, so that the material (component IX) in lanes 39 to 41 artifactually appeared to migrate with 15KII in lanes 42 to 44. In fact, 15KII migrated with 15KI and 15KIII, and all three migrated more slowly than component IX.

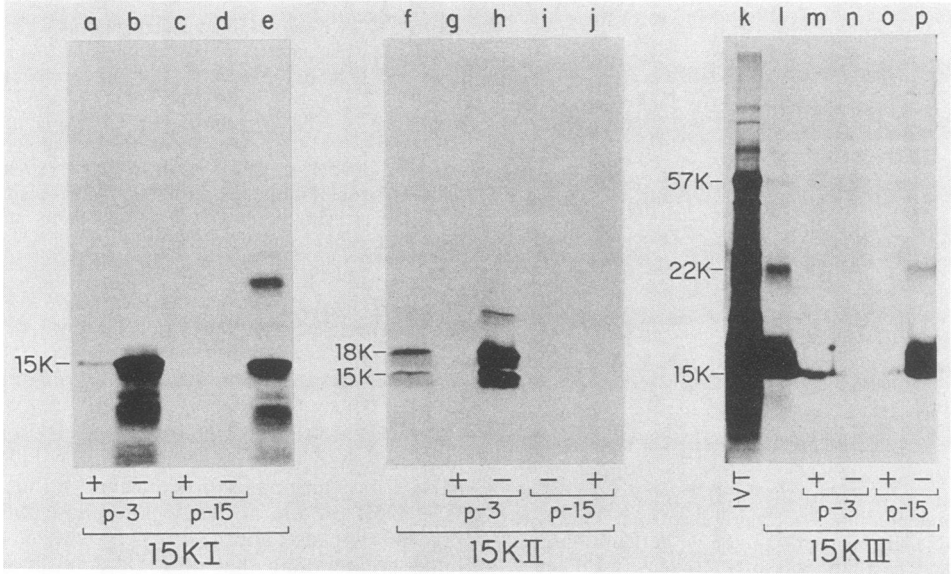

FIG. 5. Immune precipitation of E1B proteins from HPLC fractions. Fractions from an experiment similar to the one for which results are shown in Fig. 4 were analyzed by using antiserum to p-3 (N terminus of the 495R ORF) or p-15 (N terminus of the 175R ORF). Analysis by SDS-polyacrylamide gel electrophoresis of pooled fractions containing 15KI (lane e; 110,000 cpm), 15KII and 155R (lane f; 20,000 cpm), and 15KIII, 22K, and 495R (lane l; 21,000 cpm) is shown. Portions of these three fractions (containing 660,000, 100,000, and 105,000 cpm, respectively) were subjected to radioimmune precipitation analysis with antiserum to either p-3 or p-15. As a control for nonspecific precipitation, antiserum was first blocked by incubation with cognate peptide (lanes +). The apparent precipitation of a 15K protein in lane m is an artifact caused by spillover from lane l, since no 15K protein is seen in lane n, in which the antiserum was not blocked. A portion of the cell-free translation product applied to the hydroxylapatite column is shown in lane k. Fluorography was for 2 (lanes a to j) or 3 (lanes k to p) days.

sequent report by Virtanen and Pettersson (34) of two previously unmapped spliced mRNA structures, one of which would encode the 155R protein and the other of which would encode a 92R protein not yet described, and the fact that the 82R protein expected to be synthesized using the 1.02-kb mRNA had not yet been described led us to reinvestigate the identity of E1B proteins.

Extracts of HeLa cells labeled at early times after infection with Ad2 were analyzed by using antiserum prepared against peptides (Table 1) homologous to either the N or C terminus of E1B-175R (p-15 and p-27, respectively) or the N or C terminus of E1B-495R (p-3 and p-25, respectively). Antiserum to p-3 not only precipitated the 495R and 155R proteins from extracts of infected cells but also a 15K protein that migrated with the 175R protein, which was precipitated by using antiserum to either p-15 or p-27 (Fig. 2). Two-dimensional electrophoresis of the immune precipitated material demonstrated that the 15K protein that reacted with the p-3 antiserum has an isoelectric point different than that of the 175R protein (Fig. 3). Thus, the 495R-related 15K protein probably corresponds to the acidic 18K protein described by Brackmann et al. (11).

