Improved growth of enteric adenovirus type 40 in a modified cell line that can no longer respond to interferon stimulation

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Human enteric adenoviruses propagate poorly in conventional human cell lines used to grow other adenovirus serotypes. As human enteric adenoviruses have a defect in counteracting the cellular interferon (IFN) response in cell culture, to aid in growth of the virus, a 293-based cell line defective in its ability to respond to IFN was constructed. This cell line (293-SV5/V) constitutively expresses V-protein of the paramyxovirus Simian virus 5, which degrades the signal transducer and activator of transcription 1 (STAT1) and thereby prevents the STAT1-mediated IFN response. Analysis of human enteric adenovirus type 40 (HAdV-40)-infected 293-SV5/V cells compared with parental 293 cells shows that the recombinant line allows more rapid production of virus and results in higher titres. These results suggest that the defect in HAdV-40 in counteracting the IFN response can be overcome at least partially through the use of 293-SV5/V cell lines.

Human adenoviruses 40 and 41 (HAdV-40 and -41), members of the species Human adenovirus F (HAdV-F), are important aetiological agents of infantile gastroenteritis, second only to rotaviruses in prevalence (Uhnoo et al., 1984; Brandt et al., 1985). Poor replication, slow growth in cell culture and relatively low titres are characteristic of HAdV-F compared with other adenoviral species (A–E) that grow to high titre in several human cell lines (Brown, 1985). Various defects in the enteric adenovirus life cycle have been identified as possible reasons for their fastidious growth in laboratory culture. These defects include sensitivity to type I interferon (IFN), deficiencies in the ability of the HAdV-40 E1A gene to transactivate transcription of other early viral genes and a block in the release of progeny virus (van Loon et al., 1987; Brown et al., 1992; Tiemessen & Kidd, 1993; Mautner et al., 1999).

Many viruses, including adenoviruses, have evolved strategies to circumvent the antiviral actions induced by IFN (reviewed by Goodbourn et al., 2000). One such evasion strategy is to block the intracellular signalling of IFN, thereby preventing the induction of IFN-stimulated genes and the establishment of the antiviral state. V-protein of Simian virus 5 (SV5) targets the cellular protein STAT1 specifically for proteasome-mediated degradation (Didcock et al., 1999). As STAT1 is required for both type I and II IFN responses, SV5 blocks IFN induction and evades the cellular antiviral response (reviewed by Horvath, 2004). Engineering the stable expression of SV5 V-protein in laboratory cell cultures to block IFN signalling has been described and increases growth of a number of viruses (Young et al., 2003). Most human adenoviruses circumvent the IFN response, at least in part, through expression of the early region 1A protein (E1A), which binds to STAT1 to prevent phosphorylation and subsequent STAT1-mediated activation of the IFN response (Look et al., 1998). It has been suggested that the sensitivity of HAdV-40 to IFN can be mapped to a possible defect in the ability of HAdV-40 E1A to down-regulate IFN function (Takiff et al., 1981; Tiemessen & Kidd, 1993). Thus, we aimed to increase the efficiency of HAdV-40 infection by constructing stable cell lines expressing SV5 V-protein to bypass this putative defect. Cultured 293 cells are used routinely for the propagation and clinical diagnosis of HAdV-40 (Graham et al., 1977; Takiff et al., 1981; Brown, 1985; Brown & Petric, 1986). Thus, we have established a 293-based SV5 V-protein-expressing cell line.

To produce cell lines expressing SV5 V-protein, we first constructed a recombinant lentivirus (ViraPower; Invitrogen) expressing SV5 V-protein. This virus was constructed by PCR amplification of the SV5 V-protein gene.

A supplementary figure showing increased adenovirus DNA replication in HAdV-40-infected MRC5-SV5/V cells is available in JGV Online.
(N100D mutant; Chatziandreou et al., 2002) and subsequent cloning into the lentivirus vector pLenti6/V5-DEST (Invitrogen), which was then cotransfected with accessory vectors into producer cells to generate lentivirus particles. Monolayer cultures of 293 cells (European Collection of Cell Culture, Porton Down, UK) were infected with the recombinant lentivirus expressing SV5 V-protein. The resulting cells were cultured in the presence of 10 μg Blasticidin S/HCl ml⁻¹ (Invitrogen) for the selection of resistant colonies, and dilution cloning was carried out to produce nine clonal isolates.

