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Analysis of Adenovirus Early Region 4-Encoded Polypeptides Synthesized in Productively Infected Cells

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Peptide-specific antisera were developed to analyze the products encoded by adenovirus type 5 early region 4 (E4) open reading frames 6 and 7. Reading frame 6 previously was shown to encode a 34-kilodalton polypeptide (34K polypeptide) that forms a complex with the early region 1B (E1B)-55K antigen and is required for efficient viral growth in lytic infection. Antisera that were generated recognized the E4-34K protein as well as a family of related polypeptides generated by the fusion of open reading frames 6 and 7. These polypeptides shared amino-terminal sequences with the 34K protein. Short-pulse analysis suggested that the heterogeneity observed with the 6/7 fusion products resulted from differential splicing patterns of related E4 mRNAs. An antiserum directed against the amino terminus of reading frame 6 recognized only the free form of the 34K antigen that was not associated with the E1B-55K protein. This observation allowed the determination of the stability of the free and complexed form of this polypeptide. Pulse-chase analyses demonstrated that both forms of the 34K protein had half-lives greater than 24 h, suggesting that complex formation did not result in stabilization of this gene product. The half-lives of the 6/7 fusion products were approximately 4 h. The 34K protein also was shown to have a nuclear localization within infected cells. Finally, analysis of a mutant carrying deletions in both the E4-34K and E1B-55K polypeptides indicated that the complex formed between these two proteins was a functional unit in lytic infection.

The adenovirus type 5 (Ad5) early region 4 (E4) transcription unit is located between 91 and 100 map units (m.u.) in the viral chromosome and is transcribed in the leftward direction (Fig. 1A). The E4 nucleotide sequence of the closely related Ad_2 reveals seven open translational reading frames (ORFs; with coding capacities of greater than 25 amino acids) of which five contain AUG start codons (Fig. 1A, ORFs 1, 2, 3, 4, and 6) (14). A family of differentially spliced mRNAs is synthesized from this region (5, 8, 11, 24, 25). E4-specific cDNA clones have been isolated which encode all but one of the seven predicted ORFs and identify two new ORFs created by mRNA splicing (Fig. 1A, ORFs 3/4 and 6/7) (11, 25). Three E4-encoded polypeptides have been identified. An 11,000-M_r polypeptide and a 14,000-M_r polypeptide have been assigned to ORF 3 by using antisera specific for these proteins (9, 21) and are likely the same product. A 25,000-M_r polypeptide was identified by coimmunoprecipitation with the adenovirus E1B-55-kilodalton polypeptide (55K polypeptide) and assigned to ORF 6 (22; referred to in this paper as the 34K protein from the predicted molecular size based on the nucleotide sequence). The E4-34K and the E1B-55K polypeptides form a complex within adenovirus-infected human cells (22). Mutants that carry lesions in either of the polypeptides display similar lytic growth phenotypes (2, 13, 17, 20), suggesting that this complex may be functional. A number of additional polypeptides encoded by this region have been identified by in vitro translation of E4-selected mRNAs (18).

Weinberg and Ketner (27) characterized an E4 mutant, H2*dl*808 (deletion of 91.8 to 97.3 m.u.), that exhibited defects in viral DNA replication and late-gene expression. Halbert et al. (13) analyzed a set of site-directed mutants that disrupt each of the individual E4 ORFs as well as a mutant that deletes all of the E4 coding sequences. A mutant that contained a deletion in ORF6 encoding the E4-34K polypeptide (Fig. 1B, *dl*355) was moderately defective for lytic growth in HeLa cells. A mutant that lacked all of the E4 coding sequences (Fig. 1B, *dl*366) was severely defective for lytic growth. Both mutants exhibited similar, complex phenotypes, displaying perturbations in DNA replication, transcription and accumulation of late viral mRNAs, and host cell shutoff (13). Although *dl*355 and *dl*366 defects were different in degree, the similarity between their phenotypes suggests that perhaps various proteins encoded by E4 have overlapping functions. An interesting possibility is that the predicted protein encoded by an mRNA that fuses ORFs 6 and 7 (not disrupted in *dl*355 but deleted in mutant *dl*366; Fig. 1B) could overlap in function with the ORF 6 34K polypeptide.

Our studies focused on the products encoded by E4 ORFs 6 and 7. By using peptide-specific antisera in conjunction with mutants previously described (13), we identified a family of structurally related polypeptides encoded by the fusion of ORFs 6 and 7. The antisera also were used to examine the synthesis and half-lives of the related E4 proteins, to localize the E4-34K polypeptide to the nucleus of infected cells, and to study the interaction of the E4-34K and E1B-55K polypeptides. Finally, we constructed a double mutant which failed to synthesize both the E4-34K and E1B-55K polypeptides. The mutant exhibited a phenotype identical to the individual mutants, indicating that the complex formed by the two proteins was functional.

MATERIALS AND METHODS

Viruses and cells. H5*wt*300 is a plaque-purified derivative of an Ad5 stock originally obtained from H. Ginsberg, Columbia University, New York, N.Y. The construction and propagation of the E4 mutant viruses *dl*355, *dl*356, and

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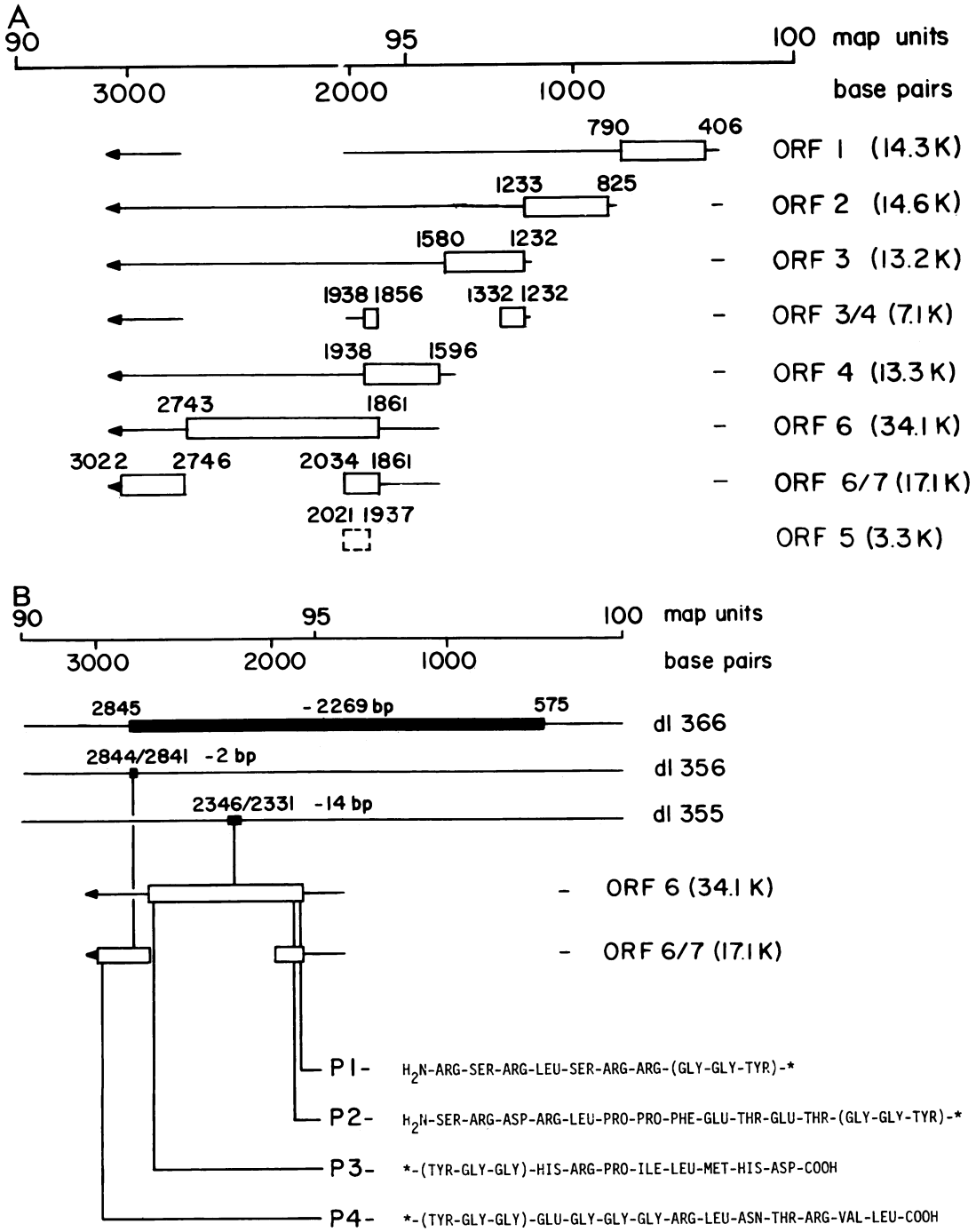

