Reduced Infectivity of Adenovirus Type 5 Particles and Degradation of Entering Viral Genomes Associated with Incomplete Processing of the Preterminal Protein

Sayuri E. Kato, Jasdave S. Chahal, and S. J. Flint

Department of Molecular Biology, Princeton University, Lewis Thomas Laboratory, Princeton, New Jersey

To investigate further the contribution of the adenovirus type 5 (Ad5) E1B 55-kDa protein to genome replication, viral DNA accumulation was examined in primary human fibroblasts and epithelial cells infected with Ad5 or the E1B 55-kDa-null mutant Hr6. Unexpectedly, all cell types were observed to contain a significantly higher concentration of entering Hr6 than of Ad5 DNA, as did an infectious unit of Hr6. However, the great majority of the Hr6 genomes were degraded soon after entry. As this unusual phenotype cannot be ascribed to the Hr6 E1B frameshift mutation (J. S. Chahal and S. J. Flint, *J. Virol.* 86:3064–3072, 2012), the sequences of the Ad5 and Hr6 genomes were compared by using high-throughput sequencing. Seven previously unrecognized mutations were identified in the Hr6 genome, two of which result in substitutions in virion proteins, G315V in the preterminal protein (preTP) and A406V in fiber protein IV. Previous observations and the visualization by immunofluorescence of greater numbers of viral genomes entering the cytosol of Hr6-infected cells than of Ad5-infected cells indicated that the fiber mutation could not be responsible for the low-infectivity phenotype of Hr6. However, comparison of the forms of terminal protein present in purified virus particles indicated that the production of mature terminal protein from a processing intermediate is impaired in Hr6 particles. We therefore propose that complete processing of preTP within virus particles is necessary for the ability of viral genomes to become localized at appropriate sites and persist in infected cells.

Successful initiation of the human adenovirus infectious cycle depends on a complex set of interactions among viral and cellular components that allow attachment, entry, and partial dismantling of virus particles prior to the transport of viral genomes to and into the infected cell nucleus. The nonenveloped, icosahedrally symmetric virus particles carry distinctive fibers that project from the penton base present at each of the 12 vertices (5, 72). The distal knob of the fiber contains the binding site for attachment to the primary cell surface receptor, the coxsackievirus and adenovirus receptor, Car, in the case of species C adenoviruses such as serotype 5 (Ad5) (4, 70, 89). Interactions of RGD sequences present in loops that project from the surface of each subunit of the pentameric penton base with α v integrins on the cell surface (14, 84) then promote the entry of virus particles by clathrin-mediated endocytosis (18, 50, 75, 80, 101; reviewed in reference 1). Subsequent escape from early endosomes into the cytoplasm is coordinated with, and dependent on, initial uncoating reactions that remove capsid proteins.

It is well established that uncoating occurs in several discrete stages (80), the first being dissociation of fibers at the cell surface (9, 30, 57, 62). Within the endosome, additional structural proteins are released, including peripentonal hexons and minor capsid proteins IIIa, VIII, and (importantly) protein VI (30, 79; reviewed in reference 80). The latter protein was implicated in endosomal escape when it was shown to be required for the ability of partially uncoated Ad5 particles to disrupt membranes *in vitro* (102). Antibodies or specific substitutions in protein VI that impair membrane lysis activity *in vitro* reduce the transduction of viral genomes into cells (56, 59, 60), indicating that this protein mediates the lysis of endosomal membranes in infected cells. The genome-containing, partially dismantled particles that enter the cytosol, which retain the majority of the hexons (30) and some protein VI (105), are transported on microtubules, with net

movement toward the microtubule organizing center (MTOC) and nucleus (8, 49, 54, 88). Such transport requires the microtubule-associated motor dynein and its regulator dynactin (8, 19, 44, 49, 54, 88). Neutralizing monoclonal antibodies (MAbs) that recognize hexons have been reported to impair the intracellular transport of partially disassembled particles and block their accumulation at the MTOC (78), suggesting that a hexon-dynein interaction is required for transport to the nucleus in infected cells. However, additional virus proteins may contribute to, or regulate, this process: substitutions in a PDxY motif present in protein VI that prevents the ubiquitinylation of this viral protein by Nedd4 family E3 ubiquitin ligases inhibited the delivery of the genome to the nucleus and the association of intracellular particles with microtubules but had no effect on endosomal escape (105).

It is well established that viral genomes enter nuclei via nuclear pore complexes (29), but whether partially uncoated particles must first traffic to the MTOC, where they have been observed to accumulate (2, 16, 49), is not clear (reviewed in reference 38). At nuclear pore complexes, the particles bind to the nucleoporin Nup214, and histone H1 becomes associated with hexons (90). Examination of the fate of proteins present in these partially disassembled particles using conformation-specific anti-hexon antibodies, anti-protein VII antibodies, or radioisotopically or fluorescently labeled proteins has established that major core protein

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Address correspondence to S. J. Flint, sjflint@princeton.edu.

S.E.K. and J.S.C. contributed equally to this work.

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VII enters nuclei with the genome while protein V and the remaining capsid subunits are removed (12, 29, 35, 42, 65, 107). Although the mechanism by which viral genomes packaged by protein VII traverse the nuclear precomplex is not well understood, it has been demonstrated recently that direct and indirect binding of the motor kinesin 1 to viral particles associated with Nup214 disrupts the particles to release capsid fragments and nucleoporins (85). This action of kinesin also increases the permeability of the nuclear envelope (85), a property that is thought to facilitate the transport of viral DNA-protein VII nucleoproteins into the nucleus via importin family receptors (39).

Viral structural proteins preIIIa, VI, VII, VIII, and Mu are synthesized as larger precursors (preIIIa, etc.) from which viral particles are assembled (reviewed in references 5 and 72). The immature particles initially assembled also contain the precursor of the terminal protein (TP), preTP, which becomes covalently attached to the 5' ends of newly synthesized viral genomes when it serves as the protein primer for initiation of DNA synthesis (36, 52). Processing of precursor proteins is essential to form mature, infectious virions: a temperature-sensitive mutation (Ad2ts1) in the L3 coding sequence for the viral cysteine protease that prevents encapsidation of this viral enzyme (67, 97) results in the accumulation at nonpermissive temperatures of noninfectious particles containing uncleaved precursor proteins (reviewed in reference 72). Such noninfectious particles enter early endosomes with normal kinetics, but in contrast to the wild type, they fail to escape from these vesicles and are transported to late endosomes and lysosomes (21, 29, 40). This intracellular fate can be attributed to failure of immature Ad2ts1 particles to induce membrane lysis, as measured by the ability of the coinertalized protein synthesis inhibitor α -sarcin to penetrate the cytoplasm (102). Immature Ad2ts1 particles are also more stable at low pHs and increasing temperatures than wild-type virions are (64, 102).

