JVI Accepts, published online ahead of print on 25 January 2012
J. Virol. doi:10.1128/JVI.06764-11

Timely synthesis of the adenovirus type5 E1B 55 kDa protein is required for efficient genome replication in normal human cells.

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Running Title: E1B 55 kDa protein and viral DNA synthesis

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Abstract word count: 186

Text word count: 4,716

Abstract

Previous studies have indicated that the adenovirus type 5 E1B 55 kDa protein facilitates viral DNA synthesis in normal human fibroblasts, but not in primary epithelial cells. To investigate this apparent difference further, viral DNA accumulation was examined in primary human fibroblasts and epithelial cells infected by the mutant AdEasyE1Δ2347, which carries the Hr6 frameshift mutation that prevents production of the E1B 55 kDa protein, in an E1-containing derivative of AdEasy. Impaired viral DNA synthesis was observed in normal human fibroblasts (HFFs), but not in normal human bronchial epithelial cells infected by this mutant. However, acceleration of progression through the early phase, which is significantly slower in HFFs than in epithelial cells, eliminated the dependence of efficient viral DNA synthesis in HFFs on the E1B 55 kDa protein. These observations suggest that timely synthesis of the E1B 55 kDa protein protects normal cells against a host defense that inhibits adenoviral genome replication. One such defense is mediated by the Mre11-Rad50-Nbs1 complex. Nevertheless, examination of the localization of Mre11 and viral proteins by immunofluorescence suggested that this complex is inactivated similarly in AdEasyE1Δ2347- and AdEasyE1- infected HFFs.

Introduction

The E1B gene of species C human adenoviruses, such as adenovirus type 5 (Ad5), encodes unrelated proteins of 19 and 55 kDa that contribute to optimizing the environment for efficient viral replication within infected cells. The 1kDa protein blocks apoptosis in infected cells (24, 66, 87, 101), and was the first viral homologue of cellular anti-apoptotic proteins to be identified (20, 86, 89). The E1B 55 kDa protein also counteracts cellular responses to infection that would be detrimental to efficient virus reproduction. One of the first properties to be ascribed to the E1B 55 kDa protein was interaction with the cellular tumor suppressor p53 (77). In rodent cells transformed by E1A and E1B gene products, this interaction can sequester p53 in juxtanuclear cytoplasmic structures (13, 42, 109). Binding of the E1B 55kDa protein to the N-terminal activation domain of p53 has also been reported to inhibit p53-dependent transcription both in *in vitro* reactions and in transient expression systems (54, 107, 108). Insertions or substitutions in the E1B protein that impaired E1B-dependent transcriptional repression were observed to reduce the ability of the E1B protein to cooperate with E1A gene products in transformation of rodent cells (55, 90, 91, 108). Inhibition of p53-dependent transcription and transforming activity have also been reported to be reduced by a substitution that prevents sumoylation of the E1B 55 kDa protein at Lys104 (28), whereas substitutions that block shuttling of this protein between the nucleus and cytoplasm (25, 47) stimulate both activities (27). These observations indicate that inhibition of the transcriptional function of p53, and presumably of induction of apoptosis by this cellular protein, are important for the transforming activity of the E1B 55kDa protein.

In adenovirus-infected cells, the E1B 55kDa protein is necessary for degradation of p53, as is the E4 Orf6 protein (15, 16, 36, 61, 64, 69, 70, 75, 78, 82). The p53 protein is a substrate of viral/cellular E3 ubiquitin ligase (the Ad E3 Ub ligase) formed by assembly of the E1B 55kDa and E4 Orf6 proteins with the cellular proteins cullin 5, elongins B and C and Rbx1, and is

targeted for proteasomal degradation by the action of this enzyme (19, 39, 53, 69). Although the Ad E3 Ub ligase is necessary to prevent accumulation of p53, the results of both genome-wide analyses of cellular gene expression (58) and examination of expression of subsets of p53-responsive genes (41, 64) indicate that, in several different cell types, the p53 protein that accumulates is transcriptionally inactive. Nor does it induce apoptosis (16, 64). It therefore appears that in infected cells one or more additional viral gene products function redundantly with the Ad E3 Ub ligase to ensure that p53 cannot trigger apoptosis or G1 arrest. One such gene product is the E4 Orf3 protein, which has been reported to induce inhibition of p53-dependent transcription in infected small airway epithelial cells (SAECs) (80).