FIG. 1. (A) E4 transcription unit. Physical map of E4 mRNAs and coding regions. The top of the figure positions the map in terms of Ad2 map unit and nucleotide sequence relative to the right end of the genome. Representative mRNAs that have been identified by cDNA analysis (11, 25) are indicated by lines and arrows; introns are indicated by spaces, and potential polypeptide-coding regions are indicated by open rectangles. ORFs were previously identified from nucleotide sequence analysis of the region (14). ORF 5 is indicated by a broken-line rectangle because this ORF contained no AUG initiation codon. Molecular sizes of predicted polypeptides encoded by each ORF and by fusion of different ORFs based on cDNA analysis are shown in parentheses. The first and last nucleotide of each ORF is indicated above the mRNAs predicted to encode these products. (B) Map of E4 deletion mutants and sequence of peptides used to generate E4 ORF 6- and 7-specific antisera. The top of the figure positions the E4 transcription unit map as described above (A). ORF 6 and the fusion of ORFs 6 and 7 are shown along with the location of E4 deletion mutants used in this study. *dl*355 carries a 14-base-pair deletion in E4 ORF 6 in a region that is unique to the 34K protein-coding sequence (13). *dl*356 contains a 2-base-pair deletion that disrupts ORF 7, and *dl*366 contains a deletion of all E4 coding sequences (13). The amino acid sequence of the peptides p1, p2, p3, and p4 that are described in the text is shown. Amino acids shown in parentheses were not derived from E4 coding sequences but were added as spacers for conjugation to carrier BSA. The site of carrier attachment is shown by an asterisk. Antisera R3 and R7 described in the text are specific for peptides p2 and p4, respectively.

*dl*366 were described previously (13). Mutant *dl*338 contains a deletion in the E1B-55K protein and has been described previously (17, 20). The mutant virus *dl*367 contains the E1B deletion present in *dl*338 and the E4 ORF 6 deletion present in *dl*355. This variant was constructed by ligating the *Eco*RI A fragment (0 to 75.9 m.u.) from *dl*338 with the *Eco*RI B fragment (75.9 to 100 m.u.) from *dl*355 followed by transfection of 293 cells. Procedures for transfection and propagation of mutants were as described previously (15). Virions purified by cesium chloride equilibrium density centrifugation were used for all infections. Virion concentration was measured by disruption of virus particles in 0.1% sodium dodecyl sulfate (SDS)–10 mM Tris (pH 7.4)–1 mM EDTA and determination of the optical density at 260 nm. An optical density of 1.0 = 10^{12} particles per ml was used. The multiplicities of infection for all experiments are given in the figure legends.

The 293 cell line (a human embryonic kidney cell line transformed with the left 11% of the Ad5 genome [12]) was obtained from C. S. H. Young, Columbia University, New York, N.Y., and maintained in Dulbecco modified Eagle medium plus 10% calf serum. HeLa cells (from American Type Culture Collection) were grown as monolayer cultures in the medium plus 10% fetal calf serum. The W162 cell line was provided by G. Ketner, Johns Hopkins University, Baltimore, Md., and maintained as described previously (28).

Analysis of polypeptides. Cultures were labeled in medium lacking methionine supplemented with 1% fetal calf serum and [^{35}S]methionine (1,100 Ci/mmol) for the times indicated in the figure legends. Labeled cells were washed twice with phosphate-buffered saline (PBS), and the cell pellets were frozen at −70°C. Cellular extracts were prepared by suspension of the cell pellets in RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 160 mM NaCl, 10 mM Tris [pH 7.4], 5 mM EDTA) and sonication for 10 s.

Immunoprecipitations were performed at 0°C by a modified version of a procedure described by Brugge and Erikson (7). Cell lysates were preadsorbed with a 1/10 volume of *Staphylococcus aureus* for 10 min. (*S. aureus* was prepared by the procedure of Kessler [16] and activated in RIPA buffer containing 0.1% bovine serum albumin [BSA]). *S. aureus* and insoluble cellular debris were pelleted by centrifugation at 10,000 × *g* for 10 min. Incubations with antibody were for 45 min followed by incubations with *S. aureus* for 15 min. The *S. aureus* pellets were washed once with a high-salt buffer (10 mM Tris [pH 7.4], 1 mM EDTA, 1 mM EGTA, 1 M NaCl, 0.5% Nonidet P-40), twice with RIPA, and once with TEN (10 mM Tris [pH 7.4], 1 mM EDTA, 50 mM NaCl). Immune complexes were released from the *S. aureus* pellet by a 10-min incubation in sample buffer (10% glycerol, 3% SDS, 5% mercaptoethanol, 50 mM Tris [pH 6.8]) at 0°C. The E1B-55K-specific monoclonal antibody (2A6) was a gift of A. J. Levine, Princeton University, Princeton, N.J., and has been described previously (23). SDS-polyacrylamide gel electrophoresis was performed as described by Sarnow et al. (21).

Peptide synthesis and immunization of rabbits. Peptides were synthesized by the solid-phase method (3, 10). Peptides 2, 3, and 4 were synthesized by S. Bakr, Brookhaven National Laboratory, Upton, N.Y. Amino acid composition analyses of the peptides were performed by acid hydrolysis and subsequent high-performance liquid chromatography analysis of the products (courtesy of M. Elzinga, Brookhaven National Laboratory). Each purified peptide was coupled to BSA by the methods of Bassiri et al. (4) and Walter et al. (26). Peptide (10-mg) was coupled to BSA at approximately 40 mol of peptide per mole of BSA. The product was then dialyzed first against water and then against PBS.