We further characterized one clonal isolate, 293-SV5/V, for the functional expression of SV5 V-protein. SV5 V-protein expression was examined by indirect immunofluorescence [V-protein was detected by using a sheep polyclonal antibody in conjunction with fluorescein isothiocyanate (FITC)-labelled donkey anti-sheep secondary antibody; Sigma]. Comparison of SV5 V-protein staining to that of DAPI and phalloidin, which mark the perimeters of the nucleus and cytoplasm, respectively, shows that SV5 V-protein is expressed at sufficiently high levels to be detected in both the cytoplasm and the nucleus (Fig. 1a). When expressed at low levels, SV5 V-protein has been reported to be located primarily in the nuclei of cells (Andrejeva et al., 2002). Furthermore, examination of SV5 V-protein expression in the modified 293-SV5/V cell line showed that SV5 V-protein co-stained with DAPI and phalloidin in all fields of view, suggesting that the cell line was clonal and that expression was stable. Functional expression of SV5 V-protein leads to proteasome-mediated degradation of STAT1 protein (Didcock et al., 1999). To confirm that SV5 V-protein was functional in the recombinant cell line, we compared the levels of IFN-induced STAT1 protein in 293-SV5/V and the parental 293 cells. Western blot analysis of cells induced with 1000 IU IFN-α ml⁻¹ for 24 h shows the presence of STAT1 protein in parental cells, but not in 293-SV5/V cells, consistent with the degradation of STAT1 via V-protein expression (Fig. 1b).

STAT1 protein is crucial for both type I and II IFN signalling; thus, 293-SV5/V cells should be generally non-responsive to IFN. To assay the IFN response, we monitored expression of an IFN-regulated reporter gene in 293 and 293-SV5/V cells. 293 and 293-SV5/V cells were each transfected with the plasmid pISRE-TA-luc (Clontech), containing the IFN-stimulated response element (ISRE) upstream of a luciferase reporter gene to measure the IFN-α response.

**Fig. 1.** Expression of functional V-protein in 293-SV5/V cell lines. (a) Fluorescence microscopy of 293 (top row) and 293-SV5/V (bottom row) cells. Monolayers were stained with DAPI (blue), TRITC-labelled phalloidin (red) or FITC-labelled SV5 V-protein (green), as indicated. Merged images (merge) are of DAPI, TRITC-labelled phalloidin and FITC-labelled SV5 V-protein. Pictures were taken on a Zeiss Axiovert 135TV fluorescence microscope. Magnification, ×1000. (b) Western blot analysis of STAT1 levels. Cytoplasmic extracts of IFN-induced 293 and 293-SV5/V cells were fractionated by SDS-PAGE. Levels of STAT1 and protein 14-3-3β protein (loading control) were detected by immunoblotting using anti-STAT1 (BD Biosciences) and anti-14-3-3β (β isoform antibody; Santa Cruz Biotechnology) antibodies, as indicated. (c) Transient-expression assay for IFN responsiveness. 293 and 293-SV5/V cells were transfected with pISRE-TA-Luc and pJacLac and then treated with IFN-α (hatched bars) or mock-treated (shaded bars). Mean ± SD luciferase levels normalized for transfection efficiency and protein content are shown. Transfections were performed in triplicate.
response, together with the plasmid pJacLac, containing the rat β-actin promoter upstream of a β-galactosidase reporter gene, to normalize for transfection efficiency (Masson et al., 1992). Cell cultures were subsequently induced with 1000 IU IFN-α ml⁻¹ or mock-treated at 30 h post-transfection; 16 h after IFN-α/mock treatment, cell lysates were harvested and firefly luciferase and β-galactosidase levels were measured [expressed as normalized luciferase units (N.L.U.); Fig. 1c]. IFN-α induced ISRE-dependent gene transcription by 18-fold (−IFN, 189 ± 3; +IFN, 3412 ± 359) in 293 cells, whereas in 293-SV5/V cells, no significant difference in ISRE-dependent gene expression was observed (−IFN, 276 ± 11; +IFN, 290 ± 8; Fig. 1c). These data indicate that 293-SV5/V cells do not respond to IFN induction (P=0.000003).

