Redundant Control of Adenovirus Late Gene Expression by Early Region 4

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A series of human adenovirus type 5 derivatives carrying deletion mutations in early region 4 (E4) were constructed and characterized with respect to viral late protein synthesis, viral cytoplasmic late message accumulation, viral DNA accumulation, and plaquing ability. Viral late protein synthesis was essentially normal in cells infected by mutants expected to produce either the E4 open reading frame (ORF) 3 product or the E4 ORF 6 product. In cells infected by mutants lacking both ORF 3 and ORF 6, late protein synthesis was dramatically reduced. The basis for this reduction appears to be a concomitant reduction in cytoplasmic late message levels. Our results suggest that the products of ORFs 3 and 6 are redundant, since they are individually able to satisfy the requirement for E4 in late gene expression. Two of the mutants examined were defective for viral late protein synthesis but showed no measurable defect in viral DNA accumulation. The defect in late gene expression is not, therefore, a reflection of a primary defect in viral DNA synthesis. Finally, mutants expected to express ORF 3 or ORF 6 formed plaques with normal or only modestly reduced efficiency, whereas mutants expected to express neither ORF formed plaques with an efficiency less than 10^{-6} that of wild-type virus. Thus, plaque-forming ability reflected late protein synthetic ability, suggesting that among these mutants late protein synthetic proficiency is the principle determinant of plaquing efficiency.

The adenovirus infectious cycle includes two programs of gene expression, early and late, which are characterized by the expression of different sets of viral genes and are separated by the onset of DNA replication. Expression of early region genes is necessary for normal progression from the early to the late program. In particular, the analysis of two large deletion mutants of early region 4 (E4) (16, 32) has implicated E4 in many of the events that occur as the late program begins; the mutants have complex phenotypes that include defects in late protein synthesis, late mRNA accumulation, DNA accumulation, and shutoff of host cell protein synthesis. Both mutants also overproduce the 72-kilodalton (kDa) DNA-binding protein of early region 2a even at late times after infection.

E4 occupies about 3,000 base pairs (bp) at the right end of the viral genome. DNA sequencing and the analysis of E4 mRNAs have identified several open reading frames (ORFs) in this region (Fig. 1) and suggest that E4 encodes at least seven distinct protein products (11, 17, 30). Three protein products encoded by E4 have been identified immunologically and include a 34-kDa protein from ORF 6 (6, 26), an 11-kDa protein from ORF 3 (8, 27), and a 19.5-kDa protein from ORF 6/7 (7). The 34-kDa protein of ORF 6 has been shown to be physically associated with the 55-kDa protein encoded by early region 1b (E1b) in productively infected cells (26), and this complex is thought to be involved in the transport of viral late messages from the nucleus to the cytoplasm of infected cells (21). The 11-kDa polypeptide product of ORF 3 is found associated with the nuclear matrix (27). The biochemical function(s) of neither the 11-kDa protein nor the 19-kDa protein is known.

To define roles for specific E4 products, Halbert et al. (16) undertook a genetic analysis of E4 in which insertion or deletion mutations were used to disrupt the sequences that encode each of the seven proposed products of this region

one at a time. Only one of these mutants was found to have a defective phenotype: H5d1355, an ORF 6 mutant, displayed mild defects in DNA accumulation and late protein synthesis. We have constructed a series of overlapping deletion mutations and several double mutations in a separate attempt to assign roles to particular E4 gene products. Here, we report (i) that the products of ORF 6 and ORF 3 can individually provide an E4 function that results in nearly normal late protein synthesis, (ii) that the E4-related protein synthetic defect is observed independently of a defect in DNA accumulation, and (iii) that although optimal plaque formation on noncomplementing cell lines requires the ORF 6 product, the ORF 3 product suffices for plaquing at a slightly lower efficiency.

MATERIALS AND METHODS

Plasmid DNA. Plasmid DNA was prepared as described previously (3, 13, 24).

Cells and viruses. W162 cells, which support the growth of adenovirus E4 mutants, have been described previously (31). HeLa cells and wild-type adenovirus type 5 (Ad5) were obtained from J. Williams, 293 cells (14) were from F. Graham, and Vero cells and wild-type adenovirus type 2 (Ad2) were from A. Lewis. The viral mutants used in this study were constructed as described below.

Viral infections. Cell monolayers were infected with mutant or wild-type virus at a multiplicity of 50 or 5 PFU per cell (32); none of the mutants examined here exhibited multiplicity-dependent variations in phenotype. Titers of mutant and wild-type virus stocks were determined on W162 cells.

Preparation and measurement of viral DNAs. DNA was prepared from whole cells or nuclei (32) by the pronase-sodium dodecyl sulfate (SDS)-phenol method (28, 32). Viral DNA-protein complex was prepared from purified virions by cesium chloride-guanidinium hydrochloride density gradient

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centrifugation (23). DNAs were labeled with ^{32}P as described previously (6).

Viral DNAs were measured by dot blot hybridization (32) with labeled Ad2 DNA (10) as the probe.