Female White New Zealand rabbits were used to generate peptide-specific antibodies. Approximately 5% of the peptide-conjugated BSA described above in a 50% emulsion of Freund adjuvant was used for each injection. The first injection was delivered intradermally to each dorsal flank. The second and third injections were delivered subcutaneously to the dorsal flanks and intramuscularly to each upper thigh at 3 and 6 weeks, respectively, after the first injection. Rabbits were bled 1 week after the last injection.

Immunofluorescence. Cells were grown on printed slides (Roboz Surgical Instrument Co., Inc.) to approximately 50% confluency and infected at 400 particles per cell. At 12 h after infection, cells were fixed in 4% formaldehyde in PBS (pH 7.2) for 10 min at room temperature followed by rinses in 50% methanol for 5 min at −20°C, in absolute methanol for 10 min at −20°C, and in 50% methanol for 5 min at −20°C. The fixed cells were rehydrated overnight in PBS at 4°C. Antisera were diluted 1:50 in PBS containing 10% fetal calf serum and incubated with cell monolayers for 1 h at 37°C. The slides were then washed six times (5 min each) with PBS, incubated with a 1:200 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) for 1 h at 37°C, washed six times with PBS (5 min each), and washed once with water. Slides were viewed and photographed with a Zeiss microscope equipped with an epifluorescence device.

RESULTS

Region E4 encoded a family of structurally related polypeptides. E4-specific cDNA clones identified mRNAs that fuse the amino terminus of ORF 6 to ORF 7 (Fig. 1A) (11, 25); the mRNAs could encode a 17,100-M_r polypeptide structurally related to the ORF 6 34K species. We prepared peptide-specific antisera to aid in the characterization of the 34K protein and to identify structurally related polypeptides that could be synthesized by alternate splicing patterns. Four peptides (Fig. 1B) were synthesized on the basis of their hydrophilicity and location in the predicted ORFs. Two peptides were specific to the amino terminus of ORF 6 (p1 and p2), one was specific to the carboxy terminus of ORF 6 (p3), and one was specific to the carboxy terminus of ORF 7 (p4). Positive antisera were obtained against peptides 2 and 4 and are designated R3 and R7, respectively. Rabbits immunized with peptides 1 and 3 failed to produce positive antisera.

Wild-type Ad5 (*wt*300) and several E4 mutants (13) were used in conjunction with the antisera to identify and assign specific polypeptides to this region. The E4 mutants are diagrammed in Fig. 1B and include *dl*355, which contains a deletion in the carboxy-terminal half of ORF 6 specific to 34K coding sequences; *dl*356, which carries a deletion in ORF 7; and *dl*366, which lacks all of the E4 coding sequences. HeLa cells were infected with wild-type and mutant viruses and labeled with [^{35}S]methionine from 12 to 16 h after infection. Cell lysates were subjected to immunoprecipitation with an E1B-55K-specific monoclonal antibody (2A6), the E4 peptide-specific antisera R3 and R7, and respective preimmune sera (NR3 and NR7). The products of immunoprecipitation were analyzed by SDS-polyacrylamide

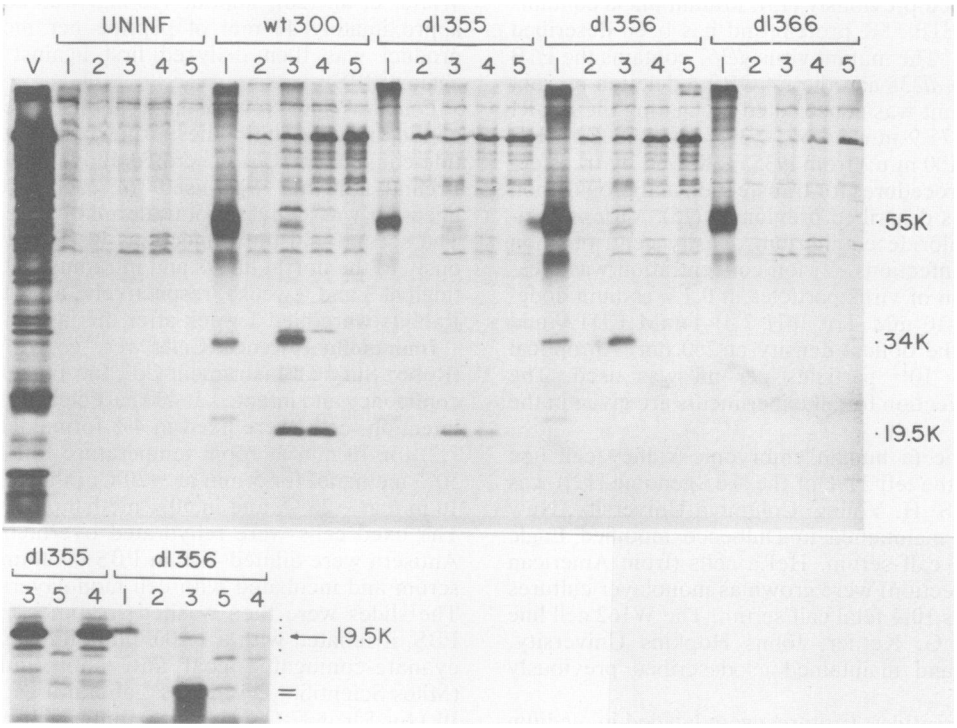

FIG. 2. Identification of a family of polypeptides synthesized from the fusion of ORFs 6 and 7 related to the ORF 6 34K protein. Uninfected HeLa cells or HeLa cells infected with wild-type or mutant viruses at a multiplicity of 5,000 particles per cell were labeled with 300 μCi of [^{35}S]methionine per ml from 12 to 16 h after infection. Cell extracts were prepared and immunoprecipitated by using preimmune rabbit sera (NR3 and NR7, lanes 2 and 5), peptide-specific antisera (R3 and R7, lanes 3 and 4), and a monoclonal antibody (2A6, lane 1) specific for the E1B-55K antigen. The products of immunoprecipitation were analyzed in a 20% SDS-polyacrylamide gel. Bands representing the E1B-55K, E4 ORF 6 34K, and E4 ORF 6/7 19.5K polypeptides are indicated beside the autoradiogram. The inset shows an overexposure of a similar gel in the region displaying the family of 6/7 fusion products. The 19.5K protein is indicated. Minor 6/7 fusion products are indicated by dots, and the truncated polypeptides synthesized by the mutant *dl*356 are designated by lines beside the autoradiograph. Lane V represents labeled markers prepared from purified Ad5 virions.

gel electrophoresis. An autoradiograph displaying the results of these analyses is shown in Fig. 2.

As previously described (22), the E1B-55K and E4-34K proteins were coimmunoprecipitated from wild-type infected cell extracts by using the E1B-55K-specific monoclonal antibody 2A6 (*wt*300, lane 1). As expected, the E1B-55K protein also was detected in *dl*355-infected cells, but the E4-34K protein was absent, owing to the deletion in ORF 6 in this mutant (*dl*355, lane 1). The 34K protein was present in cells infected with *dl*356 but was absent in cells infected with *dl*366 (*dl*356 and *dl*366, lane 1) as expected. The additional protein migrating below the 34K species in the immunoprecipitates with the 2A6 antibody (21,000 M_r) may correspond to the E1B-17K product described by Anderson et al. (1). The 34K polypeptide was immunoprecipitated by using the R3 antiserum, but not the R7 antiserum, from wild-type-infected cell extracts (*wt*300, lanes 3 and 4). Again, this species was absent in *dl*355- and *dl*366-infected cells but was observed in *dl*356-infected cells, confirming the identify of this protein (*dl*355, *dl*356, and *dl*366, lane 3).