Comparison of the structures of immature Ad2ts1 particles and mature wild-type virions at moderate resolution (≤ 10 Å) by cryo-electron microscopy has identified several differences in the interactions among structural proteins (64, 76). One unique feature of the noninfectious particles is an additional "molecular stitch" between the groups-of-nine hexons (72) and the ring of peripentonal hexons surrounding each vertex. It is thought that precursor-specific segments of proteins IIIa and VIII contribute to this structure and that its removal upon precursor cleavage would be required to facilitate the release of vertex capsomers (reviewed in reference 72). Additional protein was also observed in noninfectious Ad2ts1 particles inside the cavities of each hexon, which open on the inner surface side of the capsid, and has been attributed to preVI. As interaction with hexons blocks the membrane lysis activity of proteins VI and preVI *in vitro* (76), this more extensive hexon-preVI interaction seems likely to impair the release of protein VII from Ad2ts1 particles and hence account for their defect in endosomal escape. A third major difference is the more ordered, compact core structure (64, 76), which may be, at least in part, the result of more extensive interactions of preVII than of VII with DNA within virus particles (13).

Although these structural studies have provided plausible explanations for the increased stability of immature Ad2ts1 particles and their lack of infectivity, the relative contributions of the individual precursor-specific segments of the structural proteins, or preTP, are not known. Indeed, apart from Ad2ts1, relatively few mutations that reduce infectivity have been described. Exceptions

include the protein VI substitutions that inhibit membrane lysis activity described above (59, 60) and deletion of the protein V coding sequence (92). In addition, particles that lack the fiber or that carry fibers with substitutions in the Car binding surface of the knob or shorter or longer shafts exhibit reduced Car-dependent entry (41, 47, 55, 74, 75, 96). Here we report the serendipitous discovery of a previously unrecognized low-infectivity phenotype, degradation of the great majority of viral genomes soon after entry, and its association with a mutation in the preTP coding sequence.

MATERIALS AND METHODS

Cells and viruses. 293 cells and human foreskin fibroblasts (HFFs) were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% and 10% fetal calf serum and bovine growth serum, respectively. Primary human small airway epithelial cells (SAECs) and bronchial/tracheal epithelial cells (NHBEs) were obtained from BioWhittaker, Inc., and cultured using predefined medium and growth conditions according to the manufacturer's recommendations. Wild-type Ad5 and the E1B 55-kDa-null mutant Hr6 (34), were propagated in monolayers of 293 cells, and concentrations of infectious particles were determined by assaying plaque formation on these same cells as described previously (104).

Analysis of accumulation of viral DNA. Proliferating or quiescent cells in 35-mm or six-well dishes were infected in parallel with Ad5 or Hr6 and harvested after increasing periods of infection. DNA was purified from cells or isolated nuclei as described previously (24) or by using the DNeasy tissue kit (Qiagen) according to the manufacturer's protocol. Quantitative real-time PCR was carried out using the ABI PRISM 7900HT sequence detection system and a TaqMan probe (Applied Biosystems) of an amplicon within the ML transcription units, 90 bp long (nucleotides 7128 to 7218). The primer and probe set was as follows: ML Fwd, 5'-ACT CTT CGC GGT TCC AGT ACT C-3'; ML Rev, 5'-CAG GCC GTC ACC CAG TTC TAC-3'; ML probe, VIC-ATC GGA AAC CCG TCG GCC TCC-TAMRA. Reaction mixtures contained TaqMan Universal PCR master mix with AmpErase (Applied Biosystems), 2 μ l sample DNA (diluted as necessary), 300 nM each primer, and 200 nM TaqMan probe. In some experiments (see Fig. 3), viral DNA concentrations were assessed by using the same ML amplicon and an amplicon within the promoter of the human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter as an internal control and Sybr green detection, as described previously (11). Relative DNA concentrations were determined by the standard-curve method, and all measurements were performed in triplicate.

Illumina sequencing of viral genomes. Ad5 and Hr6 particles were purified from 293 infected-cell lysates containing $\sim 5 \times 10^{10}$ PFU by sequential centrifugation in discontinuous CsCl gradients and centrifugation to equilibrium in continuous CsCl gradients (27). The purified particles were dialyzed against two changes of 100 volumes of 0.1 M Tris-HCl, pH 8.0, prior to the addition of 1 volume of 0.01 M Tris HCl, pH 8.0, containing 10 mM EDTA, 1% (wt/vol) sodium dodecyl sulfate (SDS), and 1 mg/ml proteinase K. Following incubation for 2 h at 37°C, nucleic acids were extracted with (1:1) phenol-CHCl₃ and ethanol precipitated. The isolated DNA was resuspended in 100 μ l 0.5 M Tris-HCl, pH 8.0, and concentrations were determined from the absorbance at 260 nm measured using a NanoDrop ND-1000 spectrophotometer. Sequencing of the DNA extracted from wild-type Ad5 and Hr6 was performed at Princeton University's Lewis-Sigler Institute Microarray Facility with an Illumina Genome Analyzer II using SCS 2.3 software. One microgram of purified viral DNA was used to prepare sequencing libraries with different adapter sequences to distinguish wild-type Ad5 and Hr6 libraries, exactly according to the manufacturer's protocols. DNA from each library was sequenced over 51 cycles in a single flow cell.

Analysis of sequencing data. The Illumina output was analyzed by using tools available at the Princeton Galaxy bioinformatics local workflow system (7, 22, 23). Illumina FASTQ files were converted to a standard

file format, and 0.1% of the sequence reads acquired were selected arbitrarily and used to construct the full-length genome sequences of wild-type Ad5 and Hr6. This step was accomplished by aligning sequence reads from wild-type Ad5 against the human adenovirus C serotype 5 complete genome reference sequence AY339865 (86) by using Burrows-Wheeler Aligner (51). Variations in the resulting BAM alignments were then detected using FreeBayes (<http://bioinformatics.bc.edu/marthlab/FreeBayes>). Alignments and polymorphisms were visualized using the Integrated Genomics Viewer (69). Nucleotide identities were unambiguous in the final aligned wild-type sequence with >30-fold sequence coverage, except within ~150 bp of the genome ends, where sequence coverage dropped to no lower than 16-fold. The sequences of these terminal regions were confirmed by conventional sequencing and matched those of alignments. Once assembled, the wild-type Ad5 genome sequence was used to align the Hr6 Illumina reads as described above. This alignment was again unambiguous, with no less than 14-fold coverage, and the terminal sequences of the Hr6 genome were again verified by conventional sequencing.