Labeling and analysis of viral proteins. Viral proteins were labeled with [^{35}S]methionine as described previously (32). Labeled proteins were harvested in 10% β -mercaptoethanol–10% glycerol–4% SDS–0.05% bromophenol blue–50 mM Tris hydrochloride (pH 6.8) or 0.15 M NaCl–0.002 M EDTA–0.01 M Tris hydrochloride (pH 7.4)–1% sodium deoxycholate–1% Nonidet P-40–0.1% SDS.

Electrophoresis of cell extracts on polyacrylamide-SDS gels (19) and fluorography (4) were done as described previously (32).

Immunoprecipitations (18) were done essentially as described previously (9) with rabbit antiserum prepared against SDS-denatured Ad2 virions. Quantitation was by scintillation spectrometry of immunoprecipitates or densitometry of fluorograms of polyacrylamide-SDS gels displaying immunoprecipitated proteins.

Preparation and analysis of cytoplasmic RNA. Preparation and S1 analysis of cytoplasmic RNA were as described previously (20, 24a). The S1 probe employed was an *SphI*–*HindIII* fragment of Ad2 (nucleotides 17815 through 18316) labeled with ^{32}P on the 5' strand at the *HindIII* site. The message for the late protein pVI protects a labeled fragment of this probe 316 bp in length. Measurements of the accumulation of mutant viral mRNAs were made by densitometric comparisons of the intensities of the bands protected by mRNAs from mutant- and Ad5-infected cells.

Construction of plasmid deletion mutants. The overlapping E4 deletion mutations used in this study were derived from pEcoR1BAd5 (2). This plasmid contains the *EcoRI* B fragment of Ad5 (83.6 to 100 map units) (12, 22, 29), modified by the addition of an *EcoRI* linker to its right-hand terminus, inserted into the *EcoRI* site of pBR322. Two derivatives of pEcoR1BAd5, pHK523 and pHK524, were prepared from pEcoR1BAd5 molecules cleaved at one of their two *SmaI* sites by the addition of 8-bp *XbaI* linkers to the termini, cleavage by *XbaI*, and recircularization via the cohesive *XbaI* termini (20). Each of these plasmids contains a unique *XbaI* cleavage site at the position of one of the two *SmaI* sites present in pEcoR1BAd5: at 92.1 map units in pHK523 and at 98.4 map units in pHK524. Preparations of pHK523 and pHK524 DNA linearized with *XbaI* and of pEcoR1BAd5 DNA linearized at its unique *KpnI* site (93.5 map units) were digested with exonuclease *Bal* 31 under conditions that permitted the removal of about 2,000 bp from each molecular end over the course of a reaction. Samples taken at several times during digestion were pooled and extracted with phenol and chloroform, and the DNA was precipitated. The digested DNAs were dissolved, circularized after the addition of *XbaI* linkers to their ends, and introduced into *Escherichia coli* (20). Plasmid DNAs were prepared from the resulting colonies, and the approximate locations of the deletion endpoints in each plasmid were determined by analysis of a series of restriction enzyme digests. To produce deletions extending unidirectionally into E4 from the *SmaI* site at 92.1 map units, the smaller *BamHI*–*XbaI* fragment of pHK523, which contains pBR322 sequences bracketed by the *BamHI* and *EcoRI* sites and the viral DNA sequences from 83.6 to 92.1 map units, was ligated to the larger *BamHI*–*XbaI* fragments isolated from plasmids with desired right deletion endpoints. These ligations produce plasmids carrying deletions extending from 92.1 map units rightward to the right-hand endpoints of the original *Bal* 31-produced

FIG. 1. E4 mutations. Black bars represent the sequences deleted from mutant viruses. Filled triangles indicate the presence of an 8-bp *XbaI* linker insertion. Deletions (except 1010) extend from either of two *SmaI* sites to a centrally located position identified by its map coordinate. ORFs are indicated by open boxes and are those identified by Hérisse et al. (17) from sequence data. The fused ORFs 3/4 and 6/7 were inferred from the nucleotide sequences of cloned E4 cDNAs (11, 30). The positions of the E4 mRNA cap and polyadenylation sites are indicated. The scale is marked in map units and also in nucleotide pairs from the right genomic end.

mutations. Plasmids with deletions extending leftward into E4 from 98.4 map units were produced in an analogous fashion from derivatives of pHK524.

Deletion 1006 was produced by the ligation of an *XbaI* linker to the *SspI* end of the *KpnI*–*SspI* E4 fragment, which extends from 93.5 to 96.4 map units, and the substitution of this fragment for the corresponding *KpnI*–*XbaI* fragment of pHK524 (93.5 to 98.4 map units). Deletion 1010 was derived from 1002 and 1007. The smaller *BamHI*–*XbaI* fragment of 1007, which contains pBR322 sequences bracketed by the *BamHI* and *EcoRI* sites and the viral DNA sequences from 83.6 to 93.3 map units, was used to replace the corresponding *BamHI*–*XbaI* fragment of 1002 to create a deletion from 93.3 to 93.8 map units, defined by the overlap of the two parental deletions. Double mutations 1013, 1014, and 1015 were produced by rearrangements in which the smaller wild-type *MstII* fragment of 1005 or 1006 was replaced by the corresponding mutant fragment of pHK523 or 1002.