To facilitate studies of the different 15K E1B proteins (175R and the protein related to 495R), the translation products of E1B RNA were fractionated by hydroxylapatite HPLC (Fig. 4). Three proteins of about 15,000 molecular weight were resolved (designated 15KI, 15KII, and 15KIII in order of elution). In addition, a 16K protein (3) eluted with 15KI but could be resolved from it by reverse-phase chromatography, although with very poor recovery (data not shown). The E1B-155R and E1B-495R proteins were also apparent, as were minor species (E2A DNA-binding protein and several late virion components) arising from slight contamination of the template E1B RNA with other mRNAs. An unidentified 22K protein, which we have previously reported to be among the translation products of late E1B mRNA (14) eluted with 15KIII.

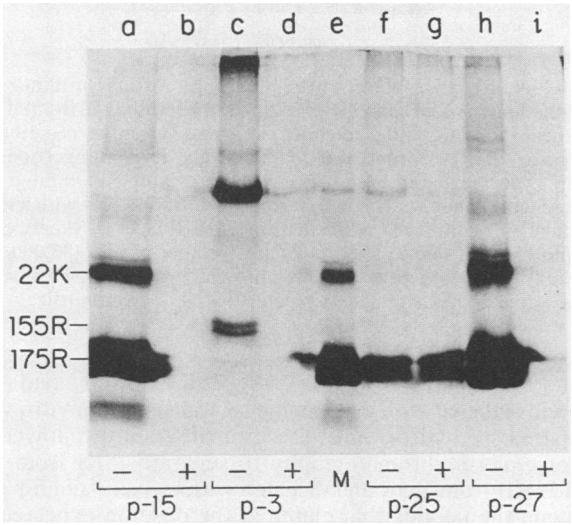

FIG. 6. Immune precipitation of fractions containing 15KIII by using N- and C-terminal-specific antisera. Fractions 54 and 55 from the experiment for which results are shown in Fig. 4 were pooled and analyzed by using antisera against the peptides listed in Table 1. In lane e, 2 μl of the pooled fractions was analyzed. Because of the different efficiencies in immunoprecipitation by the different antisera and the differences in the concentrations of the different E1B proteins, 25 μl was used for each precipitation with p-15 antiserum, 100 μl was used with p-3 antiserum, 300 μl was used with p-25 antiserum, and 200 μl was used with p-27 antiserum. Lanes +, Antiserum was first blocked by incubation with cognate peptide. (The apparent precipitation of 175R with the 495R C-terminal antiserum in lane f is an artifact, since the precipitation was not inhibited by incubation with peptide in lane g). Fluorography was for 40 days.