Given that IFN induction downregulates protein synthesis through induction and subsequent activation of the IFN-induced genes protein kinase R (PKR) and 2′,5′-oligoadenylate synthetase (OAS), we would expect an increase in relative levels of late adenoviral proteins in 293-SV5/V cells compared with 293 cells. To test this idea, we infected both cell types with HAdV-40 (Dugan strain; National Collection of Pathogenic Viruses, Porton Down, UK) and measured the fraction of cells producing the late viral capsid protein hexon, and determined its level of expression by using fluorescence-activated cell-sorting (FACS) analysis (using mAb 2Hx-2; ATCC). Fluorescence profiles were obtained by analysing 5000 viable cells using a FACSscan flow cytometer (BD Biosciences) and CellQuest software as described previously (Elsing & Burgert, 1998). The fluorescence profiles show that a larger fraction of the 293-SV5/V cells compared with 293 cells express HAdV-40 hexon (Fig. 2a–f). Also, the kinetics of expression appear to be faster in 293-SV5/V cells than in 293 cells. At 21 h p.i., 2.5 % of the 293-infected cells and 9% of 293-SV5/V-infected cells stained positive for HAdV-40 hexon (data not shown). At 44 h p.i., comparisons of 293 and 293-SV5/V cells show modest but significant average increases from 27 to 41.4 % (P=0.001) and from 51 to 75 % (P=0.02), respectively, using two separate preparations of HAdV-40 (Fig. 2g). Moreover, the amount of HAdV-40 hexon produced in 293-SV5/V cells is consistently higher than in 293 cells [compare the mean value of fluorescence in Fig. 2(b, c) with that in Fig. 2(e, f)]. These data suggest that suppression of the IFN response through degradation of STAT1 in 293-SV5/V cells promotes a modest increase in the levels of the late polypeptide hexon.

FACS analysis of the late capsid protein hexon suggested that more hexon was produced in 293-SV5/V cells than in 293 cells. We therefore wished to extend these results to determine whether the kinetics and possibly yield of infectious virus would also be altered. To investigate this, we performed one-step growth analysis of HAdV-40-infected 293 and 293-SV5/V cells. Thus, equal numbers of 293 and 293-SV5/V cells were infected in parallel with HAdV-40 at an m.o.i. of 1 and virus was harvested over a 4 day period and subsequently titred by fluorescent focus

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**Fig. 2.** Analysis of hexon expression in HAdV-40-infected 293 and 293-SV5/V cells. (a–f) Hexon staining of mock (a, d)- and HAdV-40 (b, c, e, f)-infected 293 (a–c) or 293-SV5/V cells (d–f). Two different preparations of HAdV-40 (preparation 1, b and e; preparation 2, c and f) were used for the infections. Region M1 was set to exclude staining of mock-infected cells (a, e). The percentage and mean value of fluorescence are shown within each histogram. (g) Mean percentage of cells expressing hexon over two independent experiments for two preparations of HAdV-40 (HAdV-40 and HAdV-40p2) in 293 cells (hatched bars) versus 293-SV5/V cells (filled bars), as detected by FACS analysis.
assay using 293 cells (Philipson, 1961; Fig. 3a). Overall kinetics of HAdV-40 growth in 293 cells were similar to those obtained by one-step growth analysis of clinical isolates of HAdV-40, with a characteristic extended exponential-growth phase and low yield compared with other serotypes (Brown, 1985). Comparison of the resultant virus yields in both 293 and 293-SV5/V cells shows only a modest (3.5-fold) difference in yield at 12 h p.i. However, at 12 h p.i., infection kinetics of growth are altered greatly in 293-SV5/V cells, which exhibit a steeper exponential-growth phase, reaching a near-maximal virus yield by 24 h p.i. [11.0 fluorescent focus units (f.f.u.) per cell (293), compared with a maximal yield of 45.9 f.f.u. per cell (293-SV5/V)]. At 24 h p.i., the difference between the cell lines is at its greatest, probably due to the differential effects of blocking of the IFN response at this time compared with other times, with the 293 cell yield (0.286 f.f.u. per cell) lagging by approximately 1.6 log10. At subsequent time points, the relative difference in yield decreases as HAdV-40 growth in the altered cell line approaches stationary phase. Final yield of both viruses at 83 h p.i. indicated a difference in yield of approximately one log10, 45.9 f.f.u. per cell (293-SV5/V) and 3.92 f.f.u. per cell (293).

To test the reproducibility and practical usefulness of our findings, we compared the yield of three independent HAdV-40 stocks grown in parallel on 293 and 293-SV5/V cells. Flasks of 293 and 293-SV5/V cells, grown to equivalent cell numbers, were infected in parallel with HAdV-40 at an m.o.i. of either 0.1 or 1 and harvested at 5 days p.i., a time when cytopathic effect (CPE) was complete in both cell lines (Fig. 3b). We reproducibly found titres of virus grown in 293-SV5/V cells to be approximately 1 log10 higher than in the unmodified line (P=0.007; mean fold increase, 15.9 ± 3.6; Fig. 3b). As CPE occurs more rapidly in 293-SV5/V lines than in 293 cells, this procedure would determine the minimal difference between lines, as HAdV-40 would remain in the decay phase longer in 293-SV5/V than in 293 cells. To obtain maximal virus titres, HAdV-40 is passaged routinely nine times in KB16 cells to achieve titres of up to 6 × 10^6 f.f.u. ml^-1 (Mautner, 1999). By using 293-SV5/V cells, we passaged HAdV-40 three times to produce a titre of 1.9 × 10^8 f.f.u. ml^-1.