Immunoblotting. HFFs, SAEs, or NHBEs at approximately 75 to 80% confluence were infected with Ad5 or Hr6. Cells were harvested after the periods of infection indicated and washed with phosphate-buffered saline (PBS), and extracts were prepared as described previously (11). Extracts were sonicated in 30-s bursts on ice until sample viscosity decreased, prior to the removal of cell debris by centrifugation at $10,000 \times g$ at 4°C for 5 min. Proteins were detected by SDS-polyacrylamide gel electrophoresis and immunoblotting as described previously (25), with MAbs against the E1A proteins and the E2 DNA-binding protein (DBP), M73 (33), and B6 (68), respectively. Cellular β -actin, as an internal control, was visualized with a horseradish peroxidase-labeled anti- β -actin MAb (Abcam).

To examine TPs covalently bound to viral DNA, virus particles were purified from Ad5- and Hr6-infected cells as described above. Purified particles were disrupted by incubation at 60°C for 10 min (64), and viral DNA was digested with 1,250 U/ml Benzonase nuclease (Sigma) for 30 min at 37°C. TPs were detected by immunoblotting with anti-preTP MAbs (98) kindly provided by R. Hay. Protein V, detected by immunoblotting with MAb F58#1 (53), served as an internal control.

Immunofluorescence. To examine viral replication centers, HFFs grown on coverslips to approximately 90% confluence were mock infected or infected with Ad5 or Hr6 for various periods and processed for immunofluorescence as described previously (25). The viral E2 DBP was visualized by using the B6 antibody (68) and goat anti-mouse IgG labeled with Cy5 (Jackson ImmunoResearch Laboratories Inc.). To visualize the viral genome soon after entry, HFFs on coverslips were incubated, with rocking, with Ad5, Hr6, or DMEM only (mock infection) at 4°C for 30 min. After removal of the inoculum, cells were washed twice with cold PBS prior to the addition of DMEM containing 5% (vol/vol) bovine growth serum prewarmed to 37°C. Cells were processed for immunofluorescence as described previously (25), except that they were fixed and permeabilized by incubation in prechilled methanol for 10 min at -20°C. Protein VII was examined after the periods of infection indicated by using purified rabbit anti-protein VII polyclonal antibody (42), which was kindly provided by D. Engel, and Alexa fluor 488-conjugated goat anti-rabbit IgG (Invitrogen). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), and samples were examined by confocal microscopy as described previously (25). Late endosomes and microtubules were detected by using a mouse anti-Rab7 MAb (Rab7-117; Sigma-Aldrich) with a Cy5 anti-mouse IgG secondary antibody (Jackson Immuno Research Laboratories Inc.) and a rat anti- β -tubulin antibody (Abcam) with an Alexa fluor 568-containing anti-rat IgG (Invitrogen) secondary antibody, respectively.

RESULTS

Viral DNA accumulation is impaired in normal human epithelial cells and fibroblasts in the absence of the E1B 55-kDa protein. In the Hr6 genome, deletion of bp 2347 alters the coding

TABLE 1 Comparison of viral DNA concentrations entering Ad5- and Hr6-infected cells and in virus particles

	[DNA] ^a		
Viral DNA	Hr6	Ad5	Hr6/Ad5 ratio
Entering infected cells ^b			
HFF	12.1 ± 0.09	0.28 ± 0.01	43.2
SAEC	7.96 ± 0.24	0.38 ± 0.01	20.9
NHBEC	7.25 ± 1.16	0.20 ± 0.02	36.3
Present in 1,000 PFU			
Expt 1	14,147	1,064	13.3
Expt 2	6,150	416	14.8

^a Viral DNA concentrations are in arbitrary units.

^b Viral DNA 2 h after infection of proliferating cells.

sequence of the E1B 55-kDa protein but not those of the related lower-molecular-mass proteins made from alternatively spliced mRNAs (103). The consequent shift in the reading frame of the E1B 55-kDa protein coding sequence introduces a termination codon a short distance downstream of the deletion, but no truncated E1B protein can be detected in Hr6-infected cells by using various antibodies that recognize N-terminal epitopes (46, 103). We have previously reported that Hr6 exhibits a substantial defect in viral DNA synthesis in proliferating HFFs (24). In contrast, viral DNA synthesis was reported to occur normally in quiescent human SAEs infected with a second E1B-null *dl1520* mutant, ONYX-015 (63). It is well established that replication of E1B-null mutants in established human cell lines is dependent on the host cell type (17, 26, 32, 71, 91). Consequently, it seemed possible that differences in cell type might account for the reported differences in mutant phenotypes. We therefore examined viral genome accumulation in proliferating HFFs and SAEs infected with Ad5 or Hr6. We also included NHBEs in these experiments: as subgroup C adenoviruses, such as Ad5, are associated with upper respiratory tract infections (reviewed in reference 106), these cells seemed likely to provide a closer facsimile of natural host cells than either SAEs, which are derived from the lower respiratory tract, or HFFs. Viral DNA concentrations were measured at various times after infection by using real-time PCR amplification of a sequence within the major late transcription unit, as described in Materials and Methods.