Another function of the E1B 55 kDa protein is induction of selective export of viral late mRNAs from the nucleus to the cytoplasm (67, 102). Such selective export depends on the interaction of the E1B 55 kDa protein with E4 Orf6 (see (12, 31)), and assembly of the Ad E3 Ub ligase (14, 105), although the relevant substrates have not yet been identified. Other known substrates of this enzyme include integrin α3, degradation of which may facilitate release of viral particles at the end of the infectious cycle (23), DNA ligase IV (7) and the Mre11 and Rad50 proteins (83). The latter two proteins and Nbs1 form the MRN complex, which detects, and initiates signaling in response to, double-stranded breaks in the genome, ultimately leading to non-homologous end joining or recombinational repair (reviewed in (22), (73),(96), (50), (85)). The function of the MRN complex is also blocked by the E4 Orf3 protein of species C adenoviruses, which induces recruitment of Mre11 and Rad50 to intranuclear, track-like structures that also contain cellular proteins reorganized from Pml bodies (17, 26, 30, 51, 83, 84). The E4 Orf3 protein has also been reported to colocalize with Mre11 in juxtanuclear cytoplasmic structures with the properties of aggresomes (3, 52). When both the formation of the Ad E3 Ub ligase and relocalization of Mre11 by the E4 Orf3 protein are prevented by mutation, viral DNA synthesis is impaired (29, 48, 56), and large concatemers of randomly orientated copies of the

viral genome accumulate very late in infection (83, 100). As such concatemers are far too large to be packaged into capsids, their formation presumably reduces production of progeny virus particles. This phenomenon would also impair initiation of viral DNA synthesis by sequestration of the terminal origins of replication at internal positions within concatemers. Nevertheless, several lines of evidence indicate that the inhibition of viral DNA synthesis observed when MRN components are not inactivated in Ad5 infected cells is not the result of formation of concatemers (29, 48, 79). Although the severe defects in viral DNA synthesis observed in infected cells when MRN components cannot be degraded or sequestered are relieved in cells that lack Mre11 or Nbs1 (30, 48, 56), the mechanism by which MRN components inhibit viral DNA synthesis is not yet well understood. When not relocalized and targeted for proteasomal degradation, Mre11 has been observed to associate with viral genomes in viral replication centers (56, 57, 83). It is therefore possible that recruitment of this and other damage response proteins to viral genomes blocks recognition of viral origins, or subsequent reactions in viral DNA synthesis.

With few exceptions, the studies summarized in previous paragraphs were performed using HeLa or other established lines of human cells as hosts. Such cells, most of which were derived from human tumors, are, by definition, immortal, and proliferate rapidly and under conditions (e.g. contact inhibition) in which normal cells do not. Furthermore, they are genetically abnormal, for example, carrying mutations that contribute to bypass of the circuits that regulate cell-cycle progression and checkpoint responses, and likely to differ in genotype from one another. These properties raise the possibility that some functions of adenoviral proteins necessary for efficient replication in normal host cells may be dispensable in transformed cells. Consistent with this view, the 243R E1A protein is required for efficient viral DNA synthesis in normal human lung fibroblasts, but not in HeLa cells (81). We therefore initiated investigations of the roles played by the E1B 55 kDa protein during Ad5 replication in normal human cells. One unexpected observation was that, in the absence of this protein, viral DNA

synthesis was impaired in proliferating human fibroblasts (32), although it is not in HeLa and other lines of transformed human cells (5, 34, 38, 67, 102). Furthermore, McCormick and colleagues had previously reported that no differences in viral DNA synthesis were observed in quiescent small airway epithelial cells infected by wild-type virus or the E1B 55kDa null mutant Onyx-015 (dl1520) (64). The studies reported here were initiated in an attempt to resolve this apparent discrepancy.