The endpoints of the mutations were carefully mapped using restriction enzyme digestion and gel electrophoresis. The DNA sequence of the plasmid carrying deletion 1004 was determined by the Sanger et al. technique (25), using an oligonucleotide primer (5' TCGAGCCAAACGCT 3'; nucleotides 33055 through 33068) derived from the sequence of Ad2 (22). The sequence was determined on one strand and was unambiguous. The nucleotide numbers presented in Fig. 1 correspond to the Ad2 sequence (22), which was identical to the sequence determined for 1004 in the region examined. The locations of the mutations used in this study are diagrammed in Fig. 1.

Introduction of deletion mutations into virus. A 9- μ g sample of Ad5 DNA-protein complex (23) digested with *Eco*RI and *Bam*HI, 15 μ g of pBN27 (a pBR322 clone containing Ad5 DNA from the *Bam*HI site at map position 59.5 to the *Nde*I site at position 86.4) digested with *Bam*HI and *Nde*I, and 15 μ g of *Nde*I-digested deletion mutant plasmid DNA were mixed and incubated with 5 to 7 U of T4 DNA ligase at 4°C overnight in a total volume of 70 μ l. A three-fragment ligation involving the viral left end *Bam*HI fragment (0 to 59.5 map units), the cloned Ad5 fragment (59.5 to 86.4 map units), and the appropriate *Nde*I fragment of the mutant plasmid generates an intact viral DNA molecule bearing the deletion mutation present in the mutant E4 plasmid. (Such molecules also carry pBR322 sequences attached to their right end.) DNA from the ligation mixture (2.5 μ g per 60-mm plate) was introduced into confluent W162 cells by CaPO_4 transfection (15) followed by a 25% glucose boost (5). Plaques that arose were used to infect W162 cells growing in 24-well culture dishes, and the resulting small stocks were used to produce ^{32}P -labeled DNAs, also in W162 cells. Those DNAs were analyzed by restriction enzyme digestion and agarose gel electrophoresis to identify plaques formed by deletion mutant viruses. Typically, 2.5 μ g of ligated DNA produced 10 to 30 plaques. The purity of the plasmid DNAs used in the procedure was critical: reasonable yields of mutant virus (several percent) were obtained only when the donor plasmid DNAs had been passed over a Sepharose CL2B column after CsCl_2 gradient purification. The structure of the virus obtained from transfections was verified by restriction enzyme digestion and gel electrophoresis of ^{32}P -labeled DNAs made from triply plaque-purified virus.

RESULTS

Construction of E4 mutants. The E4 mutations used in this study (Fig. 1) were constructed by in vitro manipulation of a plasmid containing the Ad5 *Eco*RI B fragment (see Materials and Methods). The mutations were subsequently introduced into virus by ligation in vitro of three viral restriction fragments: the viral left end (0 to 59.5 map units) obtained from Ad5 DNA-protein complex by digestion with *Bam*HI, a cloned Ad5 fragment containing the sequences between the *Bam*HI site at 59.5 map units and the *Nde*I site at 86.4 map units, and an Ad5 right end fragment (86.4 to 100 map units) obtained from a deletion mutant plasmid. The ligated DNAs were then introduced into W162 cells by CaPO_4 transfection, and ^{32}P -labeled DNAs prepared from the resulting plaques were screened by restriction enzyme digestion to identify viruses harboring deletion mutations. Mutant viruses were purified by three successive rounds of plaque isolation before use.

DNA accumulation by E4 mutants. E4 deletion mutants H2d/808 (32) and H5d/366 (16) accumulate reduced levels of viral DNA in infections of nonpermissive cells. To determine which members of the series of overlapping deletions that we have constructed share this defect, we prepared total DNA from mutant- and Ad5-infected HeLa cells at 24 to 27 h postinfection and compared the intensities of the bands formed by viral restriction fragments on ethidium bromide-stained agarose gels (data not shown). Cells infected by each of the mutants examined accumulated normal or nearly normal amounts of DNA with the exception of those infected by H5d/1014, which produced markedly reduced levels. When DNA was prepared from infections of the complementing cell line W162, the mutants and Ad5 appeared to accumulate equivalent levels of DNA in all cases.

TABLE 1. Comparison of the late protein synthetic defects of H5d/1004, H5d/1007, and H5d/1014

Mutant	Late protein synthesis ^a	DNA accumulation ^b	Net late protein synthesis ^c	n ^d
H5d/1004	0.077	0.83	0.09 (0.07–0.17)	4
H5d/1007	0.14	1.5	0.09 (0.05–0.17)	4
H5d/1014	0.0063	0.077	0.08 (0.07–0.11)	3

^a Measured by scintillation spectrometry for H5d/1004 and H5d/1007 or by densitometry for H5d/1014. Expressed as the geometric mean of the fraction of Ad5 values obtained for each mutant.