For these experiments, the Ad5 and Hr6 stocks were titrated in parallel and cells were infected with each virus at 30 PFU/cell. Nevertheless, we consistently observed that Hr6-infected cells contained a significantly higher concentration of input viral DNA, as illustrated in Table 1 for proliferating cells. Very similar results were obtained when quiescent cells were infected (data not shown). The concentrations of intracellular viral DNA present 2 h after the adsorption of Ad5 and Hr6 to the three types of host cell varied to a small degree. Such differences are presumably the result of variations in the efficiency of entry. Nevertheless, Hr6-infected cells invariably contained at least 20-fold more viral DNA than did the same host cells infected with Ad5. These observations imply that a significantly greater number of Hr6 particles are necessary to form a plaque, that is, that these mutant virus particles are less infectious than wild-type virions. To test this interpretation, the concentrations of viral DNA present in the same number of infectious units (1,000 PFU) of Ad5 and Hr6 were compared by using real-time PCR of viral DNA purified from the particles. The

FIG 1 Defects in the accumulation of viral DNA in proliferating SAEs, NHBEs, and HFFs infected with Hr6. Proliferating cells were infected with 30 PFU/cell Ad5 or Hr6, and DNA was isolated from cells harvested 2, 18, 24, and 30 h after infection. Viral DNA was quantified by real-time PCR as described in Materials and Methods. Relative concentrations of viral DNA were calculated as the increase in concentration at each time point over the value measured at 2 h after infection. Results represent the average of two independent experiments, and error bars represent the standard deviation.

results of this analysis of the Ad5 and Hr6 preparations used for the experiments summarized in the top part of Table 1 are shown in the bottom part of Table 1 (experiment 1), and very similar results were obtained when different stocks of the two viruses were compared in the same way (Table 1, bottom, experiment 2). These data demonstrate that an infectious unit of Hr6 contains significantly more viral DNA than does an infectious unit of Ad5, in other words, that most DNA-containing Hr6 particles are not infectious.

To permit the comparison of viral DNA accumulation in cells infected with concentrations of input viral DNA differing by more than an order of magnitude, all DNA concentrations were expressed relative to the input value measured 2 h after infection. The accumulation of viral DNA was some 10-fold less efficient in Hr6-infected, proliferating HFFs than in cells infected with Ad5 (Fig. 1A), consistent with the results of our previous experiments in which viral DNA concentrations were examined by hybridization of infected-cell DNA to ³²P-labeled viral DNA (33). Increases in viral DNA concentration were detected by 18 h after infection of proliferating SAEs or NHBEs, indicating that the early phase of infection proceeds more rapidly in these epithelial cells than in HFFs. This temporal difference may account for the higher viral DNA concentrations attained by 30 h after Ad5 infection of the two types of epithelial cells than in HFFs (Fig. 1B and C). Nevertheless, the Hr6 mutant also exhibited apparent defects in viral DNA accumulation in proliferating SAEs or NHBEs (Fig. 1).

The Hr6 mutation does not impair the synthesis of viral replication proteins. Adenoviral DNA synthesis requires the three viral replication proteins encoded within the E2 transcription unit, the viral DNA polymerase, the preTP primer, and the single-stranded DBP (see references 5, 36, and 52). An obvious explanation for the defects in viral DNA accumulation observed in Hr6-infected cells is, therefore, that viral early gene expression and synthesis of these replication proteins are impaired. To assess this possibility, the concentrations of E1A proteins, which are required for the efficient transcription of all early genes (5), and the E2 DBP were compared in proliferating normal human cells infected with Ad5 or Hr6. Total cell extracts were prepared 24 and 30 h after infection, and the viral proteins examined by using immunoblotting. No significant differences in the accumulation of the immediate early E1A proteins (data not shown) or of the E2 DBP were observed in Ad5- and Hr6-infected HFFs, NHBEs, or SAEs

(Fig. 2A). As the E2 transcription unit encodes the viral DNA polymerase and preTP protein primer, as well as the DBP (5), this result implies that the reduced accumulation of Hr6 genomes is not the result of failure to produce viral replication proteins.

We also examined the formation of viral replication centers containing the E2 DBP by immunofluorescence. In adenovirus-infected cell nuclei, the DBP forms two morphologically distinct structures, small dot-like foci and larger globular or ring-like structures (87, 95). The small foci appear early in infection, and their formation is independent of viral DNA synthesis. In contrast, the ring-like structures that are associated with newly synthesized viral DNA (61, 66, 95) do not appear when viral DNA synthesis is blocked by drugs or mutations (87, 95). Both types of DBP-containing structures were observed in proliferating HFFs infected with Ad5 or Hr6 (Fig. 2B), as well as in infected NHBEs (data not shown), but the number of DBP-containing structures was not substantially, or noticeably, higher in Hr6-infected cells than in Ad5-infected cells (Fig. 2B). When infected cells were quantified in terms of the presence of the different types of replication centers, fewer Hr6-infected than Ad5-infected HFFs and NHBEs were found to contain the larger, ring-like structures formed upon viral DNA synthesis (Fig. 2C and D), consistent with the reduced accumulation of viral DNA in the mutant-infected cells (Fig. 1).

The majority of viral genomes are degraded in Hr6-infected cells. Although Hr6-infected normal cells contained higher concentrations of input viral DNA than did cells infected with Ad5 (Table 1), neither the formation of a larger number of replication centers per infected cell nor the increased expression of early genes was observed (Fig. 2). This apparent discrepancy suggested that most Hr6 genomes might be degraded before they can serve as the template for viral gene expression and DNA synthesis within infected cell nuclei. To investigate this possibility, the concentrations of intranuclear viral DNA were measured during both the early and late phases of infection in HFFs, which are robust and simple to culture. Proliferating HFFs were infected with Ad5 or Hr6 at 30 PFU/cell, and DNA purified from isolated nuclei after increasing periods of infection. Viral DNA concentrations were measured by quantitative PCR, with cellular GAPDH DNA quantified in parallel to provide an internal control, as described in Materials and Methods. In two independent experiments, Hr6-infected HFFs contained 12.2-fold $\pm$ 1.3-fold more nucleus-asso-

FIG 2 The E1B 55-kDa protein is not required for the synthesis of E2 proteins or the formation of replication centers. (A) Proliferating normal human cells were infected with Ad5 or Hr6 or mock infected and harvested after the periods indicated. Total cell lysates were examined by immunoblotting as described in Materials and Methods, with anti-E2 DBP MAb B6 and an anti-β-actin antibody. (B) Proliferating HFFs were infected with Ad5 or Hr6 or mock infected and processed for immunofluorescence 24 h after infection, as described in Materials and Methods. The viral E2 DBP was detected with the B6 antibody and cyanine 5-labeled anti-mouse IgG and is shown false colored in green. Nuclei were stained with DAPI (blue). Expanded, ring-like replication centers and dot-like structures are indicated by the orange and white arrows, respectively. These two types of replication centers were quantified in Ad5- and Hr6-infected HFFs (C) and NHBEs (D). Shortly after the onset of viral DNA synthesis, between 100 and 200 cells were analyzed for those in which the DBP was present only in small, dot-like structures (Small foci) or present in both these foci and large, ring-like structures (Large rings).