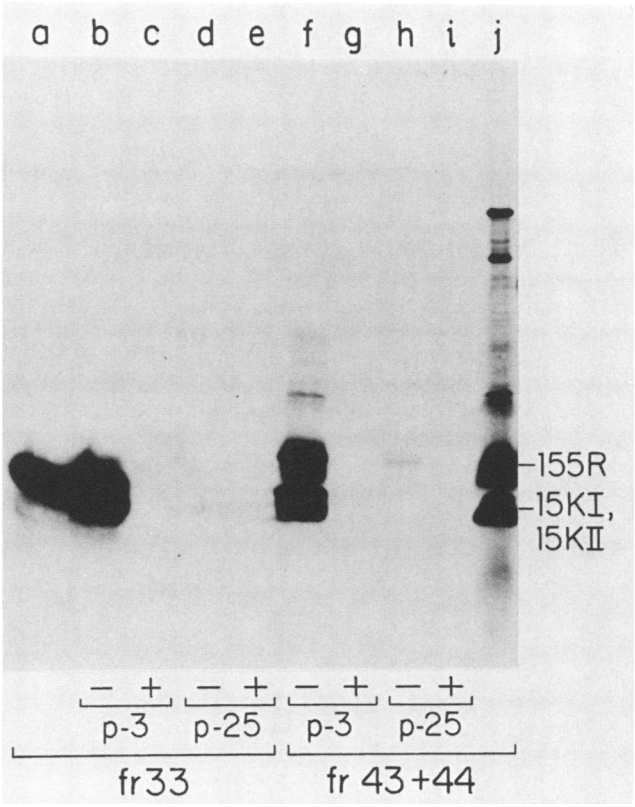

FIG. 7. Analysis of 15KI and 15KII with antisera to the N and C termini of the 495R and 155R proteins. Fraction (fr) 33 (lanes b to e) and pooled fractions 43 and 44 (lanes g to j) from the experiment for which results are shown in Fig. 4 were analyzed by immunoprecipitation. Fractions 33 (2 μl, lane a) and 43 and 44 (25 μl, lane f) are shown as markers. For each immunoprecipitation, 25 μl of fraction 33 and 50 μl of fractions 43 and 44 were used with antiserum to p-3 and 200 μl of fraction 33 and 400 μl of fractions 43 and 44 were used with antiserum to p-25. Fluorography was for 22 days.

Fractions containing the three 15K proteins resolved by hydroxylapatite HPLC were separately pooled and tested by immune precipitation by using the antisera to the peptides described in Table 1. 15KI and 15KII were both precipitated by antisera to p-3 but not to p-15, whereas the reverse was true for 15KIII and the 22K protein (Fig. 5). As expected, 155R (18K) was specifically precipitated with p-3, as was 495R (lane n, underexposed here because of the great excess of 175R, but see Fig. 6). Thus, 15KI and 15KII are both related to the 495R and 155R proteins by virtue of shared N termini, whereas 15KIII has the N terminus expected for the 175R protein.

Pooled fractions containing 15KIII, as well as 22K, 495R, and small amounts of 155R, were analyzed in more detail by using C-terminal- as well as N-terminal-specific antisera (Fig. 6). The 15KIII and 22K proteins were precipitated by antisera specific to both the N (lane a) and C (lane h) termini of 175R. Because the abundant 15KIII protein, which was

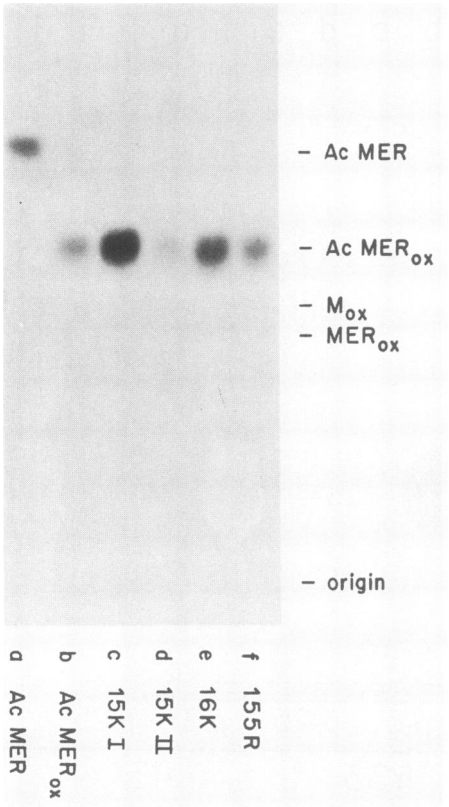

FIG. 8. Identification of the amino-terminal peptides of E1B proteins. Methionine-labeled, oxidized (ox) tryptic peptides of the E1B proteins 15KI (lane c), 15KII (lane d), 16K (lane e), and 155R (lane f) were separated by reverse-phase HPLC. The peak methionine-containing fraction corresponding to the expected elution position of acMER from each separation was applied to a cellulose thin-layer chromatography plate along with appropriate markers; chromatography was done with *n*-butanol–pyridine–acetic acid–water (300:200:60:240) as described previously (3). The position of unoxidized acMER and the positions of performic acid-oxidized acMER, MER, and M are marked. The autoradiograph shown was exposed for 3 months.