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, respectively. Primary human bronchial/tracheal epithelial cells (NHBECs) were obtained from BioWhittaker, Inc. and cultured using pre-defined media, BEGM, and growth conditions according to the manufacturer's recommendations. Cells were considered to be in the proliferative phase when ≤90% confluent, whereas cells in the quiescent state were obtained by prolonged incubation (≥ 4 days) after contact inhibition was observed. The construction of a phenotypically wild-type derivative of AdEasy (40) containing the E1A and E1B genes (AdEasy E1) was described previously (44). To introduce a GFP reporter gene into this background, the segment of pShuttleE1 (44) from the BamHI site downstream of the left arm (40) to the NotI site at bp 364 (40) was replaced with the corresponding fragment of pAdTrack-CMV (40). The resulting plasmid, pShuttle E1-G, contains the expression cassette comprising the human cytomegalovirus immediate early promoter/enhancer, the eGFP coding sequence and a poly(A) addition site from pADTrack-CMV immediately upstream of, and in inverse orientation to, the E1A transcription unit. Recovery of this modified E1A region into the AdEasy-1 genome to create AdEasyE1-G by homologous recombination in *E. coli*, introduction of the Hr6 frameshift mutation (deletion of bp2347 in Ad5 DNA (102)) into this background, and isolation of viruses were as described for AdEasyE1 and AdEasyE1Δ2347 (44). Phenotypically wild-type (AdEasyE1, and AdEasyE1-G) and E1B 55 kDa null mutants. AdEasyE1Δ2347 and AdEasyE1Δ2347-G) were propagated in monolayers of 293 cells. Viruses were titrated by plaque assay on these same cells as described (103).

142 Analysis of accumulation of viral DNA. Proliferating or quiescent cells in 6-well dishes were
143 infected in parallel with wild-type virus and the corresponding E1B 55 kDa null mutant (e.g.
144 AdEasyE1 and AdEasyE1Δ2347), and harvested after increasing periods of infection. DNA was
145 purified from nuclei isolated as described previously (32), or by using the DNeasy tissue kit
146 (Qiagen) according to the manufacturer's protocol. Quantitative real-time PCR was carried out
147 using the ABI PRISM 7900HT sequence detection system, and SyberGreen detection of an
148 amplicon within the ML transcription units, 90 base pairs long (nucleotides 7128 to 7218). The
149 primer set was as follows: ML Fwd: 5'-ACT CTT CGC GGT TCC AGT ACT C-3', ML Rev: 5'-
150 CAG GCC GTC ACC CAG TTC TAC-3'. Reactions contained 2-4 µl sample DNA (diluted as
151 necessary), 300 nM each primer, and Power SYBR Green mastermix (Applied Biosystems). To
152 provide an internal control, concentrations of cellular DNA were determined in parallel, using
153 primers for an amplicon within the promoter of the human glyceraldehyde-3 phosphate
154 dehydrogenase (GAPDH) promoter. The forward primer corresponded to positions 6513800-
155 6513820 of human chromosome 12 reference assembly, genebank accession number
156 NC_000012.10 (5□-TACTAGCGGTTTTACGGGCG-3□) and the reverse primer was
157 complementary to positions 6513942-6513965 (5□-TCGAACAGGAGGAGCAGAGAGCA-
158 3□). PCR cycles were programmed as follows: two initial steps at 50°C for 2 min and 95°C for
159 10 min, and then 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Relative DNA concentrations
160 were determined by the standard curve method. All measurements were performed in triplicate.

161

162 Immunoblotting. HFFs or NHBECs at approximately 75-80% confluence were infected with
163 wild type or E1B 55 kDa null mutant viruses. Cells were harvested at the times after infection
164 indicated, washed with phosphate-buffered saline (PBS), and extracted with 25 mM TrisHCl, pH
165 8.0, containing 50 mM NaCl, 0.5% (w/v) sodium deoxycholate, 0.5% (v/v) Nonidet P-40 (NP-40)
166 and 1 mM phenylmethylsulfonyl fluoride for 30 min at 4°C. Extracts were incubated with 125

units Benzonase® nuclease (Sigma) for 30 minutes at 37°C, and cell debris removed by centrifugation at 10,000 X g at 4°C for 5 min. The extracts were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblotting as described previously (33). The E2 DBP was detected with the monoclonal antibodies (MAb) B6 (71) and cellular β-actin, as an internal control, with an HRP-labeled anti β-actin MAb (Abcam).

Immunofluorescence. HFFs grown on coverslips to approximately 90% confluence were mock infected, or infected with wild type or E1B 55 kDa null mutant viruses for various periods, and the cells processed for immunofluorescence as described previously (33). The viral E2 DBP was visualized using the B6 antibody (71) and donkey anti-mouse IgG labeled with Alexa Fluor™488 (Jackson Immuno Research laboratories Inc.) and the E4 Orf3 protein by using the rat monoclonal antibody 6A11 (62) and Alexa Flour™ 568-conjugated goat anti-mouse IgG (Invitrogen). The cellular Mre11 protein was examined using a rabbit polyclonal antibody (GeneTex) and Cy5-donkey anti-rabbit IgG (Jackson Immuno Research Laboratories Inc) secondary antibody. The coverslips were mounted on glass slides in Aqua Polymount (Polysciences Inc.), and samples were examined by confocal microscopy as described previously (33). Images were organized using Adobe Photoshop 7.0.

