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Reduced infectivity of adenovirus type 5 particles and degradation of entering viral genomes associated with incomplete processing of the pre-terminal protein.

Running title: An unusual reduced infectivity phenotype of Ad5.

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Abstract

To investigate further the contribution of the adenovirus type (Ad5) E1B 55 kDa protein to genome replication, viral DNA accumulation was examined in primary human fibroblasts and epithelial cells infected by Ad5 or the E1B 55 kDa-null mutant Hr6. Unexpectedly, all cell types were observed to contain a significantly higher concentration of entering Hr6 then of Ad5 DNA, as did an infectious unit of Hr6. However, the great majority of Hr6 genomes were degraded soon after entry. As this unusual phenotype cannot be ascribed to the Hr6 E1B frameshift mutation (11), 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 results in substitutions in virion proteins, G315V in the pre-terminal 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 compared to 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 TP present in purified virus particles indicated that production of mature TP 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.

Introduction

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 transport of viral genomes to and into the infected cell nucleus. The non-enveloped, 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 Coxsackie 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 αv integrins on the cell surface (14, 84) then promote entry of virus particles by clatharin-mediated endocytosis (reviewed in Bai et al, 1993 (1)) (18, 50, 75, 80, 101). 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 Smith et al, 2010 (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 substitution in protein VI that impair membrane lysis activity *in vitro* reduce transduction into cells of viral genomes (56, 59, 60), indicating that this protein mediates lysis of endosomal membranes in

infected cells. The genome-containing, partially dismantled particles that enter the cytosol, which retain the majority of hexons (30) and some protein VI (105), are transported on microtubules, with net movement towards 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 that recognize hexon have been reported to impair 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 ubiquitinylation of this viral protein by Nedd4 family E3 ubiquitin ligases inhibited delivery of the genome to the nucleus, and 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 (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 the major core protein, VII, enters nuclei with the genome, while protein V and remaining capsid subunits are removed (12, 29, 35, 42, 65, 107). Although the mechanism by which viral genomes packaged by protein VII traverse the NPC 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 into the nucleus of viral DNA-protein VII nucleoproteins via importin family receptors (39).

The viral structural proteins IIIa, VI, VII, VIII and Mu are synthesized as larger precursors (preIIIa etc) from which viral particles are assembled (reviewed in (5, 72) The immature particles initially assembled also contain the precursor to 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 non-permissive temperatures of non-infectious particles containing uncleaved precursor proteins (reviewed in (72)). Such non-infectious particles enter early endosomes with normal kinetics, but, in contrast to 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 co-internalized protein synthesis inhibitor α-sarcin to penetrate into the cytoplasm (102). Immature Ad2tsl particles are also more stable to low pH and increasing temperatures than wild-type virions (64, 102).

Comparison of the structures of immature Ad2tsl particles and mature wild-type virions at moderate resolution (≤10Å) by cryo-electron microscopy has identified several differences in the interactions among structural proteins (64, 76). One unique feature of the non-infectious particles is an additional "molecular stitch" between the groups-of-nine hexons (72), and the ring of peripertonal 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 release of vertex capsomers (reviewed in (72)). Additional protein was also observed in non-infectious 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 hexon blocks the membrane lysis activity of proteins VI and preVI *in vitro* (76), this more extensive hexon-preVI interaction seems likely to impair release of pVI from Ad2 ts1 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 pre-TP, 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 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 (NHBECs) were obtained from BioWhittaker, Inc. and cultured using pre-defined media, 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 determined by plaque assay on these same cells as described (104).

Analysis of accumulation of viral DNA. Proliferating or quiescent cells in 35 mm or 6-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 base pairs 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' and ML probe: VIC-ATC GGA AAC CCG TCG GCC TCC-TAMRA. Reactions contained Taqman Universal PCR master mix with AmpErase (Applied Biosystems), 2 µl sample DNA (diluted as necessary), 300 nM of each primer and 200 nM Taqman probe. In the experiments

shown in Figure 3, viral DNA concentrations were assessed 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 SyberGreen 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 ~$5x10^{10}$ p.f.u. by sequential centrifugation in discontinuous CsCl gradients and centrifugation to equilibrium in continuous CsCl gradients (27). The purified particles were dialyzed against 2 changes of 100 volumes of 0.1 M Tris-HCl , pH 8.0, prior to addition of 1 volume of 0.01 M Tris, HCl, pH 8.0 containing 10 mM EDTA, 1% (w/v) SDS, and 1mg/ml proteinase K. Following incubation for 2 hrs at 37° C, nucleic acids were extracted with (1:1) phenol-$CHCl_3$ and ethanol precipitated. The isolated DNA was resuspended in 100 µl 0.5 M Tris-HCl, pH 8.0, and concentrations 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. 1µg 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 flowcell.