To exclude the possibility that we had serendipitously obtained a clonal variant that allowed HAdV-40 to grow better, we assessed growth of HAdV-40 in two further 293 clones expressing SV5 V-protein and compared their growth with that of 293 and 293-SV5/V cells. IFN treatment of these additional clones (293-SV5/V clone 2 and 293-SV5/V clone 3) showed no STAT1 protein [as in Fig. 1(b)]. To compare the abilities of the other SV5 V-protein-expressing lines to enhance growth of HAdV-40, we infected parallel flasks of the three clonal lines, together with 293 cells, with a single stock of HAdV-40 at an m.o.i. of 0.1. In all three recombinant lines, HAdV-40 titre was increased by approximately 1 log10 (Fig. 3c), suggesting that the increase in titre observed in 293-SV5/V cells was not due to clonal variation, but a consequence of the expression of V-protein. In addition, comparisons between HAdV-40 infections of naïve and SV5 V-protein-expressing MRC5 cell lines show increased virus replication using fluorescence microscopy of HAdV-40-infected MRC5/SV5-V lines compared with control MRC5 cells, supporting the notion that it is V-protein expression and not clonal variation that is responsible for enhanced growth of HAdV-40 (Young Fig. 3. Yield of infectious HAdV-40 is increased in 293 lines expressing SV5 V-protein compared with 293 cells. (a) One-step growth-curve analysis of HAdV-40 in 293 and 293-SV5/V cells. Petri dishes (60 mm) of 293 (▲) and 293-SV5/V (▼) cells were infected with HAdV-40 at an m.o.i. of 1. Plates were harvested at the time points indicated and amount of infectious virus was determined by fluorescent focus assay, in triplicate. (b) Flasks of equal numbers of 293 (filled bars) and 293-SV5/V (hatched bars) cells were infected at an m.o.i. of 0.1 (experiments A and B) or 1 (experiment C) with three different preparations of HAdV-40. At 5 days p.i., virus was harvested and titred by fluorescent focus assay, in triplicate. (c) Flasks containing equal numbers of 293, 293-SV5/V, 293-SV5/V clone 2 and 293-SV5/V clone 3 cells were infected in parallel with HAdV-40 at an m.o.i. of 0.1. Virus was harvested at 5 days p.i. and titred by fluorescent focus assay, in triplicate.
et al., 2003; Supplementary Fig. S1, available in JGV Online). Thus, we conclude that the increase in virus growth is due to SV5 V-protein expression and subsequent suppression of the IFN response, rather than any form of clonal selection leading to enhanced growth of HAdV-40.

Most adenoviruses can grow to high titres in cell culture, such as the well-studied HAdV-5 and HAdV-2 serotypes. However, the combined effect of slow growth and non-productive replication in cultured cell lines has impeded the study of enteric adenovirus. We show here that, by making 293 cells non-responsive to IFN by expressing the V-protein of SV5 constitutively, we can improve the growth characteristics of HAdV-40 significantly. Although titres of HAdV-40 could be increased, levels are still below those of HAdV-2 and HAdV-5, suggesting that IFN sensitivity is only partially responsible for the fastidious growth of this virus.

Inhibition of STAT1 activity improves the growth of HAdV-40 in 293 cells, although 293 cells are transformed with the HAdV-5 E1 region, of which the E1A protein has been reported to downregulate STAT1 function (Look et al., 1998). This may be explained by the observation that, in the context of 293 cells, STAT1 remains unmodulated and, as such, the cells are still responsive to IFN induction (Fig. 1c; Xie et al., 2002). Nevertheless, the introduction of a cell system that can improve virus titre significantly will allow further investigation into this intriguing adenovirus species.

As 293 cells have a number of uses in the study of a variety of viruses, the 293-SV5/V cells described here may have a number of applications, including virus propagation and the production of retroviral-packaging systems. The suitability of enteric adenovirus-based recombinant viruses as vectors for delivering therapeutic proteins to enterocytes has been described (Croyle et al., 1998). In this context, the 293-SV5/V cell line could potentially provide a system for improved yield of such recombinant viruses for gastrointestinal vector development. 293-SV5/V cells may also be of use in non-viral applications. The IFN-induced inhibitor of translation PKR is activated inadvertently during transient expression of plasmid vectors, leading to a decrease in transient expression (Kaufman & Murtha, 1987). This enzyme is induced by IFN; thus, its levels are likely to be relatively low in 293-SV5/V cells, allowing increased efficiency of translation during transient transfection. Moreover, 293-SV5/V cells may also be utilized in gene-silencing experiments where inadvertent IFN activation can potentially confound experimental design (Sledz et al., 2003).

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