ciated viral DNA at 2 h postinfection (p.i.) than did Ad5-infected cell nuclei, somewhat less than when DNA was purified from whole cells (Table 1, top). The relative concentrations of viral DNA were lower in Hr6-infected cell nuclei throughout the early and late phases of infection (Fig. 3A). However, this more detailed temporal analysis established that viral DNA concentrations declined sharply in Hr6-infected cells between 2 and 12 h p.i., decreasing by a factor of 20 (Fig. 3B). In contrast, Ad5 genomes were reduced in concentration by only 40% during the same period (Fig. 3B). As the ML amplicon used to detect viral DNA by quantitative PCR was only 90 bp in length, we conclude that the majority of Hr6 genomes, in contrast to wild-type genomes, are degraded very extensively within a few hours of entry into HFFs. Such a fate accounts for the similar numbers of viral replication centers formed in Ad5- and Hr6-infected cells (Fig. 2B). Although some input viral DNA was degraded in Ad5-infected

cells, it seemed possible that the extensive loss of mutant viral genomes might be the result of a host response(s) triggered by the high concentration of entering viral DNA molecules. To address this possibility, we compared the concentrations of viral DNA after increasing periods of infection in HFFs infected with 30 PFU/cell Ad5 but with Hr6 under conditions designed on the basis of measurements like those shown in Table 1 to yield an equal number of entering genomes. The results of a typical experiment, in which the ratio of the concentrations of entering Hr6 and Ad5 DNA was 1.8, is shown in Fig. 3D. Despite the presence of similar concentrations of viral DNA in Ad5- and Hr6-infected HFF nuclei at 2 h p.i., the mutant viral DNA was again reduced substantially in concentration from 6 to 18 h after infection, whereas only a small decrease was observed in Ad5-infected cells (Fig. 3D).

As the minimal number of genomes competent to serve as the templates for replication cannot be determined accurately from

FIG 3 Intracellular degradation of viral DNA in Hr6-infected cells. (A) Cells were infected with 30 PFU/cell Ad5 or Hr6. DNA was purified from nuclei isolated from cells harvested at the times indicated, and viral DNA concentrations were determined by quantitative PCR as described in Materials and Methods. The values, which were corrected for the cellular GAPDH DNA concentrations determined in parallel, are expressed relative to that measured at 2 h p.i. and represent the average of two independent experiments. Error bars show standard deviations. (B) The 2- to 18-h data from the experiments shown in panel A are replotted with an expanded y axis. (C) Cells were infected with equivalent numbers of Ad5 or Hr6 genomes, and intranuclear viral DNA concentrations were measured at the times indicated as described above for panel A. The corrected values, which are shown in arbitrary units, represent the mean of two independent experiments, and error bars show the standard deviations.

FIG 4 Differences between the sequences of the Ad5 and Hr6 genomes. The horizontal line at the top represents the Ad5 genome in kilobase pairs. The fold coverage (FC) of sequence reads used to assemble the sequences of Ad5 and Hr6 DNA as described in Materials and Methods is indicated below. The black lines represent silent mutations detected in the Ad5 genome compared to the reference sequence or in Hr6 compared to the Ad5 sequence (see the text). The Hr6 mutations shown in blue and red indicate the previously described E1B 55-kDa coding sequence mutations (103) and newly discovered mutations in the preTP and fiber genes that introduce substitutions, respectively.

data like those shown in Fig. 3, it was not possible to make an appropriate comparison of increases in viral DNA concentration in Ad5- and Hr6-infected cells. To circumvent this problem and assess the contribution of the E1B 55-kDa protein to viral genome replication in normal human cells, we exploited a mutant virus containing the Hr6 frameshift mutation (deletion of bp 2347 in the Ad5 genome) in an E1-containing derivative (43) of AdEasy (37). Analysis of this E1B 55-kDa-null mutant, AdEasyE1Δ2347 (43), has established that the timely synthesis of the E1B 55-kDa proteins is required for efficient viral DNA synthesis in normal human cells (11). However, greater numbers of entering viral genomes were not observed in cells infected with AdEasyE1Δ2347, compared to its wild-type parent (11), indicating that deletion of bp 2347 in the E1B 55-kDa coding sequence is not responsible for the poor infectivity of Hr6 virus particles. We therefore conclude that the Hr6 genome must contain at least one additional mutation responsible for this phenotype.

Identification of additional mutations in the Hr6 genome. To search for all mutations that might be present in the Hr6 genome, Hr6 and Ad5 DNAs were subjected to high-throughput sequencing. This host range mutant was isolated by virtue of its impaired replication in HeLa cells, compared to that in complementing 293 cells (28), following the exposure of Ad5 to nitrous acid (34). Hr6 and the Ad5 strain from which it was derived were obtained originally from J. Williams, Carnegie Mellon University, and have been maintained by preparation of master stocks from which all working stocks are amplified. Consequently, the preparations of Ad5 and Hr6 used in these experiments were derived by only a limited number of low-multiplicity passages from the stocks received originally.

Viral DNA was isolated from purified Ad5 and Hr6 particles and used to prepare Illumina genomic libraries as described in Materials and Methods. More than 370,000 reads from each virus were used to assemble complete genomic sequences. The sequence of our stock of Ad5 was mapped by alignment of reads to the human adenovirus C serotype 5 complete genome reference sequence AY339865.1 (86). A total of 98.51% of the reads were successfully mapped to this reference sequence, with the lowest coverage at the most terminal ~150-bp regions at the ends of the genome (Fig. 4). In these regions, coverage was no less than 16-fold, and their sequences determined by deep sequencing were confirmed by conventional sequencing. This analysis identified two deletions that distinguished our wild type from the reference

Ad5 genome (Fig. 4), a deletion of one A-T base pair at position 14073, from a poly(A-T) stretch of 13 bp in the reference and a deletion of one T-A base pair at position 34338 of the reference from a poly(T-A) stretch of 12 bp. Neither mutation results in any coding sequences changes. The T-A deletion at bp 34338 was found to be present in the Hr6 genome, described below. However, the A-T deletion at bp 14073 was not; at this locus, the Hr6 genome appears to be identical to the allele present in the reference sequence with accession no. AY339865.1 (15), containing a poly(A-T) stretch of 13 bp. The Hr6 genome was mapped as described above to the wild-type sequence depicted in Fig. 4, with 98.56% of the reads successfully aligned with our wild-type reference. Nine mutations unique to Hr6 were identified upon alignment with the wild type (Fig. 4; Table 2). Of these, four transition mutations and one insertion are silent, despite lying within coding sequences (Table 2). The deletion of bp 2347 and the G-to-T transversion at bp 2947 correspond exactly to the mutations previously identified in the E1B 55-kDa protein coding sequence of Hr6 by conventional sequencing (103). The remaining mutations introduce amino acid substitutions into virion proteins. The C-to-A transversion at bp 9655 results in replacement of Gly315 in preTP with Val, while a transition at bp 32252 introduces Val in place of Ala406 in fiber protein IV (Table 2).