^b Measured by dot blot hybridization. Expressed as the geometric mean of the fraction of Ad5 values obtained for each mutant.

^c Geometric mean of the ratio of late protein synthesis values to corresponding DNA accumulation values. Numbers within parentheses indicate the range of values obtained.

^d n, Number of experiments.

Viral DNA accumulation in mutant-infected cells was also measured by dot blot hybridization. HeLa cells infected by H5d/1004 and H5d/1007 accumulated nearly normal amounts of DNA: 80 and 160% of wild-type levels, respectively. In contrast, cells infected by H5d/1014 accumulated 13-fold less viral DNA than did those infected by Ad5 (Table 1). This reduction is similar to that seen in cells infected with H2d/808 and H5d/366, which are reported to make 5- to 7-fold and 10-fold less DNA than do those infected with wild-type virus, respectively (16, 32).

Late protein synthesis by E4 mutants. To examine the ability of members of this deletion series to direct the synthesis of viral late proteins, HeLa cells were infected with mutant or wild-type virus and were labeled at 24 to 26 h postinfection with [^{35}S]methionine. The cells were harvested, and the labeled proteins were analyzed on SDS-polyacrylamide gels (Fig. 2). H5d/1014-infected cells exhibited a profound defect in late protein synthesis, whereas cells infected by H5d/1004 or H5d/1007 showed a less severe but still readily detectable reduction. Other members of the deletion series showed little or no reduction in late protein synthesis. When the mutants were examined on the permissive W162 cell line, all of the mutants produced normal amounts of most viral proteins, as expected. In W162 cells infected with H5d/1004, H5d/1007, or H5d/1014, protein IV (fiber) is underrepresented. This phenotype has been observed before with H2d/808 (32); its cause is not known.

The proteins produced by cells infected with H5d/1004, H5d/1007, or H5d/1014 were also analyzed by immunoprecipitation with antiserum against Ad2 late proteins to measure the late protein synthetic defects of those mutants. Comparison by scintillation spectrometry of the amount of radioactivity immunoprecipitated from [^{35}S]methionine-labeled Ad5-, H5d/1004-, and H5d/1007-infected cell extracts (80 to 90% of which is in hexon) indicated that late protein production by the mutant-infected cells was about 1/10 that of Ad5-infected cells (Table 1). The magnitude of the late protein synthetic defect of H5d/1014 was determined by immunoprecipitating the viral proteins from equal fractions of Ad5- and mutant-infected cell extracts and by comparing the immunoprecipitates from mutant-infected cells with a dilution series of those from Ad5-infected cells on SDS-polyacrylamide gels (Fig. 3). Densitometry of the hexon bands indicated a reduction in the level of late proteins synthesized by H5d/1014-infected cells of 160-fold (Table 1).

H5d/1014 produces substantially less viral DNA than do H5d/1004, H5d/1007, and Ad5. This failure to produce normal amounts of viral DNA may contribute to the protein synthetic defect of the mutant. To make possible a direct

FIG. 2. Protein synthesis by Ad5 and E4 mutants. Proteins were labeled with [35 S]methionine from 24 to 26 h after infection of HeLa (A) or W162 (B) cells with Ad5 or E4 mutants. Labeled proteins were electrophoresed through a 10% polyacrylamide-SDS gel, and the gel was fluorographed. The positions of several viral proteins are indicated.

comparison of the magnitude of the *H5d/1014* late protein synthetic defect with those of the other mutants, we wished to normalize the sizes of the defects for the amount of viral DNA present in infected cells. The accumulation of viral DNA was therefore measured, by dot blot analysis, in an infection done in parallel with each immunoprecipitation experiment, and the ratio of late protein synthesis to DNA accumulation, both expressed as a fraction of the wild-type value, was calculated for each mutant. The values obtained for net late protein synthesis in mutant-infected cells, after normalization for the amount of viral DNA present, are presented in Table 1.

Accumulation of viral late messages by E4 mutants. Northern blot analyses of late messages produced by the E4 mutants *H2d/808* and *H5d/366* have shown that those mutants accumulate only very low levels of late mRNAs and suggest that a reduction in steady-state mRNA levels is responsible for their late protein synthetic defect (16, 32). Three (*H5d/1004*, *H5d/1007*, and *H5d/1014*) of the 12 mutants we have examined also synthesize reduced levels of viral late proteins upon infection of HeLa cells. To determine whether these reductions in late protein synthesis were accompanied by a concomitant decrease in late message levels, we measured the accumulation of viral late messages in mutant-infected cells by the S1 technique. Cytoplasmic

RNAs were prepared from mutant- and Ad5-infected HeLa cells 24 to 27 h postinfection and were analyzed by using an *SphI-HindIII* fragment of Ad2 (nucleotides 17815 through 18316) as a probe. This probe detects the message for the late protein pVI, a product of late region 3 (L3). When compared against Ad5 standards, pVI mRNA levels in *H5d/1004*- and *H5d/1007*-infected cells were found to be about 10- to 30-fold reduced, whereas *H5d/1014*-infected cells exhibited about a 260-fold reduction in that message (Fig. 4 and Table 2). These defects in late cytoplasmic RNA accumulation are sufficient to account for the late protein synthetic defect observed with these mutants.