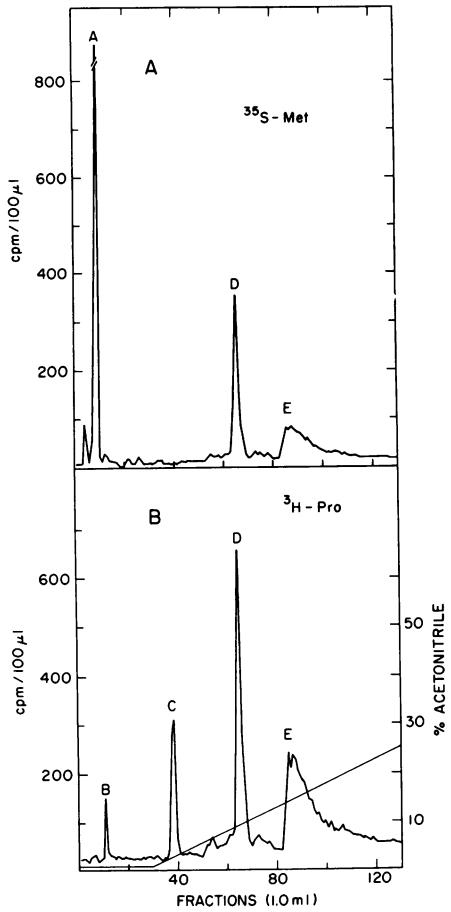

FIG. 9. Reverse-phase chromatography of [^{3}H]proline- and [^{35}S]methionine-labeled E1B-15KI tryptic peptides. E1B-15KI was synthesized by using the reticulocyte lysate system as described in the text. After performic acid oxidation and trypsin digestion, the sample was applied to a C_{18} reverse-phase column equilibrated with 10 mM ammonium acetate–1% acetonitrile; elution was with a linear gradient of acetonitrile as shown in panel B. (A) [^{35}S]methionine profile (54,000 cpm applied); (B) [^{3}H]proline profile (86,000 cpm applied). Only the first half of the chromatogram is shown since no radioactive peptides eluted at greater than 25% acetonitrile.

seen in vivo (Fig. 2 and 3), and the minor 22K protein, which was not detected in vivo, both contain the N and C termini of the 175-codon ORF, we conclude that 15KIII is 175R and 22K represents either an inefficient posttranslational modification or an artifact of electrophoresis. Hence, 22K was not further characterized.

As expected, 495R and 155R were precipitated by antisera specific to both the N (Fig. 6, lane c) and C (lane f) termini of 495R. Precipitation of 155R by p-25 is not apparent in lane f because of the lower activity of the C-specific antiserum compared with that of the N-specific antiserum and the low concentration of 155R in these fractions, but it can be seen in Fig. 7.

To determine whether 15KI and 15KII share the C terminus as well as the N terminus of the 495R and 155R proteins, these fractions were analyzed by using antiserum to p-25 (Fig. 7). Neither protein was precipitated by the C-terminal-specific antiserum (p-25), although the 155R protein, which eluted with 15KII, was precipitated as expected. Consequently, unlike 155R, neither 15KI nor 15KII has the C terminus of the 495R protein. As further confirmation of the N termini of these species, the 15KI, 15KII, and 16K proteins labeled with methionine by translation in vitro were prepared by hydroxylapatite chromatography followed by reverse-phase chromatography to separate 15KI from 16K and 15KII from 155R and digested with trypsin, and for each protein, the peptide that eluted at the position expected for the N-terminal peptide (acMER) was compared by thin-layer chromatography with authentic acMER. The three novel E1B proteins designated 15KI, 15KII, and 16K share the 495R N terminus also shared by 155R (Fig. 8). Of the three, the 15KI species is by far the most abundant, both among the products of cell-free translation and also in vivo. The 495R-related 15K protein shown in Fig. 2 and 3 behaved upon both hydroxylapatite and reverse-phase chromatography identically to 15KI synthesized in vitro (data not shown).