Analysis of sequencing data. The Illumina output was analyzed 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), 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, SAECs, or NHBECs at approximately 75-80% confluence were infected with Ad5 or Hr6. Cells were harvested after the periods of infection indicated, washed with phosphate-buffered saline (PBS), and extracts prepared as described (11). Extracts were sonicated in 30 s bursts on ice until sample viscosity decreased, prior to removal of cell debris by centrifugation at 10,000 k g at 4°C for 5 min. Proteins were detected by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblotting as described (25) with monoclonal antibodies (MAb) against the E1A proteins, and the E2 DBP, M73 (33), and B6 (68)

198 respectively. Cellular β-actin as an internal control was visualized with an HRP-labeled anti β-
199 actin MAb (Abcam).

200

201 To examine terminal proteins covalently bound to viral DNA, virus particles were purified from
202 Ad5- and Hr6- infected cells as described above. Purified particles were disrupted by incubation
203 at 60°C for 10 mins (64), and viral DNA digested with 1,250 units/ml benzonase nuclease
204 (Sigma) for 30 mins at 37°C. Terminal proteins were detected by immunoblotting with anti-
205 preTP MAbs , (98) kindly provided by R Hay Protein V, detected by immunoblotting with MAb
206 F58#1 (53) served as an internal control.

207

208 Immunofluorescence. To examine viral replication centers, HFFs grown on coverslips to
209 approximately 90% confluence were mock infected, or infected with Ad5 or Hr6 for various
210 periods, and the cells processed for immunofluorescence as described previously (25). The viral
211 E2 DBP was visualized using the B6 antibody (68) and goat anti-mouse IgG labeled with Cy5
212 (Jackson Immuno Research laboratories Inc.). To visualize viral genome soon after entry, HFFs
213 on coverslips were incubated, with rocking, with Ad5 or Hr6, or DMEM only (mock-infection)
214 at 4°C for 30 min. After removal of the inoculum, cells were washed twice with cold PBS, prior
215 to addition of DMEM + 5% (v/v) bovine growth serum pre-warmed to 37°C. Cells were
216 processed for immunofluorescence as described previously (25), except that they were fixed and
217 permeabilized by incubation in pre-chilled methanol for 10 minutes at -20°C. Protein VII was
218 examined after the periods of infection indicated using purified rabbit polyclonal anti-protein VII
219 antibody (42), kindly provided by D. Engel, and Alexafluor 488-conjugated goat anti-rabbit IgG

(Invitrogen). Nuclei were stained with DAP1, and samples were examined by confocal microscopy as described (25). Late endosomes and microtubules were detected using a mouse anti-Rab7 monoclonal antibody (Rab7-117, Sigma Aldrich), with Cy5 anti-mouse IgG secondary antibody (Jackson Immuno Research Laboratories Inc), and a rat anti-β tubulin antibody (Abcam) with Alexafluor568 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 basepair 2347 alters the coding 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 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 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 small airway epithelial cells (SAECs) infected by a second E1B null mutant, dl1520 (ONYX-015) (63). It is well established that replication of E1B null mutants in established human cells is dependent on 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 SAECs infected by Ad5 or Hr6. We also included

normal human bronchial/tracheal cells (NHBECs) in these experiments: as subgroup C adenoviruses, such as Ad5, are associated with upper respiratory tract infections (reviewed in (106), these cells seemed likely to provide a closer facsimile of natural host cells than either SAECs, 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 30 pfu/cell of each virus. 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 hrs after 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 by 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 results of this analysis of the Ad5 and Hr6 preparations used for the experiments summarized in Table 1A are shown in Table 1B, (experiment 1), and very similar results were obtained when different stocks of the two viruses were compared in the same way (Table 1B, 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 comparison of viral DNA accumulation in cells infected by 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 hrs 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 [^{32}P] – labeled viral DNA (33). Increases in viral DNA concentration were detected by 18 hrs after infection of proliferating SAECs or NHBE cells, 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 hr after Ad5 infection of the two types of epithelial cells than in HFFs (Fig.1 panels B and C). Nevertheless, the Hr6 mutant also exhibited apparent defects in viral DNA accumulation in proliferating SAECs or NHBECs (Fig. 1).