Results

The dependence of viral DNA synthesis in normal human fibroblasts and epithelial cells on the E1B 55 kDa protein.

In initial studies of viral replication in normal human cells, we observed that accumulation of viral DNA was impaired in HFFs infected by the E1B 55 kDa-null mutant Hr6 (32). In subsequent experiments, Hr6-infected normal cell nuclei have been found to contain 20 to 30-fold higher concentration of viral DNA at 2 hrs. p.i. than nuclei from cells infected in parallel with an equal multiplicity of Ad5 (S. Kato, J.S.C. and S. J.F., in preparation). This property, and the subsequent extensive degradation of Hr6 genomes, precluded meaningful interpretation and comparison of temporal changes in the concentrations of viral DNA in Ad5- and Hr6- infected cells. To investigate further the contribution of the E1B 55 kDa protein to viral DNA synthesis in normal human cells, we therefore exploited a mutant (AdEasyE1Δ2347) that carries the Hr6 frameshift mutation in the background of a phenotypically wild-type derivative of AdEasy (40), which includes the E1A and E1B genes (AdEasyE1)(44). As reported elsewhere (44), no E1B 55 kDa protein can be detected in HeLa cells or HFFs infected by AdEasy E1Δ2347, as expected, and this mutant reproduced the defects in viral late gene expression observed in cells infected by other E1B 55 kDa null mutants (6, 35, 38, 67, 102).

HFFs or NHBECs were infected in parallel with 30 pfu/cell or 5 pfu/cell, respectively, AdEasyE1 or AdEasyE1Δ2347, and the concentrations of viral DNA entering nuclei by 2 hours after infection measured as described in Materials and Methods. Similar concentrations of intranuclear DNA were observed in wild-type and mutant-infected HFFs or NHBECs (Table 1), indicating that a mutation other than the E1B 55 kDa coding sequence frameshift mutation (deletion of bp2347) is responsible for the poor infectivity of Hr6 virus particles. The closely similar infectivities of AdEasyE1 and AdEasyE1Δ2347 were therefore exploited to assess unambiguously the impact of failure to produce the E1B 55 kDa protein on viral DNA synthesis in normal human fibroblasts and epithelial cells.

Proliferating HFFs were infected with the wild-type and mutant viruses, and the concentrations of intranuclear DNA measured after increasing periods of infection by quantitative

PCR, as described in Materials and Methods. The concentration of viral DNA was observed to decrease somewhat between 2 and 18 hours after infection, but by a similar factor in AdEasyE1- and AdEasyE1Δ2347- infected cells (Fig 1A). In both cases, viral DNA concentrations increased thereafter, in agreement with results of previous analysis of the kinetics of the viral infectious cycle in HFFs (32). However, viral DNA synthesis was less efficient in AdEasyE1Δ2347-infected cells, which contained a 10-fold lower concentration of viral DNA than did wild type-infected cells at 36 hours after infection (Fig 1A). As illustrated in Figure 1B, the difference in the accumulation of viral DNA in AdEasy E1Δ2347 -compared to wild type- infected cells was lower later in infection (44 hours p.i.) than at around the time of the onset of viral DNA synthesis (22-24 hours p.i.), suggesting that this process is delayed in HFFs in the absence of the E1B 55 kDa protein. A similar impairment in viral DNA synthesis was observed when quiescent HFFs were infected by the E1B kDa-null mutant (Fig. 1C).

In an alternative approach to assess viral DNA synthesis, the formation of viral replication centers containing the E2 DNA binding protein (DBP) was compared in HFFs infected by AdEasyE1 or AdEasy E1Δ2347. In adenovirus-infected cell nuclei, the DBP forms two morphologically distinct structures, small dot-like foci and larger, globular ring-like structures (88, 98). 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 (60, 68, 98), do not appear when viral DNA synthesis is blocked by drugs or mutations (88, 97). We have reported previously that synthesis of the DBP is not impaired in AdEasyE1Δ2347-infected HFFs (44). Replication centers were therefore examined by immunofluorescence as described in Materials and Methods. Striking differences were observed: the majority of AdEasyE1- infected cells contained enlarged rings of DBP, which are formed upon initiation of viral DNA synthesis (88, 98), whereas DBP was observed either in small foci or as diffuse nuclear staining in most AdEasy E1Δ2347-infected cells (Fig. 2A). Quantification of

the different patterns of intranuclear localization of DBP indicated that the enlarged ring-like structures characteristic of replicating viral DNA developed in over 90% of AdEasyE1-infected cells, but in less than 30% of those infected by the mutant (Fig. 2B). These observations indicate that the E1B 55 kDa protein is required for efficient genome replication in Ad5-infected HFFs.