The only expected protein products of translation of the known E1B mRNAs that have not already been identified are the 82R product of translation of the abundant 1.02-kb (13S) mRNA (10) and the 92R product of the 1.31-kb mRNA (34). We therefore investigated the possibility that one or

TABLE 2. Predicted tryptic peptides of Ad2 E1B-82R and E1B-92R proteins

Peptide	Amino acid residues	
	No.	Sequence[a]
N-terminal peptides common to 82R and 92R		
t1	1–3	acMER
t2	4	R
t3	5–9	NPSER
t4	10–37	GVPAG FSGHA SVESG GETQE SPATV VFR
t5	38–68	PPGNN TDGGA TAGGS QAAAA AGAEP MEPES R
C-terminal peptide of 82R		
t6	69–82	PGPSG MNVVQ / QPPP
C-terminal peptide of 92R		
t6	69–92	PGPSG MNVVQ / EGGVP TLPMQ FESH

[a] Peptide sequences are given in the one-letter amino acid code. ac indicates an acetylated amino terminus. /, Peptide bonds resulting from splice junctions in the mRNA.

more of the novel E1B proteins might correspond to these species despite the expectation that the 82R and 92R proteins would be too small to be resolved efficiently on the gel system we used. The tryptic peptides expected from 82R and 92R are listed in Table 2. Because the 82R protein should contain no leucine, we translated E1B RNA with [^{3}H]leucine instead of [^{35}S]methionine as the label. We found that 15KII and other translation products were well labeled by leucine, whereas 15KI was barely detectable despite the fact that it was labeled much more intensely than was 15KII by methionine (data not shown). Thus, the predominant component (>99%) of 15KI contains no leucine.

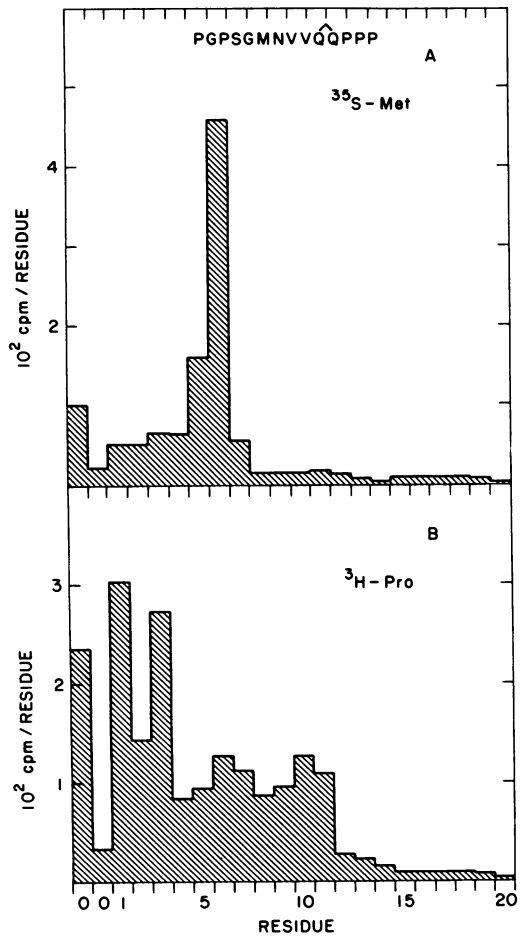

FIG. 10. Sequence analysis of the E1B-15KI carboxy-terminal tryptic peptide. The peptide labeled D in Fig. 9 was sequenced, and the yield of methionine (A) or proline (B) radioactivity at each cycle is shown. For the first two cycles (labeled 0) the coupling reagent, phenylisothiocyanate, was omitted. The predicted sequence of the C-terminal peptide is given at the top; a splice occurs in the mRNA between the two Q codons.

To further define the sequence of 15KI, protein labeled with both methionine and proline was prepared, tryptic peptides were separated by reverse-phase HPLC (Fig. 9), and individual tryptic peptides were subjected to sequence analysis. Peptide B was identified as 82R peptide t3 by virtue of a proline residue at position 2 (data not shown). Peptide D was identified as the presumptive C-terminal peptide t6 by virtue of a methionine at position 6 and prolines at positions 1 and 3 (Fig. 10). The absence of proline at positions 12 to 14, the C-terminal three residues of 82R, was not surprising because of the difficulty of sequencing to the end of a peptide, especially one with an uncharged C terminus. To confirm the identification of t6 as peptide D of 15KI, and thus the translation product of the splice junction in the mRNA for 82R, a synthetic peptide corresponding to t6 was prepared and shown to elute from HPLC identically to peptide D of 15KI (Fig. 11).