The A406V substitution in protein IV lies close to the Car-binding surface of the fiber knob, which has been identified by mutational analysis and structural studies (6, 45, 70). However, this residue is not conserved among the fibers of adenovirus serotypes that bind to Car (70, 93). Indeed, residue 406 (or its equivalent) is Asp in the fiber knob of the very closely related species C serotype Ad2 and several species A and B serotypes (93). Consistent with the lack of conservation of even amino acid similarity, an Ad5 fiber knob carrying an A406K substitution was reported to compete as efficiently as the wild type for binding to Car on Chinese hamster ovary cells (45). Furthermore, protein IV alterations that impair the fiber knob-Car interaction decrease the efficiency of genome transduction (41, 47), whereas Hr6-infected cells contain significantly higher concentrations of viral DNA immediately after infection than do those infected with Ad5 (Table 1; see also Fig. 6). As these observations argue strongly that the fiber mutation cannot account for the poor infectivity of Hr6, subsequent studies focused on the consequences of the preTP substitution.

Proteolytic processing of preTP is impaired by the G315V substitution. The 671-amino-acid E2 preTP serves as the protein

TABLE 2 Mutations identified in the Hr6 genome compared to the Ad5 genome

Site (bp)	Mutation	Genomic location	Consequence
2347	G deletion	E1B 55-kDa CDS ^a	Frameshift and truncation ^b
2947	G→T	E1B 55-kDa CDS	Substitution at aa ^c 310, TGT (C)→TTT (F) ^b
9655	C→A	PreTP CDS	Substitution at aa 315, GGC (G)→GTC (V)
14073	A insertion	IIIa CDS termination codon	Silent, TAA→TAA
18909	C→T	Hexon CDS	Silent, TAC (Y)→Tat (Y)
29183	C→T	E3 gp19k CDS	Silent, AGC (S)→AGT (S)
30794	G→A	E3 14.7k CDS	Silent, AAG (K)→AAA (K)
32252	C→T	Fiber CDS	Substitution at aa 406, GCT (A)→GTT (V)
34193	C→T	E4 Orf4 CDS	Silent, AGG (R)→AGA (R)

^a CDS, coding sequence.^b As previously reported (103).^c aa, amino acid.

primer for viral DNA synthesis when it becomes covalently attached to the 5' ends of newly replicated viral DNA molecules (36, 52). Subsequently, this precursor is processed by the viral L3 protease to the mature TP (77, 98). Initial cleavage by the protease at two closely spaced sites (Fig. 5A) generates an ~62-kDa intermediate, termed intermediate TP (iTP) (77, 98). This reaction can take place prior to the encapsidation of viral genomes during the assembly of virus particles, in contrast to the production of mature TP (99), which comprises the C-terminal 322 residues of the precursor (Fig. 5A). As the sequence of TP is not altered by the mutation in the Hr6 preTP coding sequence (Fig. 5A), the effect of this G315V substitution on preTP processing was investigated.

Equal numbers of infectious units of Ad5 and Hr6 were purified as described in Materials and Methods, and equal concentrations of viral proteins were examined by immunoblotting with MABs specific for various forms of TP (98) (Fig. 5A) or for core protein V. We

attempted to use MAB 11F11, which recognizes a C-terminal epitope in preTP (Fig. 5A) (100) to compare all of the unprocessed and partially or fully processed forms of preTP in Ad5 and Hr6 particles. However, this antibody reacted strongly with a pair of proteins migrating close to the 50-kDa molecular mass marker, as well as with three more slowly migrating species (data not shown). As it was not possible to identify the TP or its precursor unambiguously with this antibody, we exploited precursor-specific MAB 53E (Fig. 5A) to investigate preTP processing. A significantly greater concentration of the iTP processing intermediate was observed in Hr6 than in Ad5 particles (Fig. 5B). Quantification of the iTP signals shown, as described Materials and Methods, using protein V as an internal control, indicated that the concentration of iTP was 10-fold higher in the mutant virus particles. In a second experiment using Ad5 and Hr6 purified after the infection of cells with independent virus stocks, a 7.8-fold higher concentration of iTP was observed in Hr6 particles. No corresponding differences in the concentration of unprocessed preTP were observed upon longer exposure of MAB 5E3 immunoblots (Fig. 5B). These data indicate that the G315V substitution impairs the final viral protease cleavage that liberates TP but not the initial processing of preTP.

Localization of viral genomes in Ad5- and Hr6-infected cells.

Higher concentrations of viral DNA at 2 h p.i. were observed in Hr6-infected cells than in Ad5-infected cells when DNA was purified from unfractionated cells or from isolated nuclei. However, as noted previously, this difference was less pronounced when DNA was prepared from isolated nuclei. Furthermore, the mild extraction of cells with nonionic detergent used to isolate nuclei (see Materials and Methods) prevents the leakage of pre-mRNA to the cytoplasm (109) but does not remove cytoskeletal components (10, 48). It was therefore important to examine the localization of viral genomes in Ad5- and Hr6-infected cells more directly, by immunofluorescence. To promote the synchronous entry of virus particles, HFFs were incubated with Ad5 or Hr6 for 30 min at 4°C prior to removal of the inoculum and incubation at 37°C, as described in Materials and Methods. Viral genomes were visualized during the initial period of infection by immunofluorescence using polyclonal antibodies against viral core protein VII (42), which remains associated with viral genomes that enter the nucleus throughout the early phase of infection (12, 29, 35, 42, 107). Discrete foci or dots of protein VII were readily detected 2 h after infection with Ad5 in both the nucleus and the cytoplasm (Fig. 6b), but no signal was observed in mock-infected cells (Fig. 6a). By 7 h p.i., the number of protein VII foci detected was somewhat