The R3 and R7 antisera both recognized a protein migrating at 19,500 M_r (*wt*300, lanes 3 and 4). This species also was detected in *dl*355-infected cells but not in cells infected with *dl*356 or *dl*366. Thus, this protein represented an E4 ORF 6/7 fusion product that contained amino-terminal sequences from ORF 6 recognized by the R3 antiserum and carboxy-terminal sequences from ORF 7 recognized by the R7 antiserum. A longer exposure of this region of a similar gel (Fig. 2, inset) demonstrated that a number of additional minor species that were absent in *dl*356-infected cells also were specifically immunoprecipitated by using the sera from *dl*355-infected cell extracts. Identical polypeptide species also were observed in wild-type virus-infected cells (see below). In immunoprecipitations with the R3 antiserum, these species corresponded to proteins of 21,500, 20,000, 18,000, and 15,000 M_r. In immunoprecipitations with the R7 antiserums, these products were observed in addition to two smaller species that migrated at 13,500 and 11,000 M_r. The *dl*356 mutation affected all of these proteins (Fig. 2, inset, lanes 3 and 4). Two novel polypeptides were observed in immunoprecipitations from *dl*356-infected-cell extracts (13,300 and 13,000 M_r) with the R3 antiserum, but not the R7 antiserum, that likely represented truncated 6/7 fusion products synthesized by this mutant (Fig. 2 inset, *dl*356, lane 3). The predicted size of a truncated 6/7 product that could be synthesized by *dl*356 correlated well with the observed mobility of these species. The possible origin of the doublet is discussed below. We conclude from these data that the peptide-specific antisera recognized polypeptides originating from ORFs 6 and 7 of E4 and that a family of related amino- and carboxy-coterminal products were generated from the fusion of ORFs 6 and 7.

A number of the additional polypeptides observed in immunoprecipitates with the R3 and R7 antisera are viral structural proteins (Fig. 2, lane V) that in most cases also were precipitated by using preimmune sera (*wt*300, lanes 1

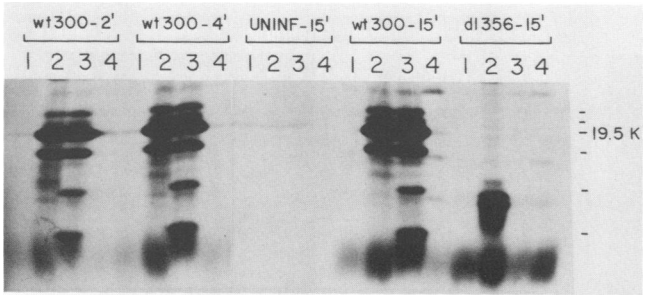

FIG. 3. Short-pulse-labeling analysis of E4 ORF 6/7 fusion polypeptides. Uninfected HeLa cells and HeLa cells infected with *wt*300 and *dl*356 at a multiplicity of 5,000 particles per cell were preincubated in medium lacking methionine for 1 h at 24 h after infection and then pulse-labeled with 500 μCi of [^{35}S]methionine per ml for the times indicated (2, 4, and 15 min). Cell extracts were prepared and immunoprecipitated by using preimmune sera (NR3 and NR7, lanes 1 and 4) and E4 peptide-specific antisera (R3 and R7, lanes 2 and 3). The products of immunoprecipitation were analyzed in a 20% SDS-polyacrylamide gel. The E4 ORF 6/7 19.5K protein is indicated. The lines show related products recognized by the E4-specific antisera and truncated in *dl*356-infected cells.

and 5). Additional species also were detected in uninfected-cell extracts by using these sera. The immunoprecipitated products described above represented polypeptide species that were consistently observed by using the R3 and R7 antisera and whose identities were confirmed genetically by using E4 mutants.

Short-pulse analysis of the ORF 6/7 fusion polypeptides. ORFs 6 and 7 are in the same E4 translational frame and are separated only by a TGA termination codon at nucleotide 2744 (14; Fig. 1B). A number of potential donor and acceptor splice sites that could fuse ORFs 6 and 7 have been predicted based on the nucleotide sequence (14) and by S1-nuclease mapping (24), but only two mRNAs that fuse ORFs 6 and 7 have been confirmed by cDNA analysis (11, 25). One mRNA fuses the amino terminus of ORF 6 (nucleotides 1861 to 2034) to ORF 7 (Fig. 1A, nucleotides 2746 to 3022) and would encode a 150-amino-acid polypeptide. Several 6/7 fusion mRNAs identified by cDNA analysis contain this splicing pattern (11, 25). A 17,100-M_r polypeptide would be encoded by this mRNA, and this product should be recognized by both the R3 and R7 antisera. The 19.5K protein described above probably corresponds to this polypeptide. A second 6/7 fusion mRNA was identified that excises the sequences between nucleotides 2035 and 2655 and would encode a protein sharing an amino terminus with the E4-34K protein but would change the reading frame after the splice site and contain a TGA termination codon at nucleotide 2727. This mRNA would encode an 82-amino-acid polypeptide with a predicted molecular weight of 9,300. This species was not observed in *wt*300-infected cells in immunoprecipitations with the R3 antiserum.

It was not clear, therefore, why a family of related 6/7 fusion products was observed in immunoprecipitates from infected-cell extracts. To address the possibility that post-translational modifications were responsible for generating this heterogeneous family of polypeptides, we performed a short-pulse-labeling experiment. Only newly synthesized, unmodified polypeptides should be identified after a very short pulse with [^{35}S]methionine. *wt*300, *dl*356, and mock-infected HeLa cells were pulse-labeled with [^{35}S]methionine, and cell lysates were subjected to immunoprecipitation with the R3 and R7 antisera. The results of electrophoretic analysis of the products are shown in Fig. 3. All of the ORF 6/7 fusion products previously detected in a 4-h labeling period (Fig. 2) were immunoprecipitated from *wt*300-infected cells by using the R3 and R7 antisera after 2-, 4-, and 15 min [^{35}S]methionine pulses. Immunoprecipitates from 6-, 8-, and 10-min pulses displayed the same pattern of proteins (data not shown). Two stable truncated products in *dl*356-infected cells were detected by using the R3 antiserum (Fig. 3, *dl*356, 15-min pulse, lane 2), confirming the identity of the polypeptides. Modification of the polypeptides by phosphorylation was not detected by labeling with $^{32}P_i$ (data not shown). The results strongly suggest that the heterogeneity observed in the family of related 6/7 fusion products is generated by alternate splicing patterns of mRNAs from this region. If posttranslational modifications are responsible for this heterogeneity, they must occur extremely rapidly on nascent or newly synthesized polypeptides.