The small amounts of the 15KII and 16K proteins, as well as poor recovery after chromatography of some of the tryptic peptides of these proteins, prevented analysis of individual tryptic peptides of these species (other than the N-terminal peptides; Fig. 8). However, of the three methionine residues within the N-terminal 78 residues of the 495-codon ORF, only the one at position 6 of t6 should be detectable by sequencing of unfractionated tryptic peptides corresponding to this region. The Met at position 1 of t1 is not sequenceable because of the N-terminal acetylation of the protein. Apparently the Arg-Pro bond between t4 and t5 is cleaved inefficiently, so the Met at position 26 of t5 is too far from the N terminus of the combined peptide to be sequenceable. Indeed, only the t6 Met at position 6 was seen upon sequencing of the total tryptic peptides of 15KI (Fig. 12A). Similar analysis of 15KII revealed methionines at positions 1, 4, and 20 (Fig. 12B). The methionine at position 1 cannot be from t1 because thin-layer chromatography (Fig. 8) showed 15KII to have the same acetylated N-terminal peptide as do the other E1B proteins, and the absence of a Met at position 6 shows that, unlike the other E1B proteins studied, 15KII does not include the entire N-terminal 78-codon domain. The only way in which we can explain this

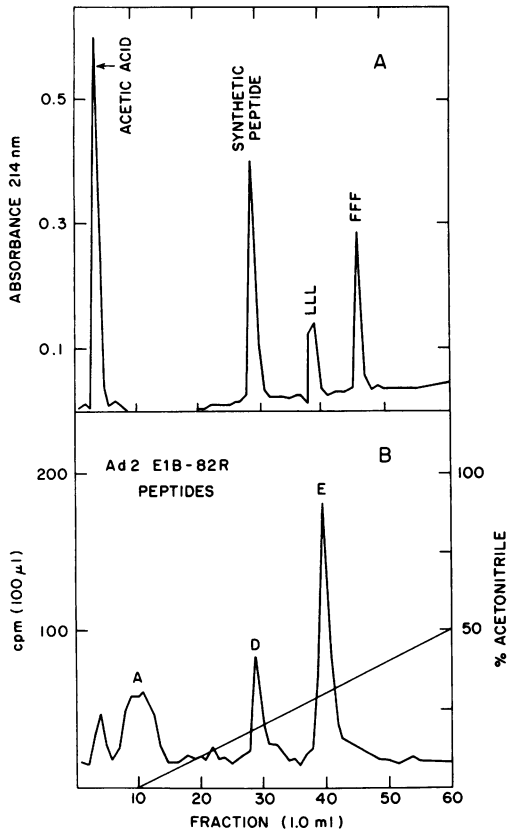

FIG. 11. Cochromatography of the E1B-15KI C-terminal tryptic peptide with a synthetic peptide corresponding to the predicted C-terminal tryptic peptide of E1B-82R. Reverse-phase HPLC was done on a Brownlee Aquapore RP-300 C_8 column equilibrated with 0.1% TFA; elution was with a linear gradient of 0.1% TFA in 80% acetonitrile–20% water as indicated in panel B. (A) Chromatography of 30 nmol of oxidized synthetic peptide; (B) chromatography of a tryptic digest of performic acid-oxidized, [^{35}S]methionine-labeled E1B-15KI (10,000 cpm were applied). The tripeptides LLL and FFF were included as markers in both experiments.