FIG 5 Comparison of TP precursors in Ad5 and Hr6 virus particles. (A) The preTP is depicted to scale by the rectangle, with the precursor-specific and mature TP sequences shown in white and gray, respectively. The vertical arrows at the top indicate the sites at which preTP is cleaved by the viral L3 protease, and the arrow at the bottom shows the position of the G315V substitution in Hr6 preTP (77, 98). The epitopes recognized by the 5E3 (residues 184 to 200) and 11F11 (residues 608 to 671) anti-preTP MABs (99) are indicated by the bars below the protein. (B) Equal concentrations of proteins recovered from Ad5 and Hr6 particles, purified from equal number of infectious units as described in Materials and Methods, were examined by immunoblotting with the MABs against protein V and preTP indicated at the top. 5E3 (long) indicates longer exposure of the blot shown to the left. The positions of molecular mass markers (sizes are in kilodaltons) are indicated at the left, and those of iTP, TP, and protein V are at the right.

FIG 6 Visualization of viral genomes during the initial period of Ad5 and Hr6 infection. HFFs were infected with 100 PFU/cell Ad5 or Hr6 for the periods indicated as described in Materials and Methods or mock infected (M), and viral genomes were visualized by immunofluorescence using an anti-protein VII polyclonal antibody (42). Nuclei were stained with DAPI (blue). Z stack projections of representative fields are shown.

lower and the majority were localized in nuclei (Fig. 6c). A strikingly larger number of protein VII-associated viral genomes were observed 2 h after Hr6 infection, with a significant decrease by 7 h p.i. (Fig. 6d and e). This result of direct observation of Hr6 genomes is in excellent agreement with the rapid initial decrease in the viral DNA concentration measured by quantitative PCR in Hr6-infected cells (Fig. 3B). At 2 h after Hr6 infection, most protein VII foci were present in the cytoplasm (Fig. 6d). This population decreased substantially by 7 h p.i. (Fig. 6, compare panels d and e), indicating that most of the entering Hr6 genomes are degraded prior to or soon after entry into the nucleus.

Transport of noninfectious Hr6 genomes to the lysosome via

late endosomes, the intracellular destination of noninfectious Ad2ts1 particles that cannot escape early endosomes (see introduction), would account readily for their degradation (Fig. 3). We therefore investigated whether Hr6 genomes were diverted to late endosomes. Cells were infected synchronously for 0.5 or 2.0 h or mock infected, and viral genomes were visualized as described in the previous paragraph, while late endosomes were detected by using a mouse MAb against the small G protein Rab7 (31, 81, 82). Microtubules were also examined, by using a rat anti- β tubulin antibody, as described in Materials and Methods. Rab7-staining late endosomes and microtubules were clearly discernible in both uninfected and infected cells (Fig. 7). However, protein VII

FIG 7 Ad5 and Hr6 genomes do not associate with late endosomes. HFFs were infected with 100 PFU/cell Ad5 or Hr6 for the periods indicated or mock infected (M), and viral genomes were visualized as described in the legend to Fig. 6. Late endosomes, some of which are indicated by orange arrows, and microtubules were stained with mouse anti-Rab and rat anti- β -tubulin-antibodies as described in Materials and Methods.

puncta representing viral genomes were not observed to be compartmentalized with late endosomes, nor did they appear to accumulate along microtubules, in Hr6- or Ad5-infected cells at either 0.5 or 2.0 h after infection (Fig. 7b to e). Furthermore, both Ad5 and Hr6 genomes were seen to congregate around juxtanuclear microtubules by 2 h after infection. Indeed, the only difference between Ad5- and Hr6-infected cells was the presence of protein VII in larger puncta in the latter (Fig. 7, compare panels d and e to panels b and c), as was also evident 2 h after infection in the experiments shown in Fig. 6.

DISCUSSION

Initial attempts to compare viral DNA synthesis when the E1B 55-kDa protein was not made in different types of normal human cells revealed a previously unreported phenotype of the mutant Hr6, a significantly larger number of genomes in an infectious unit than of its parent Ad5 (Table 1). Such poor infectivity cannot be attributed to defects in the initial reactions in the infectious cycle, such as attachment or entry into endosomes by receptor-mediated endocytosis (see introduction); much higher concentrations of viral DNA were also detected in Hr6-infected cells than in Ad5-infected cells 2 h after infection (Table 1). Rather, the majority of intracellular Hr6 DNA molecules were degraded as the infectious cycle progressed (Fig. 3B). This phenomenon was also observed when the quantities of Hr6 and Ad5 DNA that initially entered infected cell nuclei were closely similar (Fig. 3C). As the destruction of mutant DNA cannot be ascribed to induction of host responses by the much higher concentration of viral DNA in Hr6-infected than in Ad5-infected cells following infection at equal multiplicities, we conclude that Hr6 genomes are intrinsically susceptible to extensive intracellular degradation.

This unusual phenotype is not the result of the failure to accumulate the E1B 55-kDa protein in Hr6-infected cells; introduction of the Hr6 E1B frameshift mutation into the phenotypically wild-type background of AdEasyE1 (43) did not result in the reproduction of either entry of greater quantities of mutant than wild-type viral DNA into infected cells or increased degradation of mutant genomes (11). The Hr6 genome must contain at least one additional mutation outside the region previously sequenced (bp 1571 to 3679) (103) that confers the poor-infectivity phenotype. Application of high-throughput sequencing to Ad5 DNA of our laboratory strain, which was derived from that which served as the parent for Hr6 (see Results), and Hr6 DNA identified several such mutations. The Ad5 DNA sequence exhibited two differences from the reference strain (accession no. AY33986.1) (86) (Fig. 4). This small number of differences and the description 20 years ago of the insertion at bp 14073 in another Ad5 sequence (15) emphasize the stability of the genome. Both this insertion and deletion of a TA base pair near the 3' end of the genome (Fig. 4) occurred within long runs of identical base pairs, consistent with slippage errors by the viral DNA polymerase (20). The Hr6 genome was found to contain seven mutations not described previously, five silent and two that introduce amino acid substitutions (Table 2). Like the insertion and deletion in Ad5 DNA discussed above, the GC deletion responsible for the host range phenotype of Hr6 (bp 2347) may be the results of a slippage error during viral DNA synthesis, as it lies in the sequence TTGT. The five transitions (Table 2) represent mutations expected to result from base deamination by nitrous acid, the mutagen used during the derivation of Hr6 (34). The presence of two transversions (bp 2947 and 9655)

was, however, surprising in view of the overall stability of the Ad5 genome: such mutations are not induced upon the exposure of DNA to nitrous acid and must therefore have arisen spontaneously.