The Hr6 mutation does not impair 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 pre-terminal protein primer and the single-stranded DNA-binding protein (DBP) (see (5, 36, 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 efficient transcription of all early genes (5), and the E2 DBP were compared in proliferating normal human cells infected by Ad5 or Hr6.

Total cell extracts were prepared 24 and 30 hrs 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, NHBECs or SAECs (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, which 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 by Ad5 or Hr6 (Fig 2B) as well as in infected NHBECs (data not shown), but the number of DBP-containing structures was not substantially, or noticeably, higher in Hr6- then 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- 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 by Ad5 (Table 1), neither formation of a larger number of replication centers per infected cell nor increased expression of early genes were observed (Fig 2). This apparent discrepancy suggested that most Hr6 genomes might be degraded before they could serve as templates 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 ± 1.3-fold more nuclear-associated viral DNA at 2 hrs p.i. than did Ad5-infected cell nuclei, somewhat less than when DNA was purified from whole cells (Table 1A). The relative concentrations of viral DNA were lower in Hr6-infected cells 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 hrs. 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 the wild-type, 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 host response(s) triggered by the high concentration of entering viral DNA molecules. To address this possibility, the concentrations of viral DNA after increasing periods of infection were compared in HFFs infected by 30 pfu/cell Ad5, but by 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 Figure 3D. Despite the presence of similar concentrations of viral DNA in Ad5- and Hr6-infected HFF nuclei at 2 hr. p.i., the mutant viral DNA was again reduced substantially in concentration from 6-18 hrs 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 templates for replication cannot be determined accurately from data like those shown in Figure 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 AdEasy E1Δ2347 (43) has established that 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 by AdEasyEΔ12347 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 DNA were subjected to high throughput sequencing. This host-range mutant was isolated by virtue of its impaired replication in HeLa compared to complementing 293 cells (28) following 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. Over 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). 98.51% of reads were successfully mapped to this reference sequence, with the lowest coverage at the most terminal ~150 bp regions at each end of the genome (Figure 4). In these regions, coverage was no less than 16-fold, and their sequence determined by deep sequencing was confirmed by conventional sequencing. This analysis identified two deletions that distinguished our wild type from the reference Ad5 genome (Figure 4), a deletion of one A-T bp at position 14073, from a poly A-T stretch of 13 bp in the reference,

and a deletion of one T-A bp 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 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 Figure 4, with 98.56% of reads successfully aligned to our wild type reference. Nine mutations unique to Hr6 were identified upon alignment to the wild type (Figure 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 the terminal protein precursor (preTP) by 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 by 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 pre-TP is impaired by the G315V substitution

The 671 amino acid E2 preTP serves as the protein 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 encapsidation of viral genomes during assembly of virus particles, in contrast to 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 pre-TP 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 examined by immunoblotting with monoclonal antibodies 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 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 using this antibody, we exploited the precursor-specific MAb 53E (Fig 5A) to investigate preTP processing. A significantly greater concentration of the iTP processing intermediate was observed in Hr6 compared to Ad5 particles (Fig 5B). Quantification of the iTP signals shown, as described Materials and Methods and 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 infection of cells by 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 hrs p.i. were observed in Hr6- compared to 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 non-ionic detergent used to isolate nuclei (see Materials and Methods) prevents 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 immunofluoresence. To promote synchronous entry of virus particles, HFFs were incubated with Ad5 or Hr6 for 30 mins 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 hrs after infection by Ad5 in both the nuclei and cytoplasm (Fig 6, panel b), but no signal was observed in mock-infected cells (Fig 6 panel a). By 7 hr. p.i., the number of protein VII foci detected was somewhat lower, and the majority were localized in nuclei (Fig 6, panel c). A strikingly larger number of protein VII-associated viral genomes were observed 2 hrs. after Hr6 infection, with a significant decrease by 7 hrs. p.i. (Fig 6, panels d and e). This result of direct observation of Hr6 genomes is in excellent agreement with rapid, initial decrease in viral DNA concentration in Hr6-infected cells measured by quantitative PCR (Fig 3B). At 2 hrs. after Hr6 infection, most protein VII foci were present in the cytoplasm (Fig. 6, panel d). This population decreased substantially by 7 hrs. p.i. (Fig 6, compare panels d and e), indicating that most entering Hr6 genomes are degraded prior to or soon after entry into the nucleus.