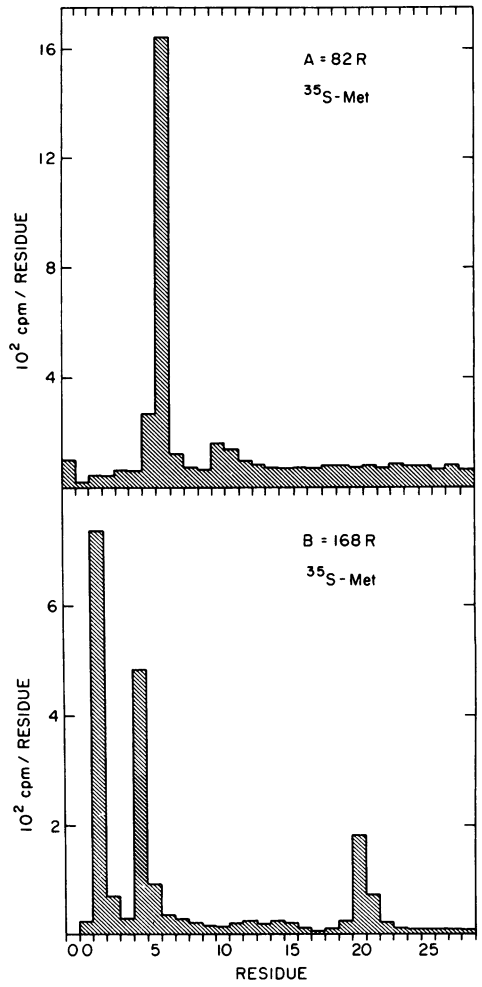

FIG. 12. Sequence analysis of mixed [^{35}S]methionine-labeled tryptic peptides of E1B-15KI and E1B-15KII. The reaction mixture from in vitro translation of E1B RNA was fractionated by SDS-hydroxylapatite chromatography followed by C_4 reverse-phase chromatography as described in the text. Appropriate fractions were pooled, oxidized with performic acid, digested with trypsin, dissolved in 50% formic acid, and applied with carrier apomyoglobin and Polybrene to a Beckman 890M protein sequencer. The total radioactivity released in each Edman cycle was plotted. (A) E1B-15KI (82R) digest (42,000 cpm applied); (B) E1B-15KII (168R) digest (4,500 cpm applied). The sequencer was run for one (panel B) or two (panel A) cycles (labeled 0) without the addition of phenylisothiocyanate to wash out any unbound radioactivity.

result is to postulate a second (and minor) splice donor sequence which connects the glycine codon at position 24 of the 495-codon ORF to the major E1B splice acceptor site just upstream of virion component IX coding sequences. Such a splice would produce an E1B-168R protein with an N-terminal 24-residue domain from 495R followed by four alanine residues and the complete sequence of protein IX. Thus, the initiating methionine of protein IX would be at position 20 in a tryptic peptide fused from t4 (Table 2) and IX. The other two methionines in protein IX fall at positions 1 and 4 in their tryptic peptides (15).

Sequence analysis of the mixed tryptic peptides of the 16K protein revealed a methionine only in position 6 (data not shown). This result, together with those of the thin-layer chromatography experiment (Fig. 8), establishes that 16K uses the same amino-terminal 78 codons as does 82R. We did not observe a significant release of methionine at residue 19 as might be expected for the 92R product; however, given the amount of material applied to the sequencer and the anticipated repetitive yield, we cannot be certain that we would have been able to detect the distal methionine in the 92R C-terminal peptide. Our results are not inconsistent with the identification of 16K as the predicted E1B-92R polypeptide, but positive identification is lacking. Alternatively, 16K could represent a posttranslational modification of 82R. The E1B gene products that have been identified are shown in Table 3 and Fig. 1.

DISCUSSION

We identified among the cell-free translation products of E1B mRNAs two 15K proteins which, because of electrophoretic mobilities similar to that of E1B-175R, have not previously been described. One of these corresponds to the previously predicted 82R translation product of the abundant 13S mRNA. We also used immune precipitation to verify the existence of E1B-82R in infected cells.

One of the difficulties that prevented earlier identification of these species is the anomalous relationship between

TABLE 3. E1B gene products[a]

E1B protein	Calculated M_r	Genomic coding location (nt)	Size of mRNA(s) (kb)
175R	20,514	1711–2236	2.28, 1.26, 1.31, 1.02
495R	54,909	2016–3501	2.28
155R	16,562	2016–2249/3270–3501	1.26
82R	8,126	2016–2249/3589–3601	1.02
168R[b]	14,186	2016–2088/3589–4020	0.86
92R[c]	9,217	2016–2249/3212–3254	1.31

[a] DNA sequence information is from references 10 and 15, RNA splice junctions (/) are from references 27 (for 82R) and 34 (for 155R and 92R), and protein sequence information is from references 2 (175R), 3 (495R and 155R), and this report (82R and 168R). mRNA structures are shown in Fig. 1.
[b] Protein, but not mRNA, identified.
[c] mRNA, but not protein, identified.

molecular weight and electrophoretic mobility. Three proteins of 82, 168, and 175 amino acid residues all migrated as 15,000-molecular-weight proteins. In addition, the previously identified 155R protein (3) migrates more slowly than either 168R or 175R.