We next compared the temporal changes in viral DNA concentration in normal human bronchial/tracheal cells (NHBECs) infected by AdEasyE1 or AdEasyE1Δ2347. As subgroup C adenoviruses, such as Ad5, are associated with upper respiratory tract infections (reviewed in (104), these cells seemed likely to provide a closer facsimile of natural host cells than either HFFs or SAECs used in other studies (63), which were derived from the lower respiratory tract. Proliferating NHBECs, which are significantly more infectable than HFFs (32), were infected with 5 pfu/cell, and viral DNA concentrations measured at various times thereafter. In these cells, significantly higher concentrations of viral DNA were detected than in HFFs (compare Figs 3A and 1A), a property also observed in SAECs (data not shown). In contrast to the results obtained in HFFs, the kinetics and efficiency of viral DNA synthesis were essentially indistinguishable in AdEasyE1- and AdEasyE1Δ2347-infected cells (Fig. 3A), nor was any significant difference detected when quiescent NHBECs were infected (Fig. 3B).

Acceleration of early phase progression in HFFs restores efficient genome replication in the absence of the E1B 55 kDa protein.

Comparison of genome replication of AdEasyE1 in HFFs and NHBEs demonstrated not only more efficient viral DNA synthesis in the latter cell type, but also differences in the kinetics of this process. In wild-type-infected NHBECs, viral DNA synthesis was well underway by 16 hours p.i., when the relative viral DNA concentration had increased some 200-fold (Fig. 3A). In contrast, no genome replication was detected at this time after AdEasyE1 infection of HFFs, and by 24 hours p.i. the quantity of intranuclear viral DNA had increased by a factor of only 4 (Fig.

1A). The delayed onset of viral DNA synthesis in HFFs is consistent with our previous observations that in these cells viral immediate early E1A proteins cannot be detected until 14 – 16 hours after infection (32). Indeed, the E2 DBP does not accumulate to a significant concentration until 24 hours after infection (Fig 4A). In contrast, this viral replication protein was readily detected by 12 hrs. after infection of NHBECs with AdEasy E1 (Fig. 4B), consistent with the earlier onset of viral DNA synthesis in these cells (Fig. 3A). Because of this difference in the rate of progression through the early phase, it was not clear whether the E1B 55 kDa protein were required to promote viral DNA synthesis specifically in fibroblasts, or whether some inhibitory mechanism, blocked by this viral protein, became activated during the extended period before initiation of genome replication in HFFs. To distinguish between these possibilities, we exploited derivatives of AdEasyE1 and AdEasyE1Δ2347 that carry the coding sequence for eGFP under the control of the human cytomegalovirus (HCMV) immediate early (IE) promoter/enhancer upstream of, and in inverse orientation to, the E1A transcription unit. As illustrated in Figure 4C, synthesis of the E2 DBP was evident by 9 hrs. after infection of HFFs by the wild-type derivative, AdEasyE1-G, and had accumulated to a much higher concentration by 12 hrs. p.i. These data indicate that insertion of the expression cassette led to considerably accelerated progression through the early phase of infection in HFFs, presumably as a result of activation of E1A transcription by the HCMV IE enhancer. We therefore compared viral DNA synthesis in proliferating HFFs infected with AdEasyE1-G or AdEasyE1Δ2347-G, by the methods described previously. In contrast to the results shown in Figure 1, no differences were observed in the kinetics or efficiency of viral DNA synthesis in mutant- compared to wild type-infected cells, and in both cases, an increase in intranuclear viral DNA concentration, albeit modest, was detected by 16 hrs. p.i. (Fig. 5). Although accelerated synthesis of viral early proteins eliminated the dependence of viral DNA synthesis in HFFs on the E1B protein, the relative quantity of viral DNA made by 36 hrs. p.i. remained lower than observed in NHBECs

(compare Figs 3A and 5), suggesting that cell type-specific differences govern the degree of amplification of the viral genome.