To compare the cytoplasmic late message defects of *H5d/1004*, *H5d/1007*, and *H5d/1014*, we again normalized the magnitude of the defects for the amount of viral DNA present in infected cells (Table 2). The values obtained for the net cytoplasmic late message defects were quite similar among the three mutants, in parallel with our observations of the protein synthetic defect.

Efficiency of plaque formation by E4 mutants. We examined the growth of E4 mutants on two noncomplementing human cell lines, 293 and HeLa, by comparing plaque

FIG. 3. Measurement of the late protein synthetic defect of *H5d/1014*. Proteins were labeled with [35 S]methionine from 24 to 26 h after infection of HeLa cells with Ad5 or *H5d/1014*. Equal amounts of radioactivity from each infected cell lysate were then immunoprecipitated, and the immunoprecipitates were fractionated on 10% polyacrylamide-SDS gels. The lane marked 1014 contains proteins precipitated from extracts of *H5d/1014*-infected cells; the remaining lanes contain Ad5 extracts immunoprecipitated and diluted as indicated. The positions of three viral proteins are indicated. U, Uninfected cells.

FIG. 4. S1 nuclease analysis of pVI message levels in *H5d/1004*-, *H5d/1007*-, and *H5d/1014*-infected cells. Total cytoplasmic RNAs were prepared from infected HeLa cells 27 h postinfection and then analyzed. The amount (micrograms) and the source of the RNA loaded in each lane is indicated. The probe employed was an *SphI-HindIII* Ad2 fragment (nucleotides 17815 through 18316) labeled on the 5' strand at the *HindIII* end, which is expected to hybridize to the late message for pVI and to produce a protected fragment of 316 bp. The full-length probe is 501 bp. Samples were fractionated on 5% polyacrylamide-7 M urea denaturing gels and then autoradiographed. m, 32 P-labeled *HinfI* fragments of pBR322; U, uninfected cells.

TABLE 2. Comparison of the cytoplasmic late RNA defects of H5dl1004, H5dl1007, and H5dl1014

Mutant	Late cytoplasmic RNA accumulation ^a	DNA accumulation ^b	Net late cytoplasmic RNA accumulation ^c	n ^d
H5dl1004	0.033	0.74	0.045 (0.035–0.071)	3
H5dl1007	0.077	1.9	0.040 (0.032–0.048)	3
H5dl1014	0.0038	0.077	0.048 (0.016–0.093)	7

^a Measured by S1 analysis. Expressed as the geometric mean of the fraction of Ad5 values obtained for each mutant.

^b Measured by dot blot hybridization. Expressed as the geometric mean of the fraction of Ad5 values obtained for each mutant.

^c Geometric mean of the ratio of late cytoplasmic RNA accumulation values to the corresponding DNA accumulation values. Numbers within parentheses indicate the range of values obtained.

^d n, Number of experiments.

formation by the mutants and Ad5 on those lines. The efficiency of plating was first determined for each mutant on each nonpermissive cell line by taking the ratio of the titer of the mutant stock on the nonpermissive line to the titer of the mutant stock on W162 cells. Then, to account for the differing plating efficiency of Ad5 on the lines, a ratio between the plating efficiency of the mutant and that of Ad5 was calculated for each nonpermissive line. These ratios, termed the relative plaquing efficiencies (RPEs), are presented in Table 3.

The plating phenotypes of the mutants can be grouped into three categories, and the mutants are listed as such in Table 3. Group 1 mutants have RPE values of $>10^{-1}$ and include H5dl1005, H5dl1006, H5dl1012, and H5dl1013. Group 2 mutants have RPEs between 10^{-1} and 10^{-3} on HeLa and 293 cells and include H5dl1001, H5dl1002, H5dl1003, H5dl1010, and H5dl1015. Group 3 mutants plate very poorly, with RPEs of $<3 \times 10^{-6}$, and include H5dl1004, H5dl1007, and H5dl1014.

Summary of mutant phenotypes. A summary of the phenotypes of the E4 mutants examined in this study is presented in Table 4.