The 168R protein, present in low amounts, results from an unexpected splice between E1B and virion component IX sequences. The 168R protein presumably incorporates the complete component IX sequence, although the methionine-containing peptides that we identified only verify the presence of the N-terminal one-fifth of component IX since the remainder of component IX contains no methionines. Thus E1B mRNAs use combinations of the two major and one minor splice donor sites and the one major and two minor splice acceptor sites to produce five mRNAs, three with one intron each and two with two introns each (Fig. 1 and Table 3). The abundant 2.28- and 1.02-kb mRNAs use the major donor sites at nucleotides (nt) 3504 and 2249, respectively, in conjunction with the major acceptor site at nt 3589. The less-abundant mRNAs use one minor donor or acceptor site; i.e., the 0.86-kb (168R) mRNA uses the proposed minor donor site at nt 2088 with the major acceptor site at nt 3589. The 1.26- and 1.31-kb mRNAs use both the major donor and acceptor sites at nts 3504 and 3589 and the major donor site at nt 2249 in conjunction with a minor acceptor site at either nt 3270 or 3212, respectively. Any potential products resulting from use of the minor donor site and either minor acceptor site would no doubt be too rare to detect by current techniques.

Thus, five E1B translation products have been described: one from the 175-codon ORF and four that use all or part of the 495-codon ORF. Of the three that do not use the entire 495-codon ORF, two use the N-terminal 78 codons and one uses only the N-terminal 24 codons. One of these two also uses the 495R C terminus, but the other uses a very short (four codons) region of another ORF. The variety of products using the 495-codon ORF is striking; our results extend previous observations of the 155R protein (3) and an 18K/20K protein that probably corresponds to the 82R protein that we identified (11, 16). An additional 92R protein is predicted to exist based on mRNA structural analysis (34), but we have failed to identify it among the E1B translation products. The variety of 495-codon-ORF-derived proteins may explain the complicated pattern of intracellular localization seen by using immunofluorescence with antisera that react with 495R (38).

Two principal issues are raised by these experiments. To what extent will 82R, 155R, and 168R substitute for particular functions of 495R? Do these different proteins have unique functions? The smaller proteins cannot substitute for 495R in RNA transport or host shutoff during productive infection because these functions are compromised by mutations that affect either 495R alone or only 495R and 155R (5, 6, 29). Furthermore, the exact form of these proteins cannot be important to any accessory roles the proteins might have during productive infection since destruction of the major splice donor site at nt 2249 does not affect virus growth (24). However, the proteins resulting from the use of cryptic splice sites in the latter instance may effectively substitute for 82R, etc., if all that is necessary is that the N-terminal domain of 495R is expressed in the absence of sequences from the middle of the 495-codon ORF and that the nature of the C terminus is unimportant. This idea suggests that the function of the smaller proteins related to 495R is to vary the expression of different domains of 495R. If so, then experiments in which large changes such as deletions or frameshifts are introduced into 495R cannot be easily interpreted in terms of separate roles for 495R and the smaller proteins. Instead, it may be necessary to alter both normal and cryptic splice sites to prevent expression of alternate proteins related to 495R.

The roles of the 495-codon reading frame in transformation are also likely to be complex. The left portion of 495R is essential for transformation (7), whereas the right portion is not (33), although various alterations within the middle and right portions of 495R often do affect transformation in different ways (4, 9).

Studies of cell lines that contain only E1A or both E1A and the left portion of E1B have implied the existence of an E1B function in increasing E1A gene expression (30, 32), and such a function has been directly demonstrated by transient expression experiments (25; A. W. Senear and J. B. Lewis, unpublished results). An interesting contrast to these observations is an opposite effect on E1A expression seen during virus infection as opposed to transient expression. In this instance, a decrease in E1A expression due to E1B function has been attributed to the 175R protein (36). Because of the very complicated pattern of E1B gene expression, a large number of mutants will probably have to be constructed and characterized to determine the extent to which the large number of 495R-related proteins represent distinct functions, but construction of such mutants should be facilitated by knowledge of how E1B is expressed at the protein level.

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