Localization of Mre11 in infected HFFs

The E1B 55kDa protein neither participates directly in viral DNA synthesis (10), nor facilitates production of viral replication proteins during the early phase of infection in transformed or normal human cells (4, 35, 44, 49, 74). The results described in the previous section therefore suggest that, when synthesized in timely fashion, this early protein protects against inhibition of viral DNA synthesis by a cellular defense mechanism, such as the double-stranded DNA break repair response. As discussed in the Introduction, the activity of the Ad E3 Ub ligase targets proteins of the cellular MRN complex for proteasomal degradation, and viral DNA synthesis is inhibited in established lines of human cells when both assembly of this infected cell-specific enzyme and synthesis of the viral E4 Orf3protein are prevented by mutation. Consistent with these previous studies, the steady state concentration Mre11 was observed to decrease more slowly in HFFs infected by AdEasyE1Δ2347 than in AdEasyE1-infected cells (44). We therefore wish to compare the relocalization of Mre11 by E4 Orf3 in normal human cells in the presence and absence of the E1B 55kDa protein. Proliferating HFFs were infected in parallel with AdEasyE1 or AdEasyE1Δ2347 for 24 hours, and viral replication centers (E2 DBP), the E4 Orf3 protein and Mre11 visualized by immunofluorescence using mouse, rat and rabbit primary antibodies, respectively, as described in Materials and Methods.

In uninfected HFFs, Mre11 was concentrated in nuclei, where it was excluded from nucleoli (Fig 6, panels a and b). Upon infection, the nuclear Mre11 signal was reduced (compare panels g and l with panel b), as expected (see Introduction). In AdEasyE1- infected cells, the cellular protein was observed in discrete fleck-like structures not present in uninfected cells (Fig 6, panel g, orange arrows). In these structures, Mre11 was localized with the E4 Orf3 protein,

and was not associated with viral replication centers (Fig 6, panels g, k, I and j), as initially observed in Ad5-infected established human cell lines (30, 83). These same changes in the properties of Mre11, reduced intranuclear concentration, reorganization to structures that contained the E4 Orf3 protein and lack of association with viral replication centers, were observed in HFFs infected by AdEasyE1Δ2347 (Fig 6A, panels l-o). Furthermore, examination of ~100 cells infected by the mutant or its wild type parent indicated that the number of infected cells in which Mre11 was localized with the E4 Orf3 proteins was not reduced in the absence of the E1B 55 kDa protein, but rather increased somewhat, from 51.2% to 73.6%. This difference is probably a consequence of the delayed degradation of Mre11 when the Ad E3 Ub ligase is not present in infected cells.

Discussion

The apparent discrepancies in the dependence of viral DNA synthesis in normal human cells on the E1B 55 kDa protein reported previously (32, 63) have been investigated further by exploiting a mutant (AdEasyE1Δ2347) that carries the Hr6 E1B frameshift mutation in the AdEasyE1 genome (44): this mutant reproduces such phenotypes of Hr6 (and other E1B 55 kDa null mutants) as impaired expression of viral late genes (44), but does not exhibit (Table 1) the low infectivity of Hr6 that will be described elsewhere (S. Kato, J.S.C. and S.J.F., in preparation). Comparison of the accumulation of viral DNA in cells infected by this mutant and its wild type parent indicated that viral genome replication is impaired in proliferating and quiescent normal human fibroblasts in the absence of the E1B 55 kDa protein (Fig. 1), consistent with our previous observation that viral DNA synthesis was reduced in HFFs infected not only by Hr6, but also by a mutant (H224) that carries a 4 amino acid insertion mutation in the E1B 55 kDa protein coding sequence (32). However, no such defect was detected in normal human bronchial/tracheal

336 epithelial cells (Fig. 3). It has been reported previously that viral DNA synthesis is not defective
337 in small airway epithelial cells infected by the E1B 55 kDa null mutant Onyx-015 (a.k.a. dl1520)
338 (63). Nevertheless, the difference in the dependence of viral DNA synthesis on the E1B protein
339 observed between normal human fibroblasts and epithelial cells does not appear to represent yet
340 another example of the well-documented variation in the efficiency of replication of E1B 55 kDa
341 null mutants with host cell type (11, 35, 38, 76, 92): when introduction of the HMCV IE
342 promoter/enhancer into the viral genome accelerated expression of early genes and progression
343 through the early phase in infected HFFs (Fig 4), no defect in viral DNA synthesis was observed
344 in the absence of the E1B 55 kDa protein (Fig 5). We therefore propose that synthesis of this
345 protein within a prescribed period after initiation of the infectious cycle is necessary to allow
346 maximally efficient viral DNA synthesis, most probably by countering a cellular defense
347 mechanism.