DISCUSSION

Products of more than one E4 ORF individually permit normal late protein synthesis. Adenovirus mutants carrying

TABLE 3. RPE of E4 mutants

Group	Mutant	Geometric mean of RPE ^a (n)	
		HeLa	293
1	1005	7.1×10^{-1} (2)	5.8×10^{-1} (3)
	1006	8.5×10^{-1} (2)	1.8×10^0 (3)
	1012	7.0×10^{-1} (2)	6.3×10^{-1} (2)
	1013	1.1×10^0 (2)	7.1×10^{-1} (2)
2	1001	1.3×10^{-1} (3)	1.9×10^{-3} (4)
	1002	1.7×10^{-1} (2)	1.5×10^{-3} (2)
	1003	6.0×10^{-2} (2)	2.0×10^{-3} (2)
	1010	8.9×10^{-2} (3)	6.3×10^{-3} (3)
	1015	2.0×10^{-1} (2)	2.0×10^{-2} (3)
3	1004	3.1×10^{-7} (1)	9.5×10^{-7} (1)
	1007	1.2×10^{-7} (1)	1.3×10^{-7} (1)
	1014	6.3×10^{-7} (1)	2.6×10^{-6} (2)

^a RPE = (titer of mutant stock on test cell/titer of mutant stock on W162 cells)/(titer of Ad5 stock on test cell/titer of Ad5 stock on W162 cells). Numbers within parentheses indicate the number of experiments.

TABLE 4. Summary of E4 mutant phenotypes^a

Mutant	Intact ORF(s)	Late protein synthesis	Late message accumulation	Viral DNA accumulation	Plaquing efficiency
1001	1, 2, 3, 3/4, 4	+	ND	+	±
1002	1, 2, 3, 3/4, 4	+	ND	+	±
1003	1, 2, 3	+	ND	+	±
1004	1	—	—	+	—
1005	3, 3/4, 4, 6, 6/7	+	ND	+	+
1006	4, 6, 6/7	+	ND	+	+
1007	None	—	—	+	—
1010	1, 2, 3, 3/4, 4, 6/7	+	ND	+	±
1012	1, 2, 3, 3/4, 4, 6	+	ND	+	+
1013	4, 6	+	ND	+	+
1014	4	—	—	—	—
1015	3, 3/4, 4	+	ND	+	±

^a +, Normal; —, defective; ±, moderately reduced; ND, not determined.

large deletions of E4 sequences exhibit defects in the synthesis of viral late proteins (16, 32; this report). One of these is H5dl1004, which carries a deletion that disrupts six of the seven E4 ORFs presumed to be functional: ORFs 2, 3, 3/4, 4, 6, and 6/7. One or more of these E4 ORFs therefore plays a necessary role in efficient viral late protein synthesis. H5dl1003 carries a deletion of the E4 sequences between 91.8 and 95.2 map units which affects E4 ORFs 3/4, 4, 6, and 6/7. This deletion does not severely affect late protein synthesis, which implies that one or more of ORFs 1, 2, and 3 can provide an E4 function that permits normal production of late proteins. Similarly, the phenotype of H5dl1006, which carries a deletion that removes the sequences between 98.4 and 96.4 map units and affects ORFs 1, 2, 3, and 3/4, implies that one or more of ORFs 4, 6, and 6/7 provides an E4 function which permits normal production of late proteins. Thus, at least two E4 ORFs must be capable of permitting late protein synthesis at normal levels: at least one of ORFs 1, 2, and 3 and at least one of ORFs 4, 6, and 6/7.

ORF 6 and ORF 3 can each provide an E4 function that permits normal late protein synthesis. Mutants H5dl1003 and H5dl1006 divide E4 into two functional halves, one which includes ORFs 1, 2, and 3 and a second which includes ORFs 4, 6, and 6/7. To identify functional ORFs within these two groups, E4 was further subdivided by construction of double mutants H5dl1013, H5dl1014, and H5dl1015. The late protein synthetic phenotypes of these mutants were then examined.

H5dl1013 contains a deletion affecting ORFs 1, 2, 3, and 3/4 and an 8-bp linker insertion mutation that disrupts ORF 6/7 near the 5' end of the ORF 7 sequences. H5dl1014 contains the same deletion as H5dl1013 and a second deletion disrupting ORF 6 in addition to ORF 6/7. H5dl1013 synthesized late proteins at normal levels, whereas H5dl1014 displayed a 160-fold reduction in late protein synthesis (Fig. 2 and 3, Table 1). Since H5dl1013 differs from H5dl1014 in that it carries ORF 6 intact, we conclude that ORF 6 provides an E4 product that permits normal late protein synthesis. This is consistent with the suggestion made by Halbert et al. (16) that ORF 6 encodes an important E4 function.

H5dl1015 carries a deletion that disrupts ORFs 1 and 2 and a deletion affecting ORFs 6 and 6/7. Mutant H5dl1014 contains the same ORF 6–ORF 6/7 disruption, but its second deletion removes portions of ORFs 3 and 3/4, in addition to ORFs 1 and 2. H5dl1015 synthesizes normal levels of late

proteins, whereas H5d/1014 is severely defective (Fig. 2, Table 1). H5d/1015 differs from H5d/1014 in that it carries ORFs 3 and 3/4 intact, indicating that one or both of these ORFs permits normal production of late proteins. H5d/1003 lacks ORF 6 and the distal end of ORF 3/4 but carries ORF 3 intact and is normal for late protein synthesis. The phenotype of this mutant suggests that of ORFs 3 and 3/4, at least ORF 3 is capable of permitting normal late protein synthesis in the absence of ORF 6.