Transport of non-infectious Hr6 genomes to the lysosome via late endosomes, the intracellular destination of non-infectious 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 hrs, or mock-infected viral genomes visualized as described in the previous paragraph, while late endosomes were detected using a mouse monoclonal antibody against the small G protein Rab7 (31, 81, 82). Microtubules were also examined, 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 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 hrs. after infection (Fig. 7, panels b-e). Furthermore, both Ad5 and Hr6 genomes were seen to congregate around juxtanuclear microtubules by 2 hrs. after infection. Indeed, the only difference between Ad5- and Hr6-infected cells was the presence of protein VII is larger puncta in the latter (Fig. 7, compare panels d and e to panels b and c), as was also evident 2 hrs. after infection in the experiments shown in Figure 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 compared to 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-, compared to Ad5-, infected cells 2hrs. 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- compared to Ad5- infected cells following infection at equal multiplicity, 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 reproduction of either entry of greater quantities of mutant compared to 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 (base pairs 1571 to 3679) (103) that confers the poor infectivity phenotype. Application of high throughput sequencing to Ad5 DNA of our lab. strain, which was derived from that that 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 (AY33986.1, (86)) (Fig. 4). This small number of differences, and the description 20 years ago of the insertion at bp14073 in another Ad5 sequence (15), emphasize the stability of the genome. Both this insertion and deletion of a TA basepair near the 3′ end of the genome (Fig. 4) occurred within long runs of identical basepairs, 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 derivation of Hr6 (34). The presence of two transversions (bp2947 and 9655) was, however, surprising in view of the overall stability of the Ad5 genome: such mutations are not induced upon exposure of DNA to nitrous acid and must therefore, have arisen spontaneously.

As mentioned in the Results, 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 3) 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 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 forms of the 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 ± 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 non-infectious. However, the G315V substitution in Hr6 preTP does not alter the TP sequence or the viral protease cleavage site that produced mature TP, but rather lies some 34 amino 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 slower than that of formation of iTP (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 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 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 using monoclonal antibodies indicated the importance of conformation 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 non-infectious 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 H6 infection (Figs 6 and 7), no difference in the localization of such mutant compared to 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 non-infectious 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 will be required to distinguish these possibilities. Nevertheless, if degradation were intranuclear, the slow 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-compared to Ad5-infected cell nuclei. Neither of these patterns were observed (Fig. 6), suggesting that degradation of Hr6

genomes within the cytoplasm is more likely. This scenario implies that incomplete processing of the covalently attached preTP impedes nuclear entry of genomes, rendering them susceptible to attack by cytoplasmic deoxyribonucleases. 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, monoclonal antibodies 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 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 expression of genes associated with immune defenses, particularly innate and anti-viral responses (58). This conclusion, which was based on comparison of cellular gene expression in HFFs infected by 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 (Figs. 3 and 6) could well be detected by the cytoplasmic sensors of foreign DNA, such as Tlr9, that initiate signaling to induce 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 production of the E1B 55 kDa protein (deletion of bp 2347) does not exhibit the reduced infectivity phenotype of Hr6, nor are higher concentrations of entering viral genome observed in cells infected by this mutant compared to its wild type parent (11). Nevertheless, expression of several interferon-inducible genes identified as increased in expression in Hr6-infected cells was also substantially higher in cells infected by AdEasyE1Δ2347 than by the wild type parent (11). Furthermore, this same response is induced by mutations that result in substitution of specific residues in the E1B 55 kDa protein (J.S.C., C. Gallagher, and S.J.F., manuscript in preparation).

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Figure 1: Defects in accumulation of viral DNA in proliferating SAECs, NHBE cells and HFFs infected by Hr6. Proliferating cells were infected with 30 pfu/cell Ad5 or Hr6, and DNA was isolated from cells harvested 2, 18, 24 and 30 hours 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 hours after infection. Results represent the average of two independent experiments, and error bars the standard deviation.