E4-34K polypeptide was located in the nucleus. The peptide-specific antisera were used to localize the E4-34K antigen within infected cells. HeLa cells were infected with *wt*300 and *dl*355, and the localization of the E4-34K antigen was determined by indirect immunofluorescence at 12 h after infection by using the R3 antiserum. Photomicrographs of the stained HeLa cells under visible and UV light are shown in Fig. 4. In *wt*300-infected cells, the R3 immunofluorescent stain was localized in the nucleus. The nuclear staining pattern in *dl*355-infected cells was reduced to near the background levels observed in the preimmune serum control (NR3) and in uninfected cells. The very low level of immunofluorescence observed in *dl*355-infected cells may be due to the synthesis of the ORF 6/7 fusion proteins or a truncated ORF 6 product (although the latter product was not detected in immunoprecipitations). A low level of nuclear fluorescence was observed by using the R7 antiserum in *wt*300-infected cells (data not shown), but these results did not conclusively permit the localization of the ORF 6/7 fusion products. Thus, the immunofluorescence observed in *wt*300-infected cells by using the R3 antiserum was due to the presence of the E4-34K antigen in the nucleus; the ORF 6/7 fusion products did not contribute significantly to the staining pattern.

R3 antiserum recognized free E4-34K polypeptides. The R3 antiserum that recognized the E4-34K polypeptide did not coimmunoprecipitate the E1B-55K protein. A protein migrating near the mobility of the E1B-55K antigen was observed in immunoprecipitates from wild-type virus-infected cells by using the R3 antiserum (Fig. 2, *wt*300, lane 3), but this species also was observed in *dl*355-infected cells (Fig. 2, *dl*355, lane 3) in which the 34K protein was absent and in cells infected with an E1B deletion mutant (*dl*338; 17, 20) that did not synthesize the 55K protein. Thus, the R3 antiserum did not immunoprecipitate the E1B-55K-E4-34K protein complex. This could have been due to the disruption of the complex by antibody binding or to masking of 34K epitopes by association with the E1B-55K protein. To examine these possibilities, an immune-clearing experiment was performed. HeLa cells infected with *wt*300 were labeled with [^{35}S]methionine, and the cell lysate was divided into two equal fractions. One fraction was consecutively immunoprecipitated three times by using the R3 antiserum before a final immunoprecipitation with the E1B-55K-specific monoclonal antibody 2A6. The other fraction of the lysate was immunoprecipitated three times by using the E1B 55K monoclonal antibody (2A6) followed by a final immunoprecipitation with the R3 antiserum. The products were

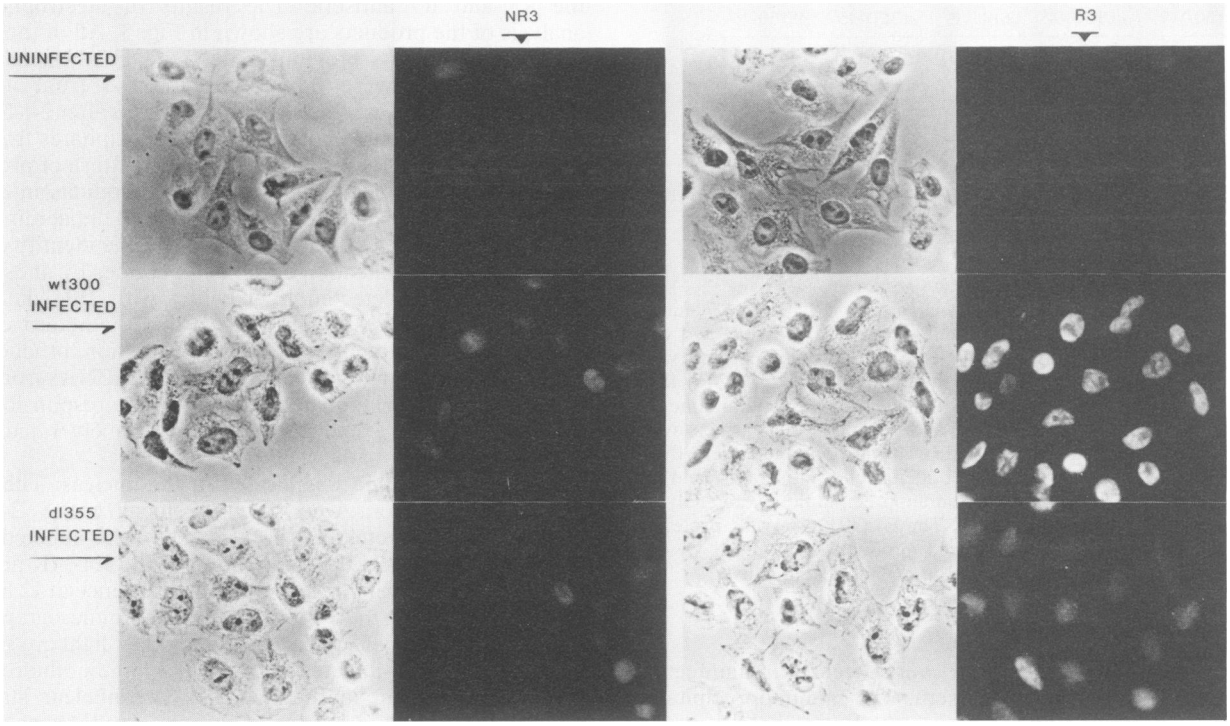

FIG. 4. Cellular localization of the E4 34K polypeptide. Uninfected HeLa cells or HeLa cells infected with *wt*300 and *dl*355 at a multiplicity of 5,000 particles per cell were stained with preimmune serum (NR3) and E4 ORF 6 peptide-specific antiserum (R3) 24 h after infection. Immunofluorescence was performed as described in the text. The figure shows phase-contrast and UV-fluorescent photomicrographs.

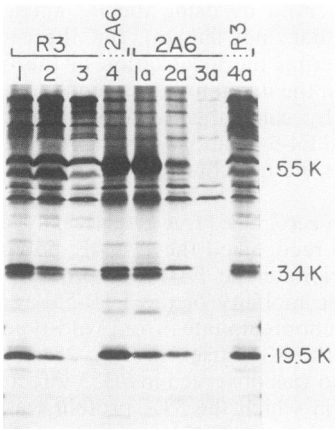

FIG. 5. Immune-clearing experiment with ORF 6 34K-specific antiserum and an E1B-55K-specific monoclonal antibody. HeLa cells were infected with *wt*300 at a multiplicity of 5,000 particles per cell and labeled with 300 μCi of [^{35}S]methionine per ml from 12 to 16 h after infection. The cell extract was divided into two equal fractions. Each fraction was subjected to three consecutive immunoprecipitations by using either the R3 antiserum (R3, lanes 1, 2, and 3) or the E1B-55K monoclonal antibody 2A6 (2A6, lanes 1a, 2a, and 3a). The lysates were subjected to immunoprecipitation by using the reciprocal antisera (R3, lane 4, with the 2A6 antibody, 2A6, lane 4a, with the R3 antiserum). The products of immunoprecipitation were analyzed in a 20% SDS-polyacrylamide gel. The bands representing the E1B-55K antigen, the E4 ORF 6 34K protein, and the E4 ORF 6/7 19.5K polypeptide are indicated. The E1B-17K protein precipitated by using the 2A6 monoclonal antibody migrated with a mobility similar to but slightly distinct from that of the E4-19.5K species.

analyzed by SDS-polyacrylamide gel electrophoresis, and the results are shown in Fig. 5.

Three consecutive immunoprecipitations with the R3 antiserum removed virtually all detectable 34K protein from the lysate (Fig. 5, R3, lanes 1 to 3). A final immunoprecipitation of this sample with the E1B-55K-specific monoclonal antibody, however, resulted in coimmunoprecipitation of a significant fraction of 34K antigen with the E1B-55K protein (2A6, lane 4). This demonstrates that 34K protein was still present in the extract in a complex with the E1B-55K protein even after apparent clearing of this antigen by using the R3 antiserum. Similarly, three successive immunoprecipitations with the E1B-55K-specific monoclonal antibody removed all traces of the E1B-55K protein and associated 34K antigen from the extract (2A6, lanes 1a to 3a). A final immunoprecipitation of the sample with the R3 antiserum, however, resulted in precipitation of a significant fraction of E4-34K polypeptide (R3, lane 4a). The results demonstrate that the R3 antiserum recognized only the free form of the E4-34K protein (not complexed with the E1B-55K polypeptide) and that the antiserum did not disrupt the physical complex between these two proteins. We performed this experiment a number of times, and we estimated that approximately 50% of the total E4-34K protein present in infected-cell extracts was associated with the E1B-55K protein. The remainder of the 34K antigen was in a noncomplexed form. The E1B-17K protein precipitated by using the 2A6 monoclonal antibody (2A6, lanes 4 and 1a to 3a) migrated in this gel with a mobility similar to but slightly distinct from the mobility of the E4-19.5K species.

Pulse-chase analysis of the ORF 6 and 6/7 fusion products. To characterize the stability of the different E4 polypeptides encoded by ORFs 6 and 7 and the effect of complex

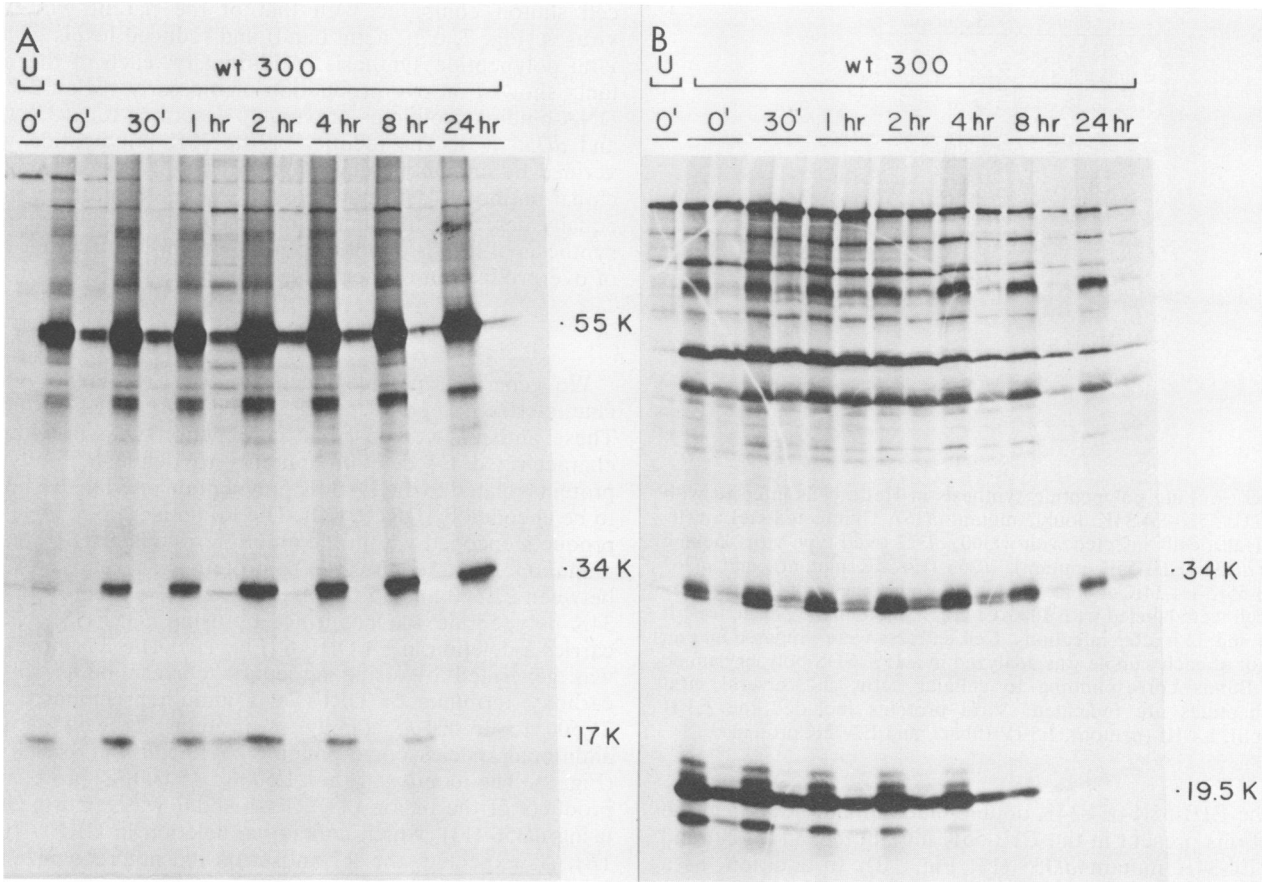

FIG. 6. Pulse-chase analysis of E4 ORF 6- and ORF 6/7-encoded polypeptides. Uninfected HeLa cells or HeLa cells infected with *wt*300 at a multiplicity of 5,000 particles per cell were preincubated in medium lacking methionine for 1 h at 12 h after infection and then pulse-labeled with 300 μCi of [^{35}S]methionine per ml for 15 min. The cells were then washed twice and incubated in medium containing an excess of unlabeled methionine. Cell extracts were prepared at 0, 0.5, 1, 2, 4, 8, and 24 h after the pulse. The samples were divided into two equal fractions and immunoprecipitated by using either the E1B-55K monoclonal antibody 2A6 (Fig. 5A) or the ORF 6 peptide-specific antiserum R3 (Figure 5B). Two successive immunoprecipitations were performed on each sample to quantitatively clear the lysate of antigen. The products of immunoprecipitation were analyzed in a 15% SDS-polyacrylamide gel. The E1B-55K antigen, E1B-17K antigen, E4 ORF 6 34K protein, and E4 ORF 6/7 19.5K polypeptides are indicated. U, Uninfected cells used as a control.

formation on the stability of the E4-34K polypeptide, we performed a pulse-chase experiment. HeLa cells infected with *wt*300 were pulse-labeled with [^{35}S]methionine for 15 min at 12 h after infection and then chased for various times in medium containing an excess of unlabeled methionine. At 0, 0.5, 1, 2, 4, 8, and 24 h after the beginning of the chase period, cell lysates were prepared and immunoprecipitations were performed by using the R3 antiserum and the E1B-55K-specific monoclonal antibody. Two consecutive immunoprecipitations were performed with each sample to quantitatively remove the antigens from the lysate. Electrophoretic analyses of the products of the immunoprecipitations are shown in Fig. 6A (E1B-55K monoclonal antibody) and 6B (R3 antiserum).

The data demonstrate differential half-lives for the E4-34K polypeptide and the polypeptides encoded by the fusion of ORFs 6 and 7. The half-life of each of the ORF 6/7 fusion polypeptides was approximately 4 h (Fig. 6B). The noncomplexed E4-34K polypeptide recognized by the R3 antiserum, on the other hand, showed essentially no turnover during the entire chase period (Fig. 6B, 24 h). In immunoprecipitations with the E1B-55K-specific monoclonal antibody to detect the complexed form of the 34K antigen, it was apparent that the complex did not form during the pulse period (Fig. 6A, 0-min chase). The complexed form of the 34K protein was observed after a 30-min chase period, with an additional increase observed after 1 h. Formation of the complex did not have any apparent effect on the stability of the 34K protein, since again no turnover of this polypeptide was detected during the entire chase period (Fig. 6A, 24-h chase). Additionally, these data demonstrate that the E1B-55K–E4-34K complex was very stable, since no apparent exchange of ^{35}S-labeled 34K for unlabeled 34K was detected during the chase period.

E4-34K and E1B-55K polypeptides functioned in concert. Mutants in the E1B-55K polypeptide (2, 17, 20) display lytic defects that are similar to those described for the E4-34K mutant *dl*355 (13), suggesting that the physical complex formed between the polypeptides may play a functional role during infection. To address this question, a double mutant (*dl*367) was constructed which contained lesions in both the E1B-55K and E4-34K coding sequences. If the individual E4-34K and E1B-55K polypeptides perform functions unassociated with the complex, then the double mutant should exhibit a more drastic phenotype compared with the individual single mutants.

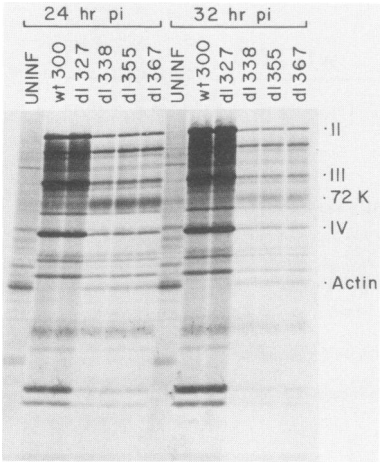

FIG. 7. Late polypeptide synthesis in HeLa cells infected with the E1B-55K–E4-34K double mutant *dl*367. Uninfected HeLa cells and HeLa cells infected with *wt*300, *dl*327 (wild-type virus lacking E3), *dl*338 (E1B-55K mutant), *dl*355 (E4-34K mutant), and *dl*367 (E1B-55K–E4-34K double mutant) at a multiplicity of 200 particles per cell were labeled with 100 μCi of [^{35}S]methionine per ml for 1 h at 24 and 32 h after infections. Cell extracts were prepared and an aliquot of each sample was analyzed in a 15% SDS-polyacrylamide gel. Bands corresponding to cellular actin and several viral polypeptides are indicated. Viral proteins included the L3-II (hexon), L4-III (penton), L5-IV (fiber), and E2-72K proteins.

The E1B-55K–E4-34K double mutant *dl*367 contained the deletions present in the E1B-55K mutant *dl*338 (17, 20) and the E4-34K mutant *dl*355 (13; Fig. 1B). In addition, as a result of the procedures used for construction of this variant, *dl*367 lacked the *Xba*I D fragment (78.5 to 84.3 m.u.) containing sequences in early region 3 (E3). E3 has been shown to be nonessential for productive growth in HeLa cells (6). A variant carrying an identical E3 deletion but intact region E1B and E4 (*dl*327) was used in the following experiments as a control for the deletion of part of E3 in *dl*367.

The severity of the *dl*367 growth defect was evaluated by comparison of its productive cycle to those of the single mutants *dl*338 and *dl*355 and to those of the wild-type viruses *wt*300 and *dl*327. Viral DNA synthesis was measured by pulse-labeling infected HeLa cells for 1 h with [^{3}H]thymidine at 16 and 24 h after infection. High-molecular-weight DNA was isolated, and incorporation of label into viral DNA was monitored by restriction endonuclease digestion with *Hin*dIII, gel electrophoresis of the resulting fragments, and subsequent autoradiography. Each of the mutants synthesized an approximately equal level of viral DNA compared with that synthesized by wild-type viruses at both times tested (data not shown).

The ability to synthesize late viral polypeptides and efficiently shut off host protein synthesis also was evaluated. HeLa cells infected with mutant and wild-type viruses were labeled with [^{35}S]methionine for 1 h at 24 and 32 h after infection. Total-cell lysates were prepared and analyzed by SDS-polyacrylamide gel electrophoresis. The results of these analyses are shown in Fig. 7. Both wild-type viruses *wt*300 and *dl*327 efficiently shut off host protein synthesis (Fig. 7, compare actin band with that for uninfected cells) and synthesized abundant levels of late viral polypeptides (Fig. 7, L3-II, L4-III, and L5-IV). In contrast, each mutant, *dl*338, *dl*355, and *dl*367, showed reduced efficiency of host cell shutoff compared with that of the parent wild-type viruses (Fig. 7, e.g., actin band) and reduced levels of late viral polypeptide synthesis. Additionally, each of the mutants showed an overproduction of the early region 2 72K DNA-binding protein as previously described for *dl*338 (17) and *dl*355 (13). The identity of the 72K polypeptide was verified by immunoprecipitation with a 72K-specific monoclonal antibody (data not shown). All three mutants were equally defective for shutoff of host protein synthesis and synthesis of late viral polypeptides and showed equal levels of overproduction of the E2-72K species.

DISCUSSION

We generated peptide-specific antisera to identify and characterize Ad5 polypeptides encoded by E4 ORFs 6 and 7. These antisera were used in conjunction with previously characterized E4 deletion mutants to define a family of proteins related to the E4-34K polypeptide previously shown to be encoded by ORF 6 (20). The R3 antiserum recognized products encoded within the amino terminus of ORF 6 and immunoprecipitated the E4-34K protein and a set of proteins between 21,500 and 15,000 M_r (Fig. 2). The identity of the 34K polypeptide was confirmed by using *dl*355 (13), which carries a deletion in E4 ORF 6 (Fig. 1B). The R7 antiserum was generated by using a peptide corresponding to the carboxy terminus of ORF 7; it immunoprecipitated the 21,500 to 15,000-M_r family of proteins as well as two additional species corresponding to 13,500 and 11,000 M_r (Fig. 2). The identity of the 21,500 to 15,000-M_r proteins as products of the fusion of ORFs 6 and 7 was confirmed by using *dl*356 (13), which contains a deletion in ORF 7 (Fig. 1B). As expected, the R7 antiserum did not recognize the E4-34K polypeptide.

The 6/7 fusion polypeptides may be functionally, as well as structurally, related to the ORF 6 34K protein. The following observations provide a genetic support for this hypothesis. Deletion of E4 ORFs 1 through 4 has no effect on virus viability in lytic infection of HeLa cells (M. M. Huang and P. Hearing, unpublished results). Mutant *dl*355 (ORF 6 deletion) is moderately defective for lytic growth, and *dl*366 (deletion of all E4 ORFs) is severely defective for viral growth (13). As previously described, the *dl*355 and *dl*366 phenotypes differ in severity but are clearly related (13). Since ORFs 1 through 4 are dispensible for virus growth, the difference in the severities of the *dl*355 and *dl*366 phenotypes may be due to the presence (*dl*355) or absence (*dl*366) of the ORF 6/7 fusion products within infected cells. We speculate that the ORF 6 34K and ORF 6/7 fusion polypeptides are functionally related and that the 6/7 fusion products allow *dl*355 to grow significantly better than *dl*366 in lytic infection. We propose that no defective phenotype was previously detected for *dl*356 (13), which contains a deletion in E4 ORF 7 (Fig. 1B), because this mutant synthesizes stable truncated products corresponding to the amino terminus of ORF 6 and part of ORF 7 (Fig. 2 and 3). Thus, the essential E4 sequences required for virus viability in lytic infection may reside solely within the amino terminus of ORF 6. An alternate possibility to explain the difference between the *dl*355 and *dl*366 phenotypes is that *dl*355 synthesizes a truncated, partly functional form of the 34K protein. We found no evidence for a stable truncated 34K product in *dl*355-infected cells by using the R3 antiserum or E1B-55K-specific monoconal antibody in either a long-pulse (Fig. 2, 4 h) or short-pulse (2 to 15 min [data not shown]) labeling period. Finally, we did not detect formation of a complex

between any of the ORF 6/7 fusion products and the E1B-55K antigen in any of the experiments with the R3 or R7 antisera or the E1B-55K-specific monoclonal antibody.

Based on cDNA analyses of E4 mRNAs that generate a fusion of ORFs 6 and 7, a 17,100-M_r polypeptide was predicted. This protein would be immunoprecipitated with both the R3 and R7 antisera. It seems likely that the 19.5 K protein shown in Fig. 2 and 3 corresponds to this product. The other related polypeptides specifically immunoprecipitated with both the R3 and R7 antisera (and truncated in cells infected with *dl*356) could have arisen by posttranslational modification of a primary product, from translation of alternately spliced E4 mRNAs, or in the case of the 13,500- and 11,000-M_r species recognized by the R7 antiserum, by proteolysis. Since all of these polypeptides were detected after a 2-min pulse-labeling period (Fig. 4), it is likely that all of the proteins represented primary translation products. Posttranslational modification also is not consistent with the generation of the smaller of these species that are still recognized by both the R3 and R7 antisera. Since only a few E4 cDNA clones have been identified that correspond to the fusion of ORFs 6 and 7 (11, 25), alternately spliced 6/7 fusion mRNAs may not have been detected owing to low abundance within infected cells. The lower levels of the proteins related to the 19.5K product are consistent with this idea. Finally, AUG initiation codons are located at amino acid positions 1 and 10 in ORF 6. Both of the codons are upstream of the peptide used to generate the R3 antiserum. Alternate use of the codons for initiation of translation could explain part of the heterogeneity observed in the related 6/7 fusion products. It is possible that the doublet observed with the truncated 6/7 products synthesized in *dl*356-infected cells (Fig. 2 and 3) may reflect the use of both of the codons for translational initiation. The 19.5K protein also appeared to migrate as a doublet in alternate gel systems.

Two other points are relevant to this discussion. First, an E4 mRNA was identified by cDNA analysis (11) that would encode a 9,300-M_r product containing the amino-terminal sequences of ORF 6 to the splice site at nucleotide 2034 (Fig. 1A) fused in an alternate frame beginning after nucleotide 2655. We found no evidence for this polypeptide by using the R3 antiserum even in short-pulse analyses (Fig. 4). Second, the R7 antiserum immunoprecipitated two additional polypeptides not recognized by the R3 antiserum (at 13,500 and 11,000 M_r; Fig. 2 and 4). Both of the proteins were absent in *dl*356-infected cells. The origin of the proteins is not known, but they may reflect products of mRNAs that fuse other E4 ORFs to ORF 7 which have not been previously identified. Alternatively, they could represent proteolytic products of the 19.5K protein or related species, although we found no evidence of an increase in the levels of these species relative to the other proteins related to the 19.5K in short-pulse experiments (Fig. 3) or in pulse-chase analyses (Fig. 5B).

The R3 and R7 antisera were used to analyze biochemically the ORF 6- and 6/7-encoded products and to localize these antigens within infected cells. Comparison of immunofluorescent patterns of cells infected with *wt*300 and *dl*355 demonstrated that the E4-34K protein was localized within the nucleus (Fig. 3). This result was not surprising, since this protein forms a complex with the E1B-55K antigen (20), which also has a nuclear localization (23). Weak nuclear fluorescence also was detected in *dl*355-infected cells by using the R3 antiserum (Fig. 3) and in *wt*300-infected cells by using the R7 antiserum (data not shown), suggesting that the proteins related to the 19.5K protein may be nuclear. We are not confident, however, that the level of fluorescence observed can be used to conclusively state this point.

The clearing experiment shown (Fig. 5) demonstrated that the R3 antiserum only recognized the free (noncomplexed) form of the 34K protein, whereas the E1B-55K-specific monoclonal antibody only coimmunoprecipitated 34K protein that was in a complex with the E1B-55K antigen. We used this observation to examine the stability of the free versus complexed form of this protein in pulse-chase experiments (Fig. 6A and B). The amount of 34K antigen synthesized during a 15-min pulse and remaining after a 24-h chase period did not suffer between the free or bound form of this protein. Thus, a stabilization of the 34K protein did not result from the interaction with the E1B-55K antigen. This is in contrast to the simian virus 40 T antigen-p53 cellular tumor antigen complex, in which T antigen stabilizes the p53 protein to produce elevated levels of p53 in transformed cells (19). The results of pulse-chase analyses also demonstrated that the 34K protein did not bind the E1B-55K polypeptide cotranslationally; instead, complex formation increased over a period of 1 to 2 h. Finally, the results demonstrate that once formed, the E1B-55K–E4-34K complex was quite stable, since the level of E4-34K protein in the complex remained constant through the entire chase period. If dissociation and reassociation of the complex was occurring during the chase period, [^{35}S]methionine-labeled 34K antigen should have been diluted from the complex by unlabeled, newly synthesized 34K protein, since this product was accumulating (unlabeled) through the course of the experiment.

E1B-55K mutants have been described which display a very similar phenotype in lytic infection to *dl*355 (2, 17, 20). A double mutant (*dl*367) carrying lesions in the E1B-55K protein (from *dl*338 [17, 20]) and E4-34K protein (from *dl*355 [13]) was constructed to test whether either of these proteins has a function in lytic infection not related to the complex of these products. Comparison of late polypeptide synthesis in cells infected with *dl*367 and the individual mutants *dl*338 and *dl*355 demonstrated that the double mutant was no more defective than either of the single mutants for growth in HeLa cells (Fig. 7). This conclusion was based on equivalent levels of viral DNA replication and equivalent reductions in host protein synthesis shutoff and late viral polypeptide synthesis, as well as overproduction of the E2-72K protein, relative to the parental wild-type viruses. The identical phenotypes of the three mutants indicate that the E1B-55K–E4-34K complex is a functional unit and that, during lytic infection, the separate E4-34K and E1B-55K polypeptides likely do not perform individual functions unrelated to the complex.

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