348 When the Ad E3 Ub ligase cannot assemble in transformed human cells, relocalization of
349 the cellular Mre11, Rad50 and Nbs1 protein by the species C adenovirus E4 Orf3 protein (84) is
350 necessary to block inhibition of viral DNA synthesis by the MRN complex (30). If the E4 Orf3
351 protein is also absent, Mre11 and other MRN proteins become localized with E2 DBP-containing
352 viral replication centers (56, 57, 83), where Mre11 binds to viral DNA (57). Although
353 degradation of Mre11 is delayed in HFFs infected by AdEasyE1Δ2347 compared to its
354 phenotypically wild type parent (44), this cellular protein was observed to be sequestered with E4
355 Orf3 in both wild type- and mutant- infected cells (Fig 6). As such relocalization of Mre11 is
356 sufficient to prevent inhibition of viral DNA by the MRN complex (30), it is highly unlikely that
357 this complex is responsible for the impaired genome replication observed in AdEasyE1Δ2347-
358 infected HFFs.

359 The E1B 55kDa protein-containing Ad E3 Ub ligase targets a number of other cellular
360 proteins for proteasomal degradation (see Introduction) and has been reported more recently to

function as a Sumo1 E3 ligase (59, 65)). It is therefore possible that timely synthesis of the enzymes that contain this E1B protein in normal human cells is required to induce removal, or inhibition by modification, of an as yet unidentified cellular protein that inhibits genome replication directly or indirectly. However, in HFFs the E1B 55 kDa protein also represses expression of a subset of cellular genes highly enriched for those associated with innate antiviral defenses and immune responses (58). The former class includes a substantial number of interferon (IFN)-inducible genes, as well as several encoding components of signaling pathways that are activated in response to infection, such as Myd88 and Irf7. One mechanism by which adenovirus infection is recognized to initiate innate immune responses is by the pathogen recognition receptor Tlr 9 (2, 8, 9, 18, 106, 110), which signals via the adaptor Myd88, Irf7 and other transcriptional activators to induce production of type I IFN and other proinflammatory cytokines (see (21, 93), (99)). Repression of expression of these genes by the E1B 55 kDa protein may therefore contribute to antagonism of antiviral defenses induced by type I IFN in Ad5-infected cells, as do the small viral RNA VA-RNAI (45, 46), the E1A proteins (1, 37, 43, 72), and the E4 Orf3 protein (94, 95). Such a function of the E1B protein could account for impaired genome replication when the early phase of infection is prolonged in HFFs infected by E1B 55kDa null mutants, as the longer period prior to induction of viral DNA synthesis might permit production of quantities of type 1 IFN sufficient to induce an effective anti-viral state by autocrine and paracrine mechanisms.

Acknowledgements

We thank Thomas Dobner for the generous gift of rat anti-E4 Orf3 monoclonal antibody, 6AII, Wenying Huang for expert technical assistance, Mohammed Selman for analysis of viral early protein synthesis in NHBECs, and Ellen Brindle-Clark for assistance with preparation of the

manuscript. This work was supported by Public Health Service grant RO1AI058172 from the National Institute of Allergy and Infectious Disease. Jasdave Chahal was partially supported by a post graduate scholarship from the National Science and Engineering Research Council of Canada.

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700 **Figure Legends.**

701

702 **Figure 1: Viral DNA synthesis in AdEasyE1- and AdEasyE1Δ2347-infected Hffs. A, B**
703 Proliferating HFFs at ~70% confluency were infected with 50 pfu/cell AdEasyE1 or
704 AdEasyE1Δ2347. At the times indicated, viral DNA concentrations were determined by
705 quantitative PCR. These values were corrected for concentrations of GAPDH DNA measured in
706 parallel, and are expressed in arbitrary units **(A)** or relative to the value measured 2 hours after
707 infection **(B)**. The values shown represent the mean of two independent experiments, with the
708 average deviations indicated by the error bars. **(C)** As B, except that quiescent HFFs were
709 infected. In all panels, WT=AdEasyE1, Δ2347 = AdEsyE1Δ2347.

710

711 **Figure 2 Formation of viral replication centers in AdEasyE1- and AdEasyE1Δ2347-infected**
712 **HFFs. A.** The E2 DBP was examined by immunofluorescence as described in Materials and
713 Methods 25 hours after infection of HFFs with 50 pfu/cell AdEasy E1 or AdEasyE1Δ2347, and
714 in mock-infected cells (M). Nuclei were stained with DAPI (blue) **B.** The appearance of DBP
715 only as diffuse nuclear staining (Diffuse), in small dot-like foci, with or without diffuse DBP
716 (Small foci) or in enlarged ring-like structures (Large rings) was counted in ≥ 100 cells infected
717 by AdEasyE1 or AdEasyE1Δ2347. The percentage of the total number of infected cells
718 containing each form of DBP are shown.

719

720 **Figure 3: Viral DNA synthesis in AdEasyE1- and AdEasyE1Δ2347-infected NHBECs.**
721 Proliferating NHBECs at ~60% confluency **(A)** or quiescent NHBECs **(B)** were infected with 5
722 pfu/cell AdEasy E1 or AdEasyE1Δ2347 and viral DNA concentrations measured at the times

indicated (see Materials and Methods). The values shown represent the mean of two independent experiments, and the error bars average deviation. In all panels, WT = AdEasyE1, Δ2347 = AdEasyE1Δ2347.

Figure 4: Synthesis of early proteins in normal human cells infected by AdEasyE1 or AdEasy E1-G. A. HFFs at ~70% confluence were infected with 50 pfu/cell AdEasyE1 for the periods indicated or mock infected (M), and the concentrations of DBP and β-actin examined by immunoblotting. **B.** As A, except that NHBECs at ~70% confluence were infected with 5 pfu/cell Ad5, or mock-infected (M). **C.** As panel A, except that HFFs at ~70% confluence were infected with 50 pfu/cell AdEasyE1-G, which contains the HCMV IE promoter/enhancer immediately upstream of the E1A transcription unit. Note the different time-scales in the different panels.

Figure 5: Viral DNA synthesis in HFFs infected by AdEasyE1-G. HFFs at 70% confluence were infected with 50 pfu/cell AdEasyE1-G (WT-G) or AdEasyE1Δ2347-G, and the concentrations of intranuclear viral DNA relative to the input value (2 hours p.i.) determined by quantitative PCR as described in the legend to Figure 1. Value shown represent the average of two independent experiments, and error bars indicate average deviations.

Figure 6: Localization of Mre11, E2 DBP and the E4 Orf3 proteins in infected HFFs.

HFFs at ~70% confluence were infected with 50pfu/cell AdEasyE1 (WT) or AdEasyE1Δ2347 (Δ2347) or mock-infected (M) for 24 hours. They were then processed for immunofluorescence and Mre11, the E2 DBP and the E4 Orf3 visualized as described in Materials and Methods. The

746 E4 Orf3 protein signal is false colored in blue. Nuclei stained with DAP1 are shown false colored
747 in cyan. The merged images do not include the nuclear stain.

748

Table 1: Comparison of viral DNA concentrations entering AdEasyE1- and AdEasyΔ2347-infected cells.

Cell Type	Entering [DNA][a]		
	AdEasyE1Δ2347	AdEasyE1	Mutant: wild-type
HFF			
Experiment 1	0.236	0.197	1.20
Experiment 2	0.099	0.07	1.41
NHBEC			
Experiment 1	0.345	0.318	1.08
Experiment 2	0.175	0.264	1.51

[a] Viral DNA concentrations (arbitrary units) 2 hours after infection of proliferating cells with 30 P.f.u./cell (HFFs) or 5 P.f.u>/cell (NHBECs) relative to those of GAPDH DNA.

Figure 1

Figure 2

Figure 3

Figure 4

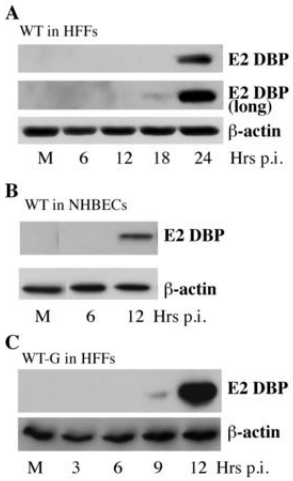

Figure 5

Figure 6