Figure 2: The E1B 55kDa protein is not required for synthesis of E2 proteins or 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 the anti-E2 DBP MAb B6 and an anti-β-actin antibody. **B.** Proliferating HFFs cells were infected with Ad5 or Hr6 or mock-infected, and processed for immunofluorescence 24 hrs after infection, as described in Materials and Methods. The viral E2 DNA binding protein was detected using the B6 antibody and cyanine 5-labeled anti-mouse IgG, and is shown false-colored in green. Nuclei were stained with DAP1 (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 NHBECs (**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).

Figure 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 determined by quantitative PCR as described in Materials and Methods. The values, which were corrected for the concentrations determined in parallel of cellular GADPH DNA, are expressed relative to that measured at 2 hrs p.i., and represent the average of two independent experiments. Error bars show standard deviations. **B.** The 2 to 18 hr data from the experiments shown in panel B are replotted with an expanded y axis. **C.** Cells were infected with equivalent numbers of Ad5 or Hr6 genomes, and intranuclear viral DNA concentrations 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 the standard deviations.

Figure 4: Differences between the sequences of the Ad5 and Hr6 genomes. The horizontal line at the top represents the Ad5 genome, in kbp. The fold coverage (FC) of sequence reads used to assemble the sequences of Ad5 and Hr6 DNA as described in Materials and Methods are 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 text). The Hr6 mutations shown in blue and red indicate the previously described E1B 55 kDa coding sequence mutations (103) and new discovered mutations in the preTP and fiber genes that introduce substitutions, respectively.

921 **Figure 5: Comparison of TP precursors in Ad5 and Hr6 virus particles. A.** The preTP is
922 depicted to scale by the rectangle, with precursor-specific and mature TP sequences shown in
923 white and gray, respectively. The vertical arrows drawn above indicate the sites at which preTP
924 is cleaved by the viral L3 protease, and the arrowhead below the positions of the G315V
925 substitution in Hr6 preTP (77, 98). Epitopes recognized by the 5E3 (residues 184-200) and
926 11FH (residues 608-671) anti-preTP MAbs (99), are indicated by the bars drawn below the
927 protein. **B.** Equal concentrations of proteins recovered from Ad5 and Hr6 particles, purified
928 from equal number of infectious units as described in Materials and Methods, were examined by
929 immunoblotting with MAbs against protein V, and preTP indicated at the top. 5E3 (long)
930 indicates longer exposure of the blot shown to the left. The positions of molecular mass markers
931 are indicated at the left, and those of the intermediate in preTP processing (iTP) and protein V at
932 the right.

933

934 **Figure 6: Visualization of viral genomes during the initial period of Ad5 and Hr6 infection.**
935 HFFs were infected with 100 pfu/cell Ad5 or Hr6 for the periods indicated as described in
936 Materials and Methods, or mock-infected (M), and viral genomes visualized by
937 immunofluorescence using a polyclonal anti-protein VII antibody (42). Nuclei were stained with
938 DAP1 (blue). Z-stack projections of representative fields are shown.

939

940 **Figure 7: Ad5 and Hr6 genomes do not associate with late endosomes.** HFFs were infected
941 with 100 pfu/cell Ad5 or Hr6 for the periods indicated, or mock-infected (M), and viral genomes
942 visualized as described in the legend to Figure 6. Late endosomes, some of which are indicated

943 by the orange arrows, and microtubules were stained using mouse anti-Rab and rat anti-β-
944 tubulin-antibodies as described in Materials and Methods.

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

	[DNA][a]		
	Hr6	**Ad5**	**Hr6:Ad5**
A. Entering Viral DNA[b]			
HFF	12.1±0.09	0.28±0.01	43.2
SAEC	7.96±0.24	0.38±0.01	20.9
NHBE	7.25±1.16	0.20±0.02	36.3
B. Viral DNA in 1000 pfu.			
Experiment 1	14,147	1064	13.3
Experiment 2	6150	416	14.8

[a]Viral DNA concentrations are in arbitrary units
[b]Viral DNA 2 hr after infection of proliferating cells

945 **Table 2: Mutations identified in the Hr6 compared to the Ad5 genome.**

Site (bp)	Mutation	Genomic location	Consequence
2347	G deletion	E1B 55 kDa CDS	Frameshift, and truncation[a]
2947	G→T	E1B 55 kDa CDS	Sbustitution aa 310: TGT (C)→TTT (F)[a]
9655	C→A	PreTP CDS	Substitution 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 aa406: GCT (A)→GTT (V)
34193	C→T	E4 Orf4 CDS	Silent: AGG (R)→AGA (R)

946

947 [a] As previously reported (103)