The simplest interpretation of the phenotypes of these mutants is that the presence of ORF 6 or of ORF 3 permits normal viral late protein synthesis. The redundancy implicit in this interpretation is consistent with the observation that E4 mutants with disruptions in a single ORF are not defective for viral growth or show only subtle defects that collectively do not account for the profound defects seen when large portions of E4 are deleted (16, 27). It should be noted that although our data do not suggest that other E4 ORFs participate in late protein synthesis, such participation is not ruled out.

The late protein synthetic defect displayed by E4 mutants is independent of defects in DNA replication. H5d/1004 and H5d/1007 display a substantial reduction in the production of late proteins in the absence of a marked deficiency in viral DNA accumulation (Table 1). This observation provides direct support for the conclusion, drawn earlier (32), that the effect on late protein synthesis of E4 mutations is not simply the result of a failure of cells infected by E4 mutants to accumulate normal amounts of viral DNA.

H5d/1014 is the only member of this mutant collection that shows a defect in viral DNA accumulation. This DNA defect (and the similar defects of H2d/808 and H5d/366) is complemented by the W162 cell line (16, 32), which indicates that the lack of a diffusible E4 product is responsible for the mutant phenotype. However, apparent inconsistencies among the available E4 mutants have prevented us from deducing a simple relationship between disruption of one of the proposed E4 ORFs and presence of the DNA defect. For example, the deletion carried by H5d/1007 affects all of the proposed E4 ORFs, but that mutant is not defective for DNA synthesis, whereas three mutants lacking the same ORFs or subsets of the ORFs affected by the 1007 deletion (H5d/1014, H2d/808, and H5d/366) do show a DNA synthetic defect. It is possible that an additional E4 ORF exists that has not yet been identified and that is involved in some aspect of DNA synthesis. If that is the case, our data seem to require that it contain sequences from more than one location in E4. Alternatively, H5d/1007 and H5d/1004, which exhibit no defect, may be capable of making fusion products that function normally in DNA synthesis. Determination of which E4 product(s) is involved in DNA synthesis and resolution of the apparent inconsistencies among the mutants described here may have to await an understanding of the biochemical nature of the defect.

The late protein synthetic defect displayed by E4 mutants is explained by reduced levels of cytoplasmic RNA. H5d/1004, H5d/1007, and H5d/1014 all display reductions in the accumulation of cytoplasmic pVI message. The magnitudes of these reductions in each case are similar to or slightly greater than the corresponding reductions in late protein synthesis. These data support the inference that a decrease in late viral mRNA accounts for the late protein synthetic defect observed in E4 mutant infections (16, 32).

Cells infected by H5d/1014 exhibit defects both in the synthesis of viral late proteins and in the accumulation of pVI mRNA that are much more severe than those of cells

infected by H5d/1004 and H5d/1007 (Tables 1 and 2). It seems likely that the failure of H5d/1014 to accumulate normal amounts of viral DNA contributes to these severe reductions. We have therefore compared the protein synthetic and mRNA accumulation defects of these three E4 mutants after correcting protein and message accumulation values for the DNA accumulation defect measured in parallel infections (Tables 1 and 2). When protein synthesis in cells infected by H5d/1014 was normalized for the decrease in viral DNA, a value of about 12-fold was obtained for the net reduction in late protein synthesis (Table 1). This value is very similar to the 11-fold reduction in net late protein synthesis observed in H5d/1004- and H5d/1007-infected cells (Table 1). The net defect in accumulation of pVI message (20-fold) in H5d/1014-infected cells is also very close to those of cells infected by H5d/1004 and H5d/1007 (22- and 25-fold, respectively). We conclude that the large apparent protein synthetic and mRNA accumulation defects of H5d/1014 involve two components: one of the same magnitude as (and presumably identical to) those of H5d/1004 and H5d/1007, superimposed upon an additional defect in viral DNA accumulation. It should be noted that this inference rests on the untested assumption that a similar fraction of the total intracellular DNA is available for transcription in cells infected by different mutants.

Mutations affecting ORF 6 have a modest effect on RPE, whereas mutations affecting both ORFs 6 and 3 show severely reduced RPEs. Members of this deletion mutant series have been divided into three groups with respect to RPE. Group 1 mutants have RPEs greater than 10^{-1} , group 2 mutants have RPEs of 10^{-1} to 10^{-3} , and group 3 mutants have RPEs less than 3×10^{-6} .

Group 1 mutants include H5d/1013 which lacks ORFs 1, 2, 3, 3/4, and 6/7. The RPE of H5d/1013 is close to 1.0 on HeLa and 293 cells, indicating that these ORFs are not essential for normal viral plaquing ability. H5d/1005, H5d/1006, and H5d/1012 all lack subsets of the ORFs disrupted by the mutations present in H5d/1013 and, as would be expected, also display nearly normal plaquing ability.

Group 2 mutants include H5d/1010, which carries a deletion disrupting only ORF 6. Its RPE falls between 10^{-1} and 10^{-2} on HeLa cells and between 10^{-2} and 10^{-3} on 293 cells, indicating that ORF 6 is important for optimal plaque formation. Mutants lacking ORF 6 and, in addition, several combinations of ORFs 1, 2, 3/4, 4, and 6/7 are similar to H5d/1010 in their RPEs. This suggests that even in the absence of ORF 6, none of these ORFs plays a role in plaque formation in the cells examined.

Group 3 mutants include H5d/1004, H5d/1007, and H5d/1014, all of which lack multiple ORFs including both ORFs 6 and 3. These mutants show severely reduced RPEs on both nonpermissive cell lines tested. By reasoning similar to that applied above to late protein synthesis, our data indicate that it is the combination of an ORF 3 mutation and an ORF 6 mutation that is responsible for the profound defect in the plating ability of group 3 mutants. Thus, as in late protein synthesis, either ORF 6 or ORF 3 can individually provide a function that permits plaque formation, although ORF 6 appears to be required for optimal plaquing ability.

The parallel between late protein synthesis and plaque formation makes it likely that proficiency in late protein synthesis is the principal determinant of plaquing ability among these mutants. Quantitatively, plaquing seems to be a much more sensitive indicator of the disruption of the viral life cycle than does our assay of late protein synthesis: in the

cases of H5d1004 and H5d1007, late protein synthetic defects of about 10-fold are reflected in plaquing deficiencies 5 orders of magnitude greater. Since other studies (7, 16) have reported a small late protein synthetic defect for the ORF 6 mutant H5d1355, the defect observed in the growth of the ORF 6 mutants described here, which do not display an obvious deficiency in late protein synthesis, may also be the result of a small reduction in late protein synthesis and the sensitivity of the plaque assay to such reductions. Our data do not rule out, of course, the possibility that ORFs 3 and 6 have roles in the viral life cycle apart from the one that they play in late protein synthesis.

Models for ORF 3 and ORF 6 function. In the absence of E4 ORFs 3 and 6, adenoviruses exhibit a profound defect in the synthesis of viral late proteins due to a failure to accumulate viral late cytoplasmic RNAs. E4 ORFs 3 and 6 are able individually to provide a function that permits both the accumulation of essentially normal levels of late message and normal late protein synthesis. This observation suggests to us that the products of ORF 3 and ORF 6 act independently and in parallel in late message accumulation: either at the same step in the pathway to normal late mRNA accumulation or in two different pathways, either of which can lead to normal accumulation of viral late messages. The ORF 3 and 6 products might act instead in sequential steps along a single pathway to late message accumulation. This seems less likely, since a defect in either step would be expected to disrupt the pathway and lead to a pronounced late protein synthetic defect. Such a defect is not observed in mutants lacking only one of the ORF 3 or ORF 6 products. It remains possible that small defects in each of two sequential steps in one pathway might act synergistically to produce a large effect.

We have analyzed transcription, nuclear accumulation, and cytoplasmic accumulation of late message in cells infected by H2d1808 and H5d1004. The results of these studies, to be presented elsewhere (24a), suggest that the DNA-independent defect in late viral RNA metabolism in cells infected by these mutants is due primarily to the rapid degradation of the precursors of viral late messages in the nucleus of the infected cells. Thus, events that affect stability directly or, like processing or transport, might affect stability indirectly are reasonable candidates for steps at which the ORF 6 and ORF 3 gene products exert their effect.

The 55-kDa protein product of E1b is required for the optimal accumulation of late viral mRNA in the cytoplasm of infected cells and functions either in the transport of message to the cytoplasm or in the stabilization of mRNA as it first reaches the cytoplasm (1, 21). Sarnow et al. (26) have established that the E4 ORF 6 34-kDa protein physically interacts with the E1b 55-kDa protein, and they have suggested that those proteins function in concert. The defect which has been observed with the E1b 55-kDa mutant examined (H5d1338) is modest: the mutant is only three- to fourfold less efficient than wild-type virus in the accumulation of late viral messages in the cytoplasm (21). In contrast, E4 mutants lacking both ORFs 3 and 6 show a 20-fold decrease in the levels of cytoplasmic late message. Thus, the defect due to the disruption of the complex of the 34- and 55-kDa proteins does not seem to fully account for the deficiency observed in the accumulation of cytoplasmic late mRNA by ORF 6⁻ ORF 3⁻ mutants. Further, ORF 6⁻ ORF 3⁻ mutants display a large defect in nuclear RNA accumulation (24a) that is not observed with H5d1338. This suggests that ORFs 6 and 3 are important in at least one process that is not affected by disrupting the complex of the 55- and

34-kDa proteins. Although we have concluded that the ORF 3 and 6 products are individually sufficient to permit normal late protein synthesis, it is important to note that they are not completely interchangeable. For example, ORF 6 deletions have a much greater effect on plating efficiency than do those of ORF 3. The role played by the ORF 6 product as a member of the complex of the 55- and 34-kDa proteins might account for this observation. A more detailed analysis of the mutants generated in this study is underway and may help to determine the extent to which the effect of E4 mutations on late gene expression is due to an effect on the function of the complex of the 55- and 34-kDa proteins.

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