As mentioned previously the results of several previous studies and the initial entry of higher concentrations of Hr6 than of Ad5 DNA (Table 1) provide strong evidence that the mutation that results in an A406V substitution in fiber protein IV (Table 2) cannot be responsible for the low infectivity of Hr6 particles. The entry of Hr6 genomes into the cytosol upon escape from endosomes (Fig. 7), a late reaction that depends on the exposure of protein VI upon partial disassembly triggered by the initial loss of fibers bound to Car (see introduction), provides additional support for this conclusion. Furthermore, analysis of the forms of TP present in viral particles indicated that processing of preTP from the intermediate formed by initial cleavage by the viral L3 protease to mature TP (Fig. 5A) is impaired in Hr6 particles (Fig. 5B). Quantification using protein V as an internal control indicated that, relative to Ad5, Hr6 particles contained, on average, an 8.9-fold $\pm$ 1.1-fold higher concentration of iTP. This value is in reasonable agreement with the greater number of genomes per infectious unit of Hr6 (Table 1), particularly if an incompletely processed TP at only one end of a viral DNA molecule is sufficient to render that genome noninfectious. However, the G315V substitution in Hr6 preTP does not alter the TP sequence or the viral protease cleavage site that produces mature TP but rather lies some 34 amino acids nearer the N terminus (Fig. 5A).

Previous studies have established that the sites at which preTP is cleaved by the viral protease are accessible in *in vitro* reactions (98). However, the rate of production of mature TP was much lower than that of iTP formation (100). Furthermore, in infected cells, preTP that is not covalently linked to viral DNA can be processed only to iTP (100). These observations indicate that a conformational change is required upon the packaging of preTP (or iTP) covalently linked to the viral genome to confer access of the protease to the cleavage site (MTGG-V) that forms the N terminus of mature TP. The substitution in Hr6 preTP introduces a bulky Val residue in place of Gly in a sequence that comprises three Gly residues in the wild type. This substitution would be expected to decrease the flexibility of this segment of the precursor (or iTP) and hence impair conformational change. In this context, it is perhaps noteworthy that analysis of the domain organization of preTP with MAbs indicated the importance of conformational change to the ability of the protein to bind to DNA containing the viral origin of replication and to the viral DNA polymerase (99).

The results of quantification of intracellular viral DNA molecules (Fig. 3) and their visualization (Fig. 6) established unequivocally that the majority of Hr6 genomes are degraded within a relatively short period after infection. Nevertheless, mutant genomes were not observed to associate with late endosomes, the destination of noninfectious Ad2ts1 particles (see introduction), indicating that escape from early endosomes into the cytosol was not impaired by the preTP 3915V substitution. Although a significantly larger number of viral genomes, concentrated in the cytosol, was readily visualized early after Hr6 infection (Fig. 6 and 7), no difference in the localization of such mutant versus Ad5 genomes could be discerned (Fig. 7). Furthermore, attempts to visualize specifically Hr6 genomes covalently attached to iTP were not successful. Consequently, the data currently available cannot establish whether noninfectious Hr6 DNA molecules are degraded

prior to nuclear entry or soon after transport into that organelle. Tracking of the movement and intracellular destination of individual viral genomes in real time in living cells is required to distinguish these possibilities. Nevertheless, if degradation were intranuclear, the low rate of loss of entering Hr6 genomes (Fig. 3B) predicts that, during the initial period of infection, a significantly larger number of Hr6 genomes would be present in nuclei than in the cytoplasm, and in Hr6-infected than in Ad5-infected cell nuclei. Neither of these patterns was observed (Fig. 6), suggesting that degradation of Hr6 genomes within the cytoplasm is more likely. This scenario implies that incomplete processing of covalently attached preTP impedes the nuclear entry of genomes, rendering them susceptible to attack by cytoplasmic DNases. Such enzymes include the abundant 3' → 5' exonuclease Trex1, which is responsible for the cytosolic degradation of DNA products of HIV-1 reverse transcription (108), as well as cytoplasmic DNA molecules that can activate innate immune responses (83, 110).

Our conclusion that the fate of entering Ad5 genomes is governed by processing of the covalently attached TP is consistent with previous observations. For example, MAbs that react with only preTP or iTP detected the protein(s) only in discrete nuclear foci thought to be viral replication centers, whereas antibodies that bind to other regions revealed TP throughout the nucleus (100). Furthermore, mutations in precursor-specific segments of preTP have been reported to block the association of entering viral genomes with the operationally defined structure termed the nuclear matrix and transcription of viral intermediate-early and early genes (73), although whether preTP processing was impaired was not determined.

We have reported previously that the E1B 55-kDa protein represses the expression of genes associated with immune defenses, particularly innate and antiviral responses (58). This conclusion, which was based on comparison of cellular gene expression in HFFs infected with Ad5 and Hr6, assumed that the only difference in Hr6-infected cells was the absence of the E1B protein. The phenotypes of Hr6 reported here suggest an alternative mechanism of activation of these cellular genes: the large quantities of viral DNA, probably substantially degraded, that persist in the cytoplasm for a considerable period following Hr6 infection (Fig. 3 and 6) could well be detected by the cytoplasmic sensors of foreign DNA, such as Tlr9, that initiate signaling to induce the transcription of innate immune response genes (3, 94). However, the properties of additional mutants carrying alterations in the E1B 55-kDa protein coding sequence establish unequivocally that the E1B protein acts as a repressor of expression of these genes. The null mutant (AdEasyE1Δ2347) engineered to carry only the Hr6 mutation that prevents the production of the E1B 55-kDa protein (deletion of bp 2347) does not exhibit the reduced-infectivity phenotype of Hr6, and higher concentrations of entering viral genome are not observed in cells infected with this mutant than in those infected with its wild-type parent (11). Nevertheless, the expression of several interferon-inducible genes identified as increased in expression in Hr6-infected cells was also substantially higher in cells infected with AdEasyE1Δ2347 than in cells infected with the wild-type parent (11). Furthermore, this same response is induced by mutations that result in the substitution of specific residues in the E1B 55-kDa protein (J. S. Chahal, C. Gallagher, and S. J. Flint, unpublished data).

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