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Development of a novel DNA based reverse genetics system for classic human astroviruses

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ABSTRACT

Human astroviruses (HAstVs) are a frequent cause of gastroenteritis in young children and immunocompromised patients. The current report describes a new approach to recover genetically defined HAstVs through the use of a reverse genetics system based on a single DNA plasmid. This plasmid, carrying the full-length virus genome under a T7 promoter, is directly transfected into cells expressing T7 RNA polymerase, resulting in the rapid and robust recovery of infectious HAstV. The efficiency of the system was tested with the generation of a chimeric astrovirus having the HAstV serotype 1 replication machinery and the capsid derived from a HAstV serotype 8 virus. This new system provides an efficient and reproducible method to deepen our knowledge of astrovirus biology.

1. Introduction

Diarrheal diseases are one of the leading causes of morbidity and mortality in young children, with human astrovirus (HAstV) being an important etiological agent (Jeong et al., 2012; Mitchell et al., 1999).

Astrovirus infections have also been reported in the elderly and in immunocompromised patients, and recently, novel HAstVs have been associated with neurological disorders such as encephalitis and meningitis (Cordey et al., 2016; Lum et al., 2016; Quan et al., 2010; Vu et al., 2017). To date, no antiviral drugs or vaccines are available for these viruses (Cordey et al., 2016; Lum et al., 2016; Quan et al., 2010; Vu et al., 2017). To increase our knowledge about the biology and pathogenesis of HAstVs, an accessible and efficient reverse genetics system is fundamental.

The HAstV genome is a 6.8–7.0 kb positive-sense single-stranded RNA (ssRNA). The 5′-terminus of the genomic RNA is linked to a VPg protein and the 3′-terminus is polyadenylated (Guo et al., 2010; Mendez-Toss et al., 2000). The genome is organized in 3 open reading frames named ORF1a, ORF1b, and ORF2. ORF1a and ORF1b are translated from the genomic RNA as two large polyproteins. ORF1a encodes the nonstructural proteins, including the viral protease and VPg (Speroni et al., 2009), while ORF1b encodes the RNA dependent-RNA polymerase. The VPg protein has an important role in the HAstV replication cycle, since treatment of the viral genome with proteases abolishes infectivity (Fuentes et al., 2012). This suggests that VPg is important for the early steps of infection such as translation, possibly by recruiting translation factors. The RNA replication process of HAstV has not been fully described but it is assumed to be similar to other positive-sense ssRNA viruses. Thus, a negative-sense full-length copy of the genome is transcribed and used as a template for subsequent genomic and subgenomic RNA synthesis. The subgenomic RNA comprises ORF2 that codes for the structural precursor polyprotein of about 780 amino acid residues (Bass and Qiu, 2000).

Reverse genetics systems allow the manipulation of the genome offering a crucial tool for the study of mechanisms of viral replication and infection, the structure and function of genes, and the recovery of viral strains extinct for years. Similarly, they open the way for new therapies, expression vectors, vaccine development, and gene therapy, creating a wide range of possibilities for basic science and future and immediate applications (Kobayashi et al., 2007; Pekosz et al., 1999; Stobart and Moore, 2014).

The first reverse genetics system reported for astrovirus consisted of a full-length cDNA clone of a HAstV serotype 1 genome, which was transcribed *in vitro* with a cap structure added at the 5′-end instead of the native VPg (Geigenmuller et al., 1997). This system generates an RNA that can be transfected into permissive cells to allow the recovery of recombinant infectious virus (Geigