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Adenovirus Early Region 4 Encodes Two Gene Products with Redundant Effects in Lytic Infection

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In order to assign specific functions to individual gene products encoded by adenovirus type 5 early region 4 (E4), we have constructed and analyzed a set of mutant viruses that express individual E4 open reading frames or combinations of open reading frames. The results of these analyses demonstrate that the gene products of E4 open reading frames 3 and 6 have redundant effects in viral lytic infection. These E4 products independently augment viral DNA replication, viral late protein synthesis, the shutoff of host cell protein synthesis, and the production of infectious virus. The product of open reading frame 6 is more efficient in the regulation of these processes than is the product of open reading frame 3. The regulation of viral DNA replication and the control of viral and cellular protein synthesis appear to be separable functions associated with both E4 gene products. The role of early region 4 in adeno-associated virus helper function, however, is mediated only by the product of open reading frame 6. Finally, we demonstrate that E4 mutant viruses display a multiplicity-leakiness phenotype which is consistent with the regulatory role that this region plays in viral infection.

Early region 4 (E4) of adenovirus type 5 (Ad5) is located between 91 and 100 map units (mu) on the viral chromosome and is transcribed in a leftward direction (see Fig. 1). A complex family of overlapping E4 mRNAs is generated during viral infection due to alternate splicing of a primary transcript (10, 29, 30). A number of different proteins are likely encoded by E4, corresponding to seven different translational open reading frames (ORFs). E4-specific cDNA clones have been isolated which could encode all but one (ORF5) of the seven predicted ORFs and identify two new ORFs created by mRNA splicing (see Fig. 1, ORF1, -2, -3, -4, -6, -3/4, and -6/7; 10, 29, 30). Three E4-encoded polypeptides have been identified. An 11,000- to 14,000-M_r polypeptide (referred to as the 11K protein) was identified by using tumor antisera that recognize this product; the 11K protein is encoded by ORF3 (7, 24). A 25,000- to 34,000-M_r polypeptide (referred to as the 34K protein) was initially identified by coimmunoprecipitation with the adenovirus E1B 55,000-M_r protein, using a monoclonal antibody directed against the 55K product (23). The 34K protein subsequently has been analyzed by using peptide-specific antiserum directed against this polypeptide (6). The 34K protein is encoded by ORF6 (23). A family of related products (15,000 to 21,500 M_r) is encoded by the ORF6/7 mRNA(s); a prominant 19,500-M_r polypeptide (19.5K protein) is encoded by the fusion of these two ORFs (6). A number of additional E4-specific polypeptides have been identified by in vitro translation of E4-selected mRNAs, but these proteins have not been assigned to specific ORFs (17, 28).

Genetic analyses have demonstrated that E4 is involved in a number of different aspects of regulation during viral lytic infection. Mutant viruses which carry a deletion of the entire E4 region display profound defects in viral DNA replication, viral late mRNA accumulation, viral late protein synthesis, and the shutoff of host cell protein synthesis (12, 33). Additionally, these mutant viruses overexpress the E2 72,000-M_r DNA-binding protein (72K protein; 12) and they are partially defective for viral particle assembly (8). E4 is also involved in helper function for adeno-associated virus (AAV; 20; for a review, see reference 3). It is not clear whether all of the phenotypes associated with the disruption of E4 coding sequences are primary effects of the mutations. Viral mutants that carry mutations in E4 may be propagated on a complementing cell line, W162 (32).

Site-directed mutagenesis of individual E4 ORFs has identified only one gene product, the ORF6 34K protein, that is required for E4 function within infected cells (12, 13). An ORF6 mutant virus (*dl*355) displays a similar, but very moderate, defect in viral lytic growth compared with that of a mutant virus that lacks all of the E4 coding sequences (*dl*366; 12). Disruption of the other E4 ORFs does not reduce virus viability (12, 13). These results suggest that multiple products encoded by E4 are functionally compensatory. In this study, we report the construction and characterization of a series of E4 mutant viruses that express individual ORFs or combinations of ORFs. Our results demonstrate that the products of ORF3 and ORF6 have redundant effects in viral infection. Both gene products independently augment viral DNA replication, viral late protein synthesis, the shutoff of host cell protein synthesis, and the production of infectious virus. The product of ORF6 is more efficient in the regulation of these processes than is the product of ORF3. AAV helper function, however, is only associated with the ORF6 product. Finally, we demonstrate that E4 mutant viruses display a multiplicity-leakiness phenotype.

MATERIALS AND METHODS

Plasmids, viruses, and cells. The recombinant plasmid, p75-100, contains the Ad5 right terminal *Eco*RI fragment (75.3 to 100 mu) inserted between the unique *Bam*HI (Ad5 100 mu) and *Eco*RI (Ad5 75.3 mu) sites of pBR322. Cleavage with *Bam*HI linearizes the plasmid at 100 mu. All of the E4 mutations that were constructed for these analyses were introduced into the Ad5 p75-100 background. The nucleotide numbers used below are from the sequence of the Ad2 genome (14), since the sequence of Ad5 early region 4 has

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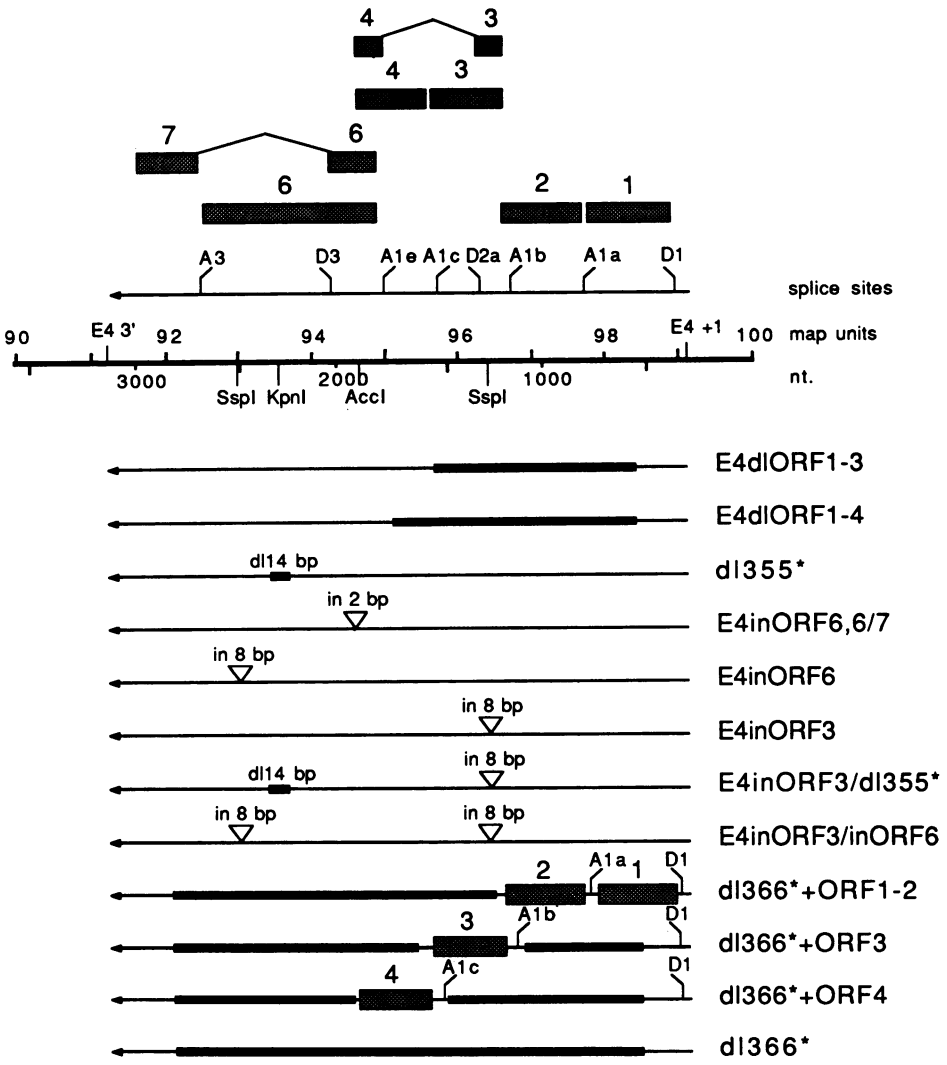

FIG. 1. E4 transcription unit map and mutant viruses. A physical map of E4 is shown at the top. The Ad5 genomic map between 90 and 100 mu is shown as a dark bar with map units indicated above the line; nucleotide numbers relative to the right terminus are indicated below the line. The E4 cap site is indicated as E4 +1, and the E4 polyadenylation signal is indicated as E4 3'. Relevant restriction endonuclease cleavage sites that were used for the construction of E4 insertion mutations are indicated below the line (*Ssp*I, *Kpn*I, and *Acc*I). E4 mRNA splice donor and acceptor sites determined from the nucleotide sequence of E4 cDNAs (30) are indicated directly above this line and are designated according to the published cDNA sequences. E4 translational ORFs, described in the text, are indicated by shaded boxes and correspond to ORF1, -2, -3, -4, and -6 and the fusion of ORF3/4 and ORF6/7 due to mRNA splicing; the locations of these ORFs are taken from the Ad2 nucleotide sequence (14). The E4 mutant viruses that are described in the text are shown below the E4 transcription unit schematic drawing. Solid bars correspond to deletion mutations. Triangles above the lines correspond to insertion mutations. Mutant viruses that contain the insertion of ORFs in a *dl*366* background are also shown.

not been completely determined. Where necessary, we determined the Ad5 E4 sequence to verify the presence of certain restriction endonuclease cleavage sites.

The following deletion and insertion mutations were introduced into p75-100 in Ad5 E4. Mutant virus E4*dl*ORF1-3 (Fig. 1) contains a deletion of the sequences between a *Sma*I site at 98.4 mu (nucleotide [nt] 575) and a *Bgl*II site at 95.7 mu (nt 1543). Mutant virus E4*dl*ORF1-4 (Fig. 1) contains a deletion of the sequences between the *Sma*I site at 98.4 mu and a *Bgl*II site at 94.9 mu (nt 1818). Both of these mutations were constructed by partial digestion with *Bgl*II, followed by a repair reaction using Klenow polymerase and subsequent cleavage with *Eco*RI (75.3 mu). These fragments were ligated with a fragment spanning the sequences between the *Bam*HI (100 mu) and *Sma*I (98.4 mu) sites. Mutant virus *dl*355 (Fig. 1) was described previously (12) and contains a 14-base-pair (bp) deletion at the *Kpn*I site at 93.5 mu (nt 2340). The *dl*355 mutation was originally constructed into a plasmid that lacks the sequences between 78.5 and 84.3 mu (Ad5 E3); the *dl*355 mutation was reconstructed into p75-100 to restore E3 and is newly designated as *dl*355*. Mutant virus E4*in*ORF6,6/7 (Fig. 1) contains a 2-bp insertion at an *Acc*I site in Ad5 E4 at 94.6 mu (nt 1947). This mutation was generated by partial digestion of p75-100 with *Acc*I, followed by a repair reaction using Klenow polymerase and subsequent ligation. Mutant virus E4*in*ORF6 (Fig. 1) contains an 8-bp insertion at an *Ssp*I site at 93.0 mu (nt 2514). This mutation was constructed by partial digestion of p75-100 with *Ssp*I, followed by insertion of an 8-bp *Cla*I linker and subsequent ligation. Mutant virus E4*in*ORF3 (Fig. 1) con-

tains an 8-bp insertion at an *Ssp*I site at 96.4 mu (nt 1300). This mutation was constructed in the same manner as that described for mutant E4*in*ORF6. Mutant virus E4*in*ORF3/*in*ORF6 (Fig. 1) was constructed by combining the individual mutations described above into one plasmid utilizing the *Kpn*I site (93.5 mu) and the *Bam*HI site (100 mu) to interchange restriction endonuclease fragments from the parental mutant plasmids. Mutant virus E4*in*ORF3/*dl*355* was constructed in the same manner as that described for mutant *in*ORF3, using p75-100 containing the same *dl*355 mutation as the parental plasmid. Mutant virus *dl*366 (Fig. 1) was previously described (12) and contains a deletion of E4 coding sequences between the *Sma*I sites at 98.4 mu and 92.1 mu (nt 2843). This mutation restored a *Sma*I site at the junction of the deletion; a *Cla*I linker was inserted at this *Sma*I site for subsequent cloning manipulations. The *dl*366 mutation was originally constructed into a plasmid that lacks the sequences between 78.5 and 84.3 mu ($E3^-$); the *dl*366 mutation was reconstructed into p75-100 ($E3^+$) and is newly designated *dl*366*. Mutant viruses *dl*366*+ORF1-2, *dl*366*+ORF3, and *dl*366*+ORF4 (Fig. 1) were constructed by inserting restriction endonuclease fragments containing these ORFs into the *Sma*I site (converted to *Cla*I) of *dl*366*. These fragments were: ORF1-2, *Sma*I at 98.4 mu (nt 575) to *Ssp*I at 96.4 mu (nt 1300); ORF3, *Alu*I at 96.9 mu (nt 1133) to *Alu*I at 95.9 mu (nt 1608); ORF4, *Hae*III at 96.0 mu in Ad5 (nt 1436) to *Acc*I at 94.6 mu (nt 1947). Each of these mutations was constructed such that the acceptor splice signals present upstream of ORF1, -2, -3, and -4 were maintained for proper splicing to the E4 leader sequence (see Fig. 1). Each of the insertion and deletion mutations described above was confirmed by nucleotide sequence analysis.

These mutations were rebuilt into intact Ad5 viral chromosomes by ligation or, alternatively, by overlap recombination. For reconstruction by ligation, the 0-to-75.3-mu (0-to-*Eco*RI) fragment of Ad5 *wt*300 was ligated to mutant plasmid DNA cleaved with *Eco*RI and *Bam*HI and subsequently used for transfection. For reconstruction by overlap recombination, mutant plasmids were cleaved with *Eco*RI and *Bam*HI and used in cotransfections with the 0-to-93.5-mu fragment from virus *dl*310-Xba^+. Virus *dl*310 is a phenotypically wild-type Ad5 variant that lacks *Xba*I cleavage sites (16); *dl*310-Xba^+ was derived from *dl*310 and contains a unique *Xba*I cleavage site at 93.5 mu (M. Leza and P. Hearing, unpublished data). Viral plaques were isolated after transfection of either 293 cells (11) or W162 cells, following established procedures (12, 27, 33). 293 cells were efficiently transfected and were used to isolate viable E4 mutant viruses. W162 cells are a derivative of the established Vero cell line that express E4; the W162 line efficiently complements defective E4 mutant viruses (32). Correct viral reconstruction was verified by extensive restriction endonuclease mapping and by comparison with the sequences of the parental mutant plasmid DNAs. Purified viral particles were prepared and quantitated for each mutant virus as previously described (12). The following mutations were reconstructed into a *wt*300 background: E4*dl*ORF1-3, E4*dl*ORF1-4, and E4*in*ORF6,6/7. The following mutations were reconstructed into a *dl*310 background: E4*in*ORF3, E4*in*ORF6, *dl*355*, E4*in*ORF3/*dl*355*, E4*in* ORF3/*in*ORF6, *dl*366*, *dl*366*+ORF1-2, *dl*366*+ORF3, and *dl*366*+ORF4.

The HeLa cell line was obtained from the American Type Culture Collection and was maintained in Dulbecco modified minimal essential medium (DME) containing 10% calf serum. The W162 cell line was kindly provided by Gary Ketner and was maintained in DME containing 10% calf serum. The 293 cell line was maintained in DME containing 10% calf serum. All virus infections were performed at a multiplicity of 200 particles per cell, unless otherwise noted, at 37°C for 1 h. Viral single-step growth curves were performed as follows. After infection, the cells were washed and fresh medium was added. Infected cells were collected in the medium at various times after infection and were lysed by repeated freeze-thawing; the virus titer in the culture supernatant was determined by plaque assay. The infectious virus yield per cell for the wild-type virus was approximately 1,000 when titers were determined in 293 cells, and the yield was 10 when titers were determined in W162 cells. Reduced plaquing efficiency in W162 cells was previously noted (12).

Analysis of DNA replication, polypeptide synthesis, and AAV helper function. For analysis of DNA accumulation, HeLa cells were infected with the wild-type or mutant viruses, and total cellular and viral DNA (31) was isolated at various times after infection. The DNA was denatured in 0.3 N NaOH at 65°C, neutralized using 2 M ammonium acetate (pH 7.0), diluted as indicated in the legend to Fig. 4, and applied to nitrocellulose by using a slot blot apparatus (31). The blots were then probed by Southern hybridization analysis (26), using Ad5 DNA ^{32}P-labeled by random priming (9). The results were visualized by autoradiography, and quantitated by excising the individual bands and direct scintillation analysis.

Immunoprecipitation of E4 polypeptides by using an ORF6 peptide-specific antiserum (R3 serum; 6) and a tumor antiserum that recognizes the ORF3 product (C1; 7) was performed as described previously (6). Briefly, infected cultures were radiolabeled with 200 μCi of [^{35}S]methionine per ml in DME lacking methionine from 12 to 16 h after infection. Labeled cells were washed twice with phosphate-buffered saline, and cellular extracts were prepared by lysis in RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 160 mM NaCl, 10 mM Tris [pH 7.4], 5 mM EDTA) and sonication. Immunoprecipitation experiments were performed at 0°C, following a modified version (6) of the procedure described by Brugge and Erikson (5). Immune complexes were released from the *Staphylococcus aureus* pellet by a 10-min incubation in sample buffer (10% glycerol, 3% SDS, 5% 2-mercaptoethanol, 50 mM Tris [pH 6.8]) at 0°C. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Sarnow et al. (24). Analyses of proteins in total cellular lysates were performed as described above, except that cultures were radiolabeled with 25 μCi of [^{35}S]methionine per ml from 24 to 25 h after infection. After lysis of the cells in RIPA buffer, the extracts were diluted into sample buffer and analyzed by SDS-PAGE.

AAV helper function was assayed after DNA transfection by using a recombinant plasmid containing the AAV genome, pSM620 (21; a kind gift of S. McLaughlin and N. Muzyczka), or after virus infection by using intact AAV virus (a kind gift of S. McLaughlin and N. Muzyczka). For AAV plasmid rescue analysis, subconfluent monolayers of 293 cells were transfected with a mixture of plasmid pSM620 (1 μg/100-mm-diameter dish) and E4 mutant virus particles (200 particles per cell) by the calcium phosphate precipitation procedure (34). Salmon sperm DNA (19 μg/100-mm-diameter dish) was added as a carrier. After incubation overnight with the mixture, the cells were washed and fresh medium was added. Low-molecular-weight DNA was isolated by the procedure of Hirt (15) 40 h later. For AAV virus

rescue analysis, monolayers of HeLa cells were coinfected with a mixture of AAV virus (10 focus-forming units per cell) and E4 mutant viruses (200 particles per cell) for 1 h at 37°C. Then, the cells were washed and fresh medium was added. Low-molecular-weight DNA (15) was isolated at 40 h after infection. AAV replicative forms were analyzed by Southern hybridization (26), using AAV DNA (pSM620) ^{32}P-labeled by random priming (9).

RESULTS

Construction and propagation of E4 mutants. Previous analyses suggested that multiple gene products encoded by E4 may share redundant properties (12). To test this idea, we constructed a series of E4 mutant viruses that express individual E4 ORFs or combinations of ORFs (Fig. 1). Defective viruses were propagated in W162 cells, a Vero-derived cell line transformed with E4 that complements E4 mutant viruses (32). Mutant viruses E4*dl*ORF1-3 and E4*dl*ORF1-4 contain deletions spanning ORF1 through ORF3 and ORF1 through ORF4, respectively. As shown below, these viruses express the products of ORF6 and ORF6/7. Mutant virus *dl*355 has been described previously and contains a 14-bp deletion at 93.5 mu that disrupts ORF6 but not ORF6/7 (12). Mutant virus E4*in*ORF6,6/7 contains a 2-bp insertion at 94.6 mu that results in a frameshift mutation in the coding region common to ORF6 and ORF6/7. Mutant virus E4*in*ORF6 contains an 8-bp insertion at 93.0 mu that results in a frameshift mutation in ORF6 but does not affect the ORF6/7 products (see below). Mutant virus E4*in*ORF3 contains an 8-bp insertion at 96.4 mu that results in a frameshift mutation in ORF3. Mutant virus *dl*366 has been described previously and contains a deletion that disrupts all of the E4 ORFs (12). The *dl*355 and *dl*366 mutations were originally constructed into viruses that lack E3 (E3$^-$; 12). These mutations were reconstructed into viruses with a wild-type E3 background; these viruses are designated *dl*355* and *dl*366*. For the reasons described below, two viruses with double mutations were constructed that combine lesions introduced into ORF3 and ORF6 (E4*in*ORF3/*dl*355* and E4*in*ORF3/*in*ORF6). Three mutant viruses were constructed that insert specific ORFs into a *dl*366 background. Mutant virus *dl*366*+ORF1-2 contains ORF1 and ORF2, mutant virus *dl*366*+ORF3 contains ORF3, and mutant virus *dl*366*+ORF4 contains ORF4. Each of these mutant viruses contains the donor and acceptor splice signals necessary for splicing of the presumptive mRNAs that encode the ORF1, ORF2, ORF3, and ORF4 products (30; Fig. 1).

We used a peptide-specific antiserum directed against the N-terminal region shared by ORF6 and ORF6/7 (serum R3; 6) to analyze expression of these products in cells infected with the wild-type and mutant viruses. HeLa cells were infected with Ad5 *wt*300 or E4 mutant viruses, radiolabeled at 12 h after infection with [^{35}S]methionine, and the ORF6 and ORF6/7 products were immunoprecipitated from cellular lysates by using the R3 serum. The products were analyzed by SDS-PAGE (Fig. 2).

As previously described (6), the ORF6 34K protein and the ORF6/7 19.5K protein were specifically immunoprecipitated with the R3 serum from *wt*300-infected cellular extracts (Fig. 2, compare lanes *wt*300 + R3 with *wt*300 + preimmune). The authenticity of these products were previously confirmed (6). The R3 serum does not coimmunoprecipitate the E1B 55K protein in a complex with the E4 ORF6 34K protein; this antiserum apparently only recognizes the uncomplexed form of the ORF6 34K product (6). Mutant viruses *dl*ORF1-3 and *dl*ORF1-4 contain intact ORF6 and ORF6/7; both the ORF6 34K protein and the ORF6/7 19.5K protein were synthesized in cells infected with these viruses. As previously reported, *dl*355* did not express the ORF6 34K product but efficiently produced the ORF6/7 19.5K protein (6). Mutant virus *in*ORF6,6/7 contains a 2-bp insertion in the N-terminal region common to ORF6 and ORF6/7. As seen in Fig. 2, this mutation disrupted expression of both E4 gene products. Mutant virus *in*ORF6 contains a frameshift mutation in the region unique to ORF6. As expected, this mutation disrupted expression of the ORF6 34K protein but not that of the ORF6/7 19.5K protein. Mutant virus *in*ORF3 synthesized both the ORF6 and ORF6/7 products, as predicted. Finally, the viruses containing double mutations in ORF3 and ORF6, *in*ORF3/*dl*355* and *in*ORF3/*in*ORF6, would be expected to synthesize the ORF6/7 19.5K protein but not the ORF6 34K protein. As seen in Fig. 2, these results were confirmed. These two viruses likely expressed reduced levels of the ORF6/7 product because of reduced viral DNA replication (see below).

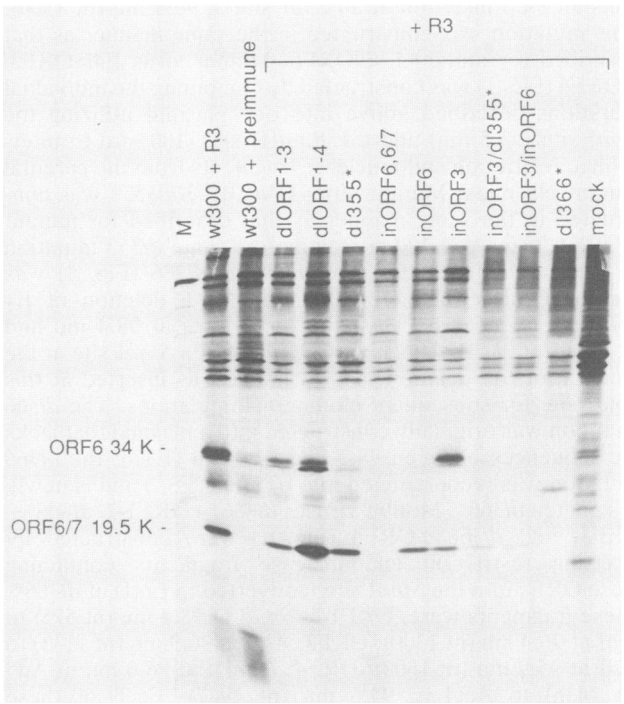

FIG. 2. Analysis of E4 ORF6 and ORF6/7 expression. HeLa cells were infected with wild-type Ad5 (*wt*300) or mutant viruses (indicated at the top) at 5 PFU per cell. The cells were labeled with 200 μCi of [^{35}S]methionine per ml from 12 to 16 h after infection. Cellular extracts were prepared, and proteins were immunoprecipitated by using the ORF6,6/7-specific antiserum (R3) or preimmune serum. The products of immunoprecipitation were analyzed on an SDS-20% polyacrylamide gel. Lane M corresponds to labeled virion markers prepared from purified Ad5 virions. Mock represents an immunoprecipitation experiment using an extract from uninfected HeLa cells. The positions of the ORF6 34K protein and the ORF6/7 19.5K protein are indicated at the left.

We also tested expression of the E4 ORF3 11K product with the mutant viruses by immunoprecipitation with a tumor antiserum (C1; 7) that recognizes this protein. Each of the mutant viruses that carries an intact ORF3 (*dl*355*, *in*ORF6,6/7, *in*ORF6, and *dl*366*+ORF3) expressed the 11K protein to a level that was comparable to that of *wt*300, while

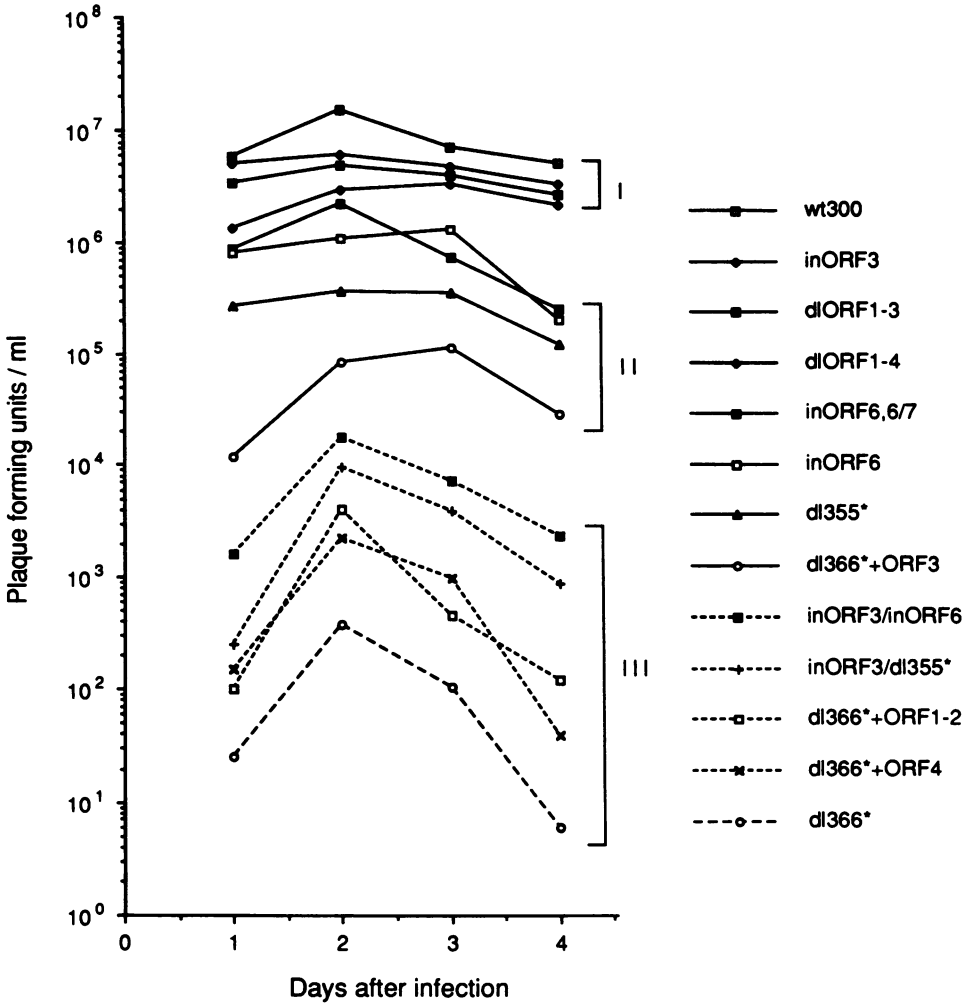

FIG. 3. Growth kinetics in HeLa cells. HeLa cells were infected with the wild-type or mutant viruses at a multiplicity of 200 particles per cell. Virus yields were measured at the times indicated by plaque assay on W162 cells. The symbols that represent the individual viruses are indicated adjacent to the growth curves. The results represent the averages of three independent experiments. The data from the day 0 time point are not presented to simplify the plot; these values averaged about 10^1 for the different mutant viruses. The three classes of mutants that are referred to in the text (I, II, and III) are indicated.

each of the viruses with mutations in ORF3 (*dl*ORF1-3, *dl*ORF1-4, *in*ORF3, *in*ORF3/*dl*355*, *in*ORF3/*in*ORF6, *dl*366*, *dl*366*+ORF1-2, and *dl*366*+ORF4) did not express the 11K product (data not shown). No other antisera are currently available that may be used to immunoprecipitate E4 products encoded by ORF1, -2 and -4.

ORF3 and ORF6 gene products are redundant. The growth kinetics of the individual E4 mutant viruses were analyzed in HeLa cells by a single-step growth curve (Fig. 3). As previously described, a mutant virus that lacks E4 coding sequences (*dl*366*) was extremely defective for growth (reduced 10^5 to 10^6 in final virus yield). Mutant viruses with disruptions in ORF3, ORF1 through ORF3, and ORF1 through ORF4 (*in*ORF3, *dl*ORF1-3, and *dl*ORF1-4) grew with comparable efficiencies to that of the wild-type virus, *wt*300. These results confirm previous analyses (12, 13) and demonstrate that viruses that express only the ORF6 and ORF6/7 products are completely viable in HeLa cells. Viruses with frameshift mutations only in ORF6 or in both ORF6 and ORF6/7 (*dl*355*, *in*ORF6, and *in*ORF6,6/7) were reduced 5- to 10-fold in final virus yield compared with the wild-type virus. These results demonstrate that the ORF6/7 product(s) does not contribute functionally to the intermediate viability observed with the ORF6 mutant, *dl*355*. Interestingly, virus *dl*366*+ORF3, which expressed the ORF3 11K protein, was only partially defective for growth in HeLa cells; this virus was reduced only 100-fold in yield compared with that of the wild-type virus. This result suggested that the ORF3 gene product could compensate for the loss of the ORF6 product and that the moderately defective phenotype observed with viruses containing mutations in ORF6 may reflect ORF3 expression and function.

To test this point, we constructed and analyzed the viability of viruses that contain double mutations in ORF3 and ORF6 (*in*ORF3/*dl*355* and *in*ORF3/*in*ORF6). The results in Fig. 3 show that the yields of both of these mutant viruses were reduced at least 100-fold greater than that of the ORF6$^-$ parental viruses. We concluded from these results that the ORF3 and ORF6 products are functionally compensatory. It is clear from the results in Fig. 3, however, that neither of the viruses that contain mutations in both ORF3 and ORF6 were as defective as *dl*366*. This suggests that

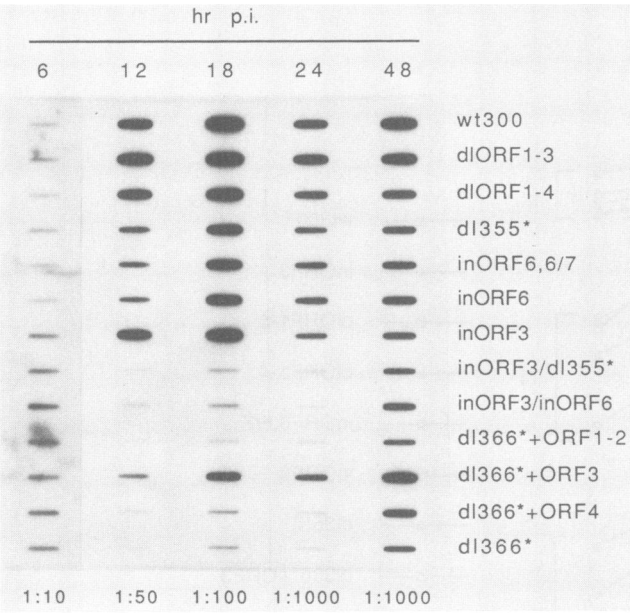

FIG. 4. Viral DNA accumulation in HeLa cells. HeLa cells were infected with the wild-type or mutant viruses at a multiplicity of 200 particles per cell. Total cellular and viral DNA was isolated at the times indicated, denatured, neutralized, diluted as indicated (1:10, 1:50, 1:100, and 1:1,000), and applied to nitrocellulose using a slot blot apparatus. The blots were hybridized with a ^{32}P-labeled adenovirus genomic probe. Autoradiography was for 10 h for the 6-h time point and for 6 h for the other time points.

other E4 products provide additional functional properties. This may represent expression of products from ORF1, -2, and -4, since viruses that were constructed to express these proteins (*dl*366*+ORF1-2 and *dl*366*+ORF4) also grew better than *dl*366*, although these viruses were considerably reduced in final yield compared with that of *wt*300.

We have grouped these mutant viruses into three classes (I, II, and III; Fig. 3). Mutant viruses in class I expressed the product of ORF6 and exhibited comparable viability to that of the wild-type virus. The results with these viruses demonstrated that the ORF6 product provided full E4 function in the absence of the other E4 gene products. Mutant viruses in class II lacked the ORF6 product but expressed the product of ORF3. These mutant viruses were only moderately defective for growth; the ORF3 gene product appears to accout for most of the E4 function observed with the ORF6$^-$ mutant viruses. In addition, the products from ORF1, -2, and -4 may have provided additional, albeit very minor, E4 function. The ORF6/7 product(s) did not appear to contribute to E4 function. Mutant viruses in class III lacked both the ORF3 and ORF6 gene products and were severely defective for growth. These classifications are based on the results of single-step growth curves presented above as well as the further characterization of these viruses described below.

Viral DNA replication was assayed at various times after infection of HeLa cells with the wild-type or mutant viruses. Total cellular and viral DNA was isolated at 6, 12, 18, 24, and 48 h after infection, and viral DNA accumulation was measured by slot blot analysis, using a ^{32}P-labeled adenovirus genomic probe (Fig. 4). The levels of viral DNA at 6 h after infection, which is before the onset of viral DNA replication, reflect the input viral genomes. These levels were within twofold of each other in infections with the different viruses; identical results were obtained at 4 and 8 h after infection (data not shown). The levels of viral DNA accumulation observed at later times after infection with the mutant viruses were consistent with the results obtained in the single-step growth curves (Fig. 3). Viruses with mutations that disrupt ORF3, ORF1 through ORF3, and ORF1 through ORF4 (*in*ORF3, *dl*ORF1-3, and *dl*ORF1-4) accumulated viral DNA at levels that were comparable to that observed with the wild-type virus, *wt*300. A lag in the onset of viral DNA replication with viruses that carry mutations in ORF6 (*dl*355*, *in*ORF6,6/7, and *in*ORF6) was detected, as previously described for *dl*355 (12); this lag is most easily observed at the 12-h time point. The level of viral DNA replication with these mutant viruses was reduced three- to fourfold at 12 h after infection; the levels of viral DNA were similar to that of the wild-type virus at later times. The virus that expresses only ORF3 (*dl*366*+ORF3) accumulated viral DNA with similar kinetics to those of the viruses with mutations in ORF6; this result is consistent with the result of a growth curve using this virus (Fig. 3). Viruses carrying double mutations that disrupt ORF3 and ORF6 (*in*ORF3/*dl*355* and *in*ORF3/*in*ORF6) were as defective as *dl*366* in DNA accumulation. Similar results were obtained with mutant viruses *dl*366*+ORF1-2 and *dl*366*+ORF4. All of these defective viruses, however, accumulated nearly wild-type levels of viral DNA by 48 h after infection, demonstrating that region E4 is not absolutely required for viral DNA replication but rather plays an auxilliary role in this process. These results support the conclusion that the ORF3 and ORF6 products have redundant effects in the regulation of viral DNA replication.

We next analyzed viral late protein synthesis and the shutoff of host cell protein synthesis in infected cells. HeLa cells were infected with the wild-type or mutant viruses and radiolabeled with [^{35}S]methionine at 24 h after infection. Total cellular lysates were prepared, and the proteins were analyzed by SDS-PAGE (Fig. 5A). Mutant viruses with disruptions of ORF3, ORF1 through ORF3, and ORF1 through ORF4 (*in*ORF3, *dl*ORF1-3, and *dl*ORF1-4) synthesized viral late proteins at levels that were comparable to that of the wild-type virus, *wt*300. Viruses with mutations in ORF6 (*dl*355*, *in*ORF6,6/7, and *in*ORF6) were moderately reduced in the synthesis of viral late proteins, as previously described for *dl*355 (12). These viruses also displayed a reduced efficiency in the shutoff of host cell protein synthesis; this effect is most easily observed with the cellular actin band which was reduced considerably in cells infected with *wt*300 compared with that in uninfected cells. Virus *dl*366*+ORF3, which expresses only ORF3, clearly synthesized viral late proteins but at a level that was somewhat reduced compared with those of the ORF6 mutant viruses. In addition, this mutant virus synthesized very reduced levels of the fiber polypeptide (IV; Fig. 5A and B and 7B). Reduced fiber protein synthesis is a characteristic of mutant viruses that contain large deletions in E4 even with infection of the complementing cell line, W162 (12, 33); this decrease may reflect a *cis* effect on the processing of fiber mRNA. Infection of HeLa cells with virus *dl*366*+ORF3 did not reduce the level of host protein synthesis. The viruses with double mutations in ORF3 and ORF6, *in*ORF3/*dl*355* and *in*ORF3/*in*ORF6, were as defective as *dl*366* for viral late protein synthesis and the shutoff of host cell protein synthesis. The same results were obtained with the other mutant viruses, *dl*366*+ORF1-2 and *dl*366*+ORF4. Identical results were obtained with all of the viruses when protein synthesis was examined at 48 h after infection (data not shown).

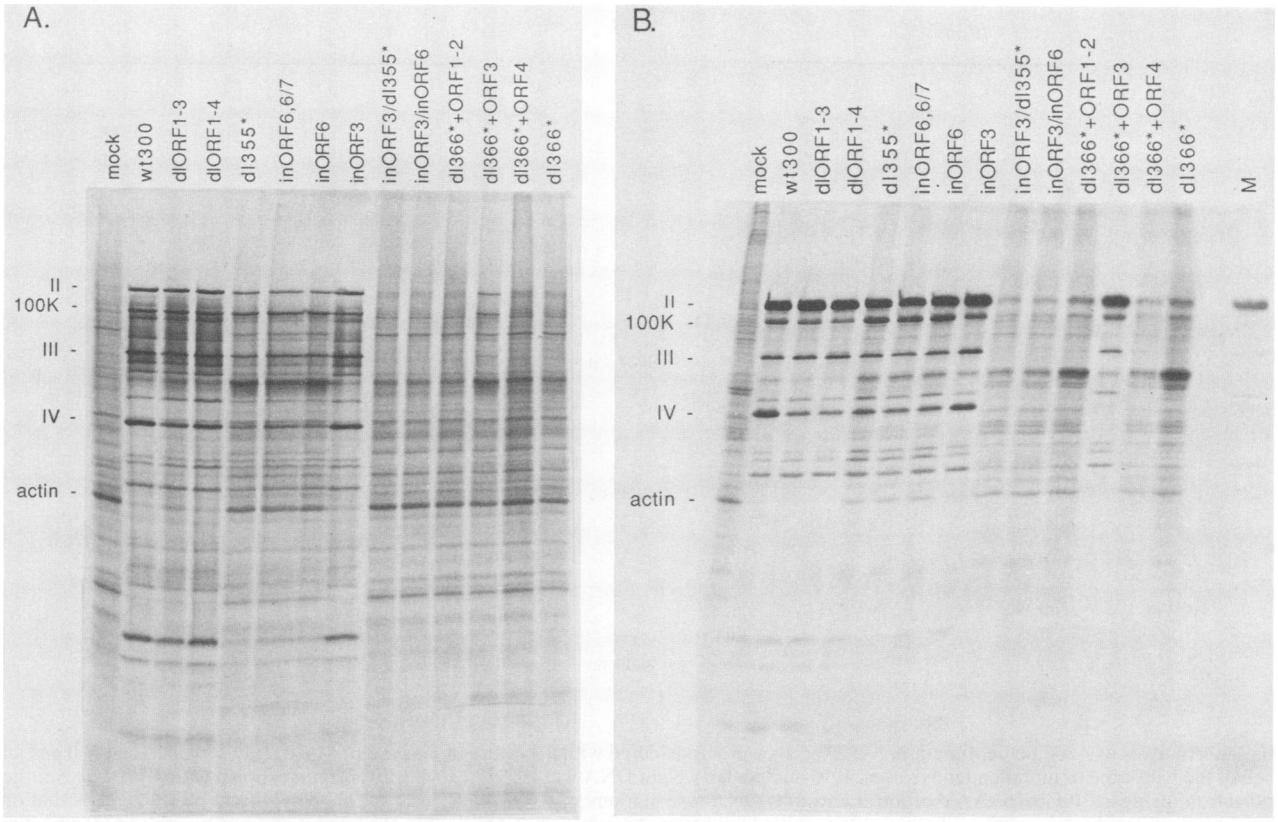

FIG. 5. Viral late protein synthesis in HeLa and 293 cells. HeLa (A) and 293 (B) cells were infected with the wild-type or mutant viruses at a multiplicity of 200 particles per cell. Cultures were labeled with 25 μCi of [^{35}S]methionine per ml for 1 h at 24 h after infection. Cellular extracts were prepared and analyzed on an SDS-12.5% polyacrylamide gel. Bands corresponding to cellular actin and several viral polypeptides are indicated. The viral proteins are: II, hexon; 100K; III, penton; and IV, fiber. Lane M corresponds to labeled Ad5 virion proteins. Mock represents uninfected HeLa and 293 cells.

E4 mutant viruses have been shown to overexpress the E2 72K DNA-binding protein (12). We found in our analyses, however, that overproduction of the 72K protein was variable from experiment to experiment and between the different mutant viruses. Therefore, we have not drawn any conclusions with these viruses concerning overproduction of this protein.

We also examined viral late protein synthesis and the shutoff of host cell protein synthesis with the mutant viruses in infected 293 cells. The 293 cell line was used, since these cells are more permissive for the growth of E4 mutant viruses than the HeLa cell line (12). We reasoned that mutant viruses that were severely defective for growth in HeLa cells may be only partially defective in 293 cells, and therefore the phenotype of these viruses would be more readily detectable. 293 cells were infected with the wild-type or mutant viruses and were radiolabeled with [^{35}S]methionine at 24 h after infection. Proteins present in total cellular extracts were analyzed by SDS-PAGE (Fig. 5B). Viruses containing mutations in ORF6 (*dl*355*, *in*ORF6,6/7, and *in*ORF6) synthesized levels of viral late proteins that were comparable to that of the wild-type virus, but they were partly defective for the shutoff of host cell protein synthesis. Virus *dl*366*+ORF3 synthesized viral late proteins (except fiber, see above) and shut off host cell protein synthesis to a level that was similar to *wt*300. As was observed in infected HeLa cells, viruses with mutations in ORF3 and ORF6, *in*ORF3/*dl*355* and *in*ORF3/*in*ORF6, were as defective as *dl*366* for viral late protein synthesis and the shutoff of host cell protein synthesis. Comparable results were obtained with mutant viruses *dl*366*+ORF1-2 and *dl*366*+ORF4. We conclude from these analyses that the ORF3 and ORF6 products independently regulate viral late protein synthesis as well as the shutoff of host cell protein synthesis.

ORF6 gene product is responsible for AAV helper function. Expression of E4 augments rescue of AAV (20, reviewed in reference 3). Adenovirus helper function for AAV has been assayed transiently, using a plasmid that carries the AAV genome, as well as in AAV virus-infected cells (3). E4 helper function is required for efficient AAV DNA replication, cytoplasmic accumulation of AAV mRNA, and AAV capsid protein synthesis (22). We analyzed the role of the E4 gene products in AAV helper function. In a transient assay, 293 cells were transfected with pSM620, a plasmid that carries an intact AAV genome (20), and they were infected with the wild-type or mutant adenoviruses. In a virus assay, HeLa cells were coinfected with AAV and the wild-type or mutant adenoviruses. AAV rescue was measured by analyzing the AAV replicative forms present in low-molecular-weight DNA (15) by Southern hybridization (26). The results of plasmid rescue experiments are shown in Fig. 6A, and the results of virus rescue experiments are shown in Fig. 6B.

Double-stranded monomer-length and dimer-length replicative forms of AAV were observed in these analyses when

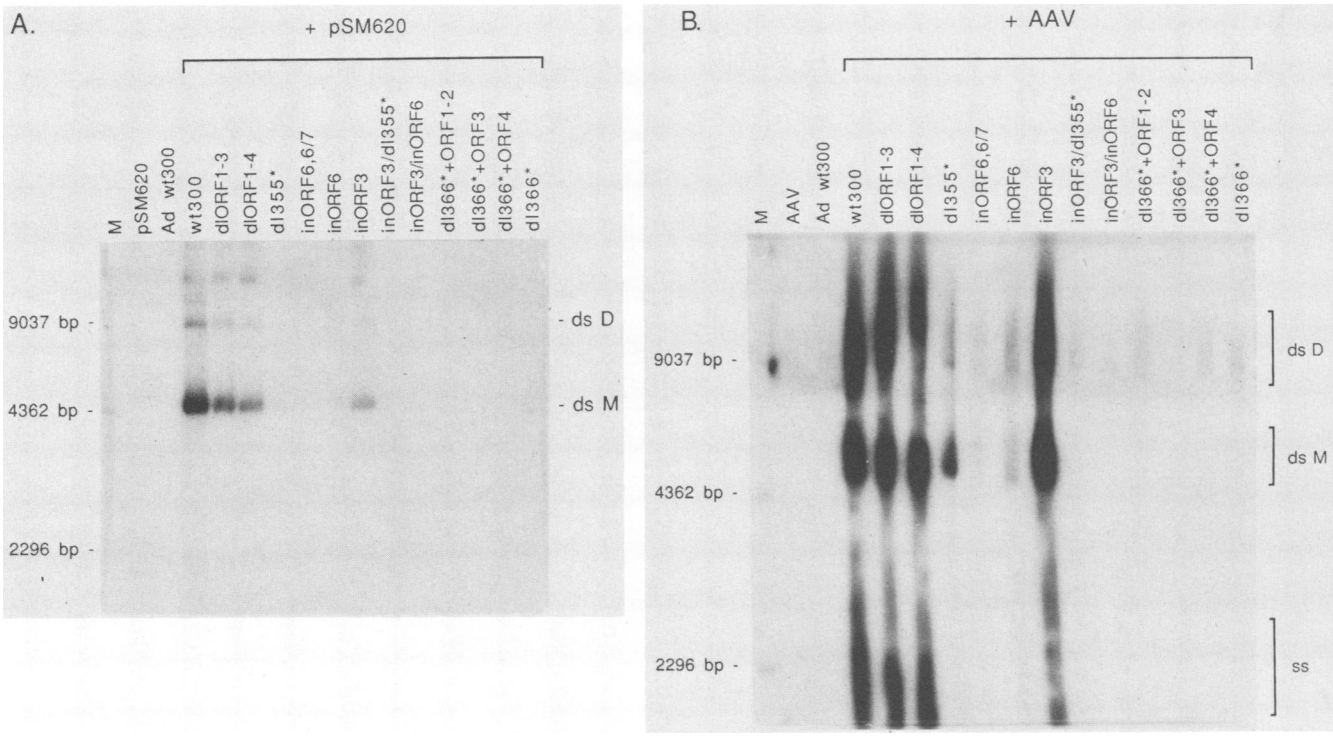

FIG. 6. Analysis of AAV helper function. (A) 293 cells were transfected with a mixture of plasmid pSM620 (1 μg per dish) and 200 particles per cell of the wild-type or mutant adenoviruses. Low-molecular-weight DNA was isolated at 40 h after transfection and analyzed by Southern hybridization, using a ^{32}P-labeled AAV probe. Lanes: M, DNA size markers of 9,037, 4,362, and 2,296 bp in length; pSM620, cells that only received plasmid DNA; Ad *wt*300, cells that only received wild-type Ad5 virions. AAV double-stranded monomer length (ds M) and dimer length (ds D) replicative forms are indicated adjacent to the autoradiogram. (B) HeLa cells were coinfected with AAV (10 focus-forming units per cell) and the wild-type or mutant adenoviruses (200 particles per cell). AAV replication was analyzed as described for panel A. Lanes: AAV, cells that only received AAV; Ad *wt*300, cells that only received wild-type Ad5 virions. AAV single-stranded DNA (ss) was observed in this experiment and is indicated at the right.

AAV-transfected or AAV-infected cells were infected with wild-type adenovirus (*wt*300 plus pSM620, Fig. 6A; *wt*300 plus AAV, Fig. 6B). Single-stranded AAV genomes were also detected in cells coinfected with AAV and *wt*300 in which AAV replication was very efficient (Fig. 6B). The results of both analyses demonstrated that only the ORF6 product was involved in AAV rescue, both in a transient assay measuring AAV excision and DNA replication and in virus infection measuring AAV DNA replication. Mutant viruses *in*ORF3, *dl*ORF1-3, and *dl*ORF1-4 were as efficient as the wild-type virus for AAV rescue. In contrast, all of the other mutant viruses were severely reduced or defective for AAV rescue function. This includes virus *dl*366*+ORF3, which was only moderately defective in each of the other assays described above. The transient assay experiments were performed in 293 cells in which this virus was as efficient as the wild-type virus for viral late protein synthesis and the shutoff of host cell protein synthesis (Fig. 5B). We conclude from these analyses that the ORF6 gene product is solely responsible for E4 AAV helper function; this assay distinguishes a unique property of the ORF6 gene product.

Multiplicity leakiness of defective E4 mutant viruses. Analysis of replication with the mutant viruses at 48 h after infection demonstrated that E4 expression was not absolutely required for viral DNA replication; E4 mutant viruses defective for E4 function in a single-step growth curve (Fig. 3) accumulated nearly wild-type levels of viral DNA by 48 h after infection (Fig. 4). This result suggested that E4 may have a catalytic function in the regulation of viral DNA replication and perhaps viral late protein synthesis. By analogy to the function of the E1A gene products (25), we tested whether mutant viruses defective for E4 function displayed a multiplicity-leakiness phenotype. We analyzed this possibilty in HeLa cells in two ways. First, virus yield in a single-step growth curve was analyzed by using cells infected with *dl*366* at low (200 particles per cell) and high (2,000 particles per cell) multiplicities of infection (Fig. 7A). Second, we analyzed viral late protein synthesis at 24 h after infection in cells infected at low and high multiplicities of infection with several of the mutant viruses (Fig. 7B).

The results from a single-step growth curve demonstrated that *dl*366* displayed a multiplicity-leakiness phenotype; infection at a high multiplicity of infection resulted in a 10^2- to 10^3-fold increase in virus yield compared with that of cells infected at a low multiplicity of infection. Similar results were observed when viral late protein synthesis was examined. Cells infected with defective viruses *dl*366* and *in*ORF3/*dl*355* displayed considerably increased levels of viral late proteins compared with those of cells infected with these viruses at a low multiplicity of infection. Partial shutoff of host cell protein synthesis was observed with these mutant viruses at a high multiplicity of infection. We conclude from these analyses that the E4 gene products are not absolutely required for viral DNA replication and late gene expression but rather that they play a facilitating role within viral infection in these processes.

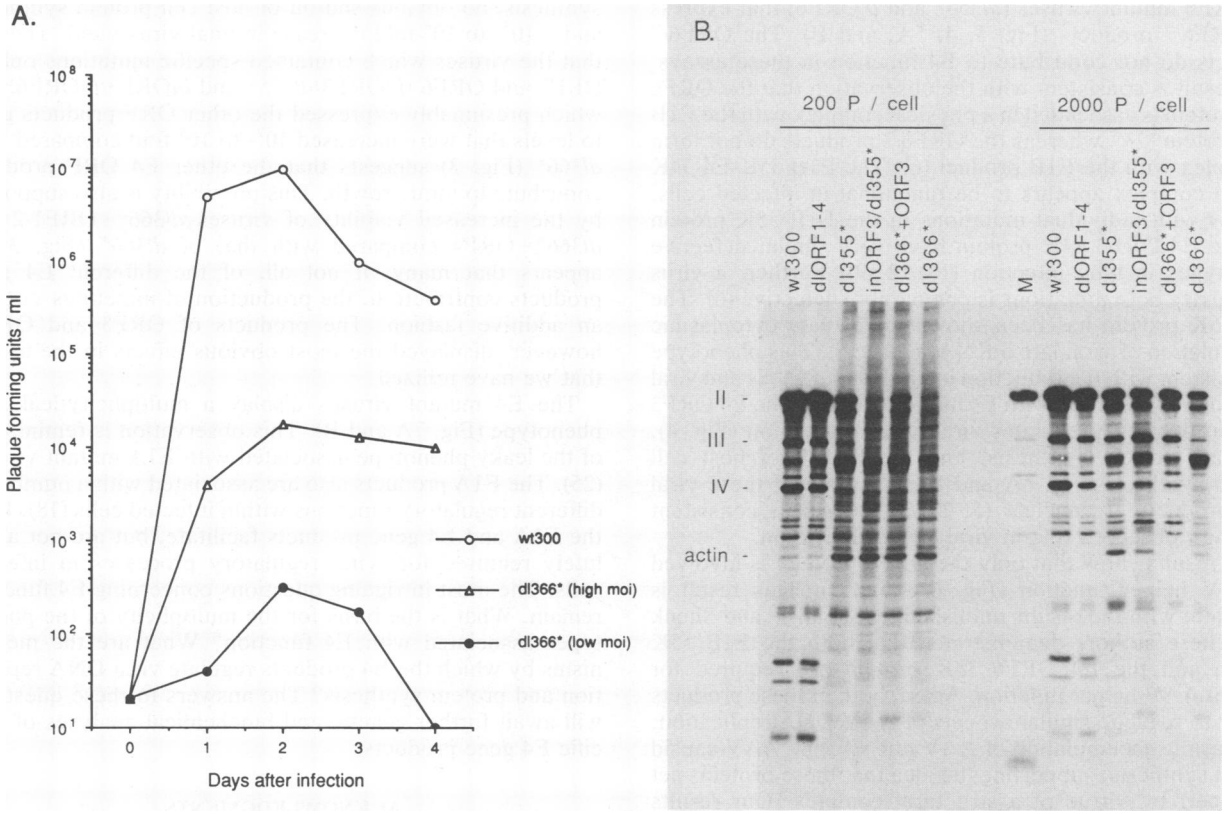

FIG. 7. Multiplicity leakiness of E4 mutants. (A) HeLa cells were infected with *wt*300 (200 particles per cell) or mutant virus *dl*366* at low (200 particles per cell) or high (2,000 particles per cell) multiplicities of infection (moi). Virus yields were measured at the times indicated by plaque assay on W162 cells. (B) HeLa cells were infected with wild-type or mutant viruses at a multiplicity of 200 or 2,000 particles per cell, as indicated at the top. Cultures were labeled with 25 μCi of [^{35}S]methionine per ml for 1 h at 24 h after infection. Cellular extracts were prepared and analyzed on an SDS-12.5% polyacrylamide gel. Lane M corresponds to labeled Ad5 virion proteins. Bands corresponding to cellular actin and several viral polypeptides (defined in the legend to Fig. 5) are indicated.

DISCUSSION

The results of these analyses demonstrate that the gene products of E4 ORF3 and ORF6 similarly affect a number of events in viral lytic infection. These include the regulation of the onset of viral DNA replication (Fig. 4), viral late protein synthesis (Fig. 5A and B), the shutoff of host cell protein synthesis (Fig. 5A and B), and the production of infectious virus (Fig. 3). The latter property likely reflects one or more of the other aspects of regulation. The ORF6 product appears to be more efficient than the ORF3 product in the regulation of these processes. The observed redundancy of the ORF3 and ORF6 products in lytic infection likely reflects one of three alternatives. First, these two proteins may share functional properties in lytic infection, e.g., a common enzymatic activity. Second, these proteins may independently or coordinately be involved in a common pathway that augments virus production, e.g., viral DNA replication. Finally, the ORF3 and ORF6 products may affect independent processes in infected cells that each contribute to virus yield. Our current analyses do not distinguish among these possibilities.

Mutant viruses that are defective for E4 function (e.g., *dl*366) synthesize early viral mRNAs at levels that are comparable to that of the wild-type virus (12). The earliest defect that is observed with E4 mutant viruses is a lag in the onset of viral DNA replication (12; Fig. 4). However, the DNA synthesis defect may not fully account for the other aspects of the complicated phenotype associated with E4 function. By 48 h after infection of HeLa cells, all of the E4 mutant viruses accumulated levels of viral DNA that were comparable to that detected with the wild-type virus (Fig. 4), yet viral late protein synthesis at this time was still dramatically reduced with these viruses (data not shown) and was identical to the pattern observed at 24 h after infection (Fig. 5A). This observation is consistent with a similar result reported by Weinberg and Ketner with another mutant virus defective for E4 function, *dl*808 (33). Therefore, the DNA synthesis defect appears to be a separable phenotype from the defect in viral late protein synthesis. It is possible that an alteration in the normal program of viral DNA replication leads to a diverse set of secondary alterations during infection. Alternatively, the E4 gene products may have multiple functions within infected cells.

Viruses that are defective for ORF6 function (e.g., *dl*355) display the same range of phenotypes as mutant viruses that entirely lack E4 coding sequences (e.g., *dl*366), but the defects observed with ORF6 mutant viruses are only moderate (12; these results). It was previously suggested that the ORF6/7 fusion products may share functional properties with the ORF6 34K protein by virtue of the overlapping coding sequences (6, 12). The results obtained with mutant virus *in*ORF6,6/7 argue against this possibility. This virus is defective for expression of the ORF6 and ORF6/7 products (Fig. 2), yet it was not found to be any more defective than

the ORF6 mutant viruses (*dl*355* and *in*ORF6) that express the ORF6/7 products (Fig. 3, 4, 5A, and B). The ORF6/7 products do not contribute to E4 function in these assays. This result is consistent with the observation that the ORF6 34K protein is associated in a physical complex with the E1B 55K protein (23), whereas the ORF6/7 products do not form a complex with the E1B product (6). The E1B 55K-E4 34K protein complex appears to be functional in infected cells. Viruses with individual mutations in the E1B 55K protein and the E4 ORF6 34K protein have very similar defective phenotypes in lytic infection (1,2,12,19). Further, a virus that carries both mutations is comparably defective (6). The E1B 55K protein has been shown to regulate cytoplasmic accumulation of viral late mRNAs (2,19,35). This phenotype is consistent with the reduction in viral late mRNAs and viral late proteins observed with E4 mutant viruses. The E4 ORF3 gene product also regulates viral DNA replication (Fig. 4), viral late protein synthesis, and the shutoff of host cell protein synthesis (Fig. 5A and B). All three of these viral gene products are nuclear (5, 23, 24), which is consistent with their observed role in viral DNA replication.

Our results show that only the ORF6 product is involved in AAV helper function (Fig. 6A and B). This result is consistent with the results published by Samulski and Shenk (22). These authors demonstrated that both the E1B 55K protein and the E4 ORF6 34K protein are required for efficient AAV helper function. Again, both of these products appear to regulate similar aspects of AAV DNA replication, cytoplasmic accumulation of AAV mRNA, and AAV capsid protein synthesis, supporting the idea that these proteins act in concert by virtue of a structural complex. Our results demonstrate that the E4 ORF3 product is not involved in this process. AAV helper function is the only phenotype that we have detected to date that functionally distinguishes the E4 ORF3 and ORF6 gene products. This result is surprising, because AAV appears to utilize adenovirus products to perform the same functions that these products perform in adenovirus infection. The AAV transient replication assay was performed in 293 cells in which the E4 ORF3 product efficiently compensated for the loss of other E4 products for adenovirus viral late protein expression (Fig. 5B). Therefore, we feel confident that the ORF3 product is not involved in AAV helper function.

We have grouped the E4 mutant viruses into three classes (I, II, and III; Fig. 3) on the basis of analyses of viral DNA replication, viral late protein synthesis, the shutoff of host cell protein synthesis, and the production of infectious virus. Viruses in class I (*dl*ORF1-3, *dl*ORF1-4, and *in*ORF3) behaved like the wild-type virus in all of the assays employed. These viruses expressed the ORF6 gene product. Viruses in class II (*dl*355*, *in*ORF6, *in*ORF6,6/7, and *dl*366*+ORF3) lacked the ORF6 product and expressed the ORF3 product. These viruses displayed a lag in the onset of viral DNA replication, reduced levels of viral late protein synthesis, inefficient shutoff of host cell protein synthesis, and a 10- to 20-fold decrease in final virus yield. The fact that these viruses accumulated levels of viral DNA at 24 h after infection that were comparable to that of the wild-type virus (Fig. 4) yet displayed a reduction in viral late protein synthesis and the shutoff of host protein synthesis at this time point (Fig. 5A) again suggests that these phenotypes are separable. Mutant viruses in class III (*in*ORF3/*dl*355*, *in*ORF3/*in*ORF6, *dl*366*+ORF1-2, *dl*366*+ORF4, and *dl*366*) contained mutations in ORF3 and ORF6. These viruses displayed a significant lag in the onset of viral DNA replication, dramatically reduced levels of viral late protein synthesis, no obvious shutoff of host cell protein synthesis, and a 10^3- to 10^5-fold decrease in final virus yield. The fact that the viruses which contained specific mutations only in ORF3 and ORF6 (*in*ORF3/*dl*355* and *in*ORF3/*in*ORF6) and which presumably expressed the other ORF products grew to levels that were increased 10^2- to 10^3-fold compared with *dl*366* (Fig. 3) suggests that the other E4 ORF products contribute to viral growth. This possibility is also supported by the increased viability of viruses *dl*366*+ORF1-2 and *dl*366*+ORF4 compared with that of *dl*366* (Fig. 3). It appears that many, if not all, of the different E4 gene products contribute to the production of infectious virus in an additive fashion. The products of ORF3 and ORF6, however, displayed the most obvious effects in the assays that we have utilized.

The E4 mutant viruses display a multiplicity-leakiness phenotype (Fig. 7A and B). This observation is reminiscent of the leaky phenotype associated with E1A mutant viruses (25). The E1A products also are associated with a number of different regulatory functions within infected cells (18). Both the E1A and E4 gene products facilitate, but are not absolutely required for, viral regulatory processes in infected cells. The most intriguing questions concerning E4 function remain. What is the basis for the multiplicity of the phenotypes associated with E4 function? What are the mechanisms by which the E4 products regulate viral DNA replication and protein synthesis? The answers to these questions will await further genetic and biochemical analyses of specific E4 gene products.

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ADDENDUM

Bridge and Ketner (4) recently described the redundant control of adenovirus late-gene expression by the E4 ORF3 and ORF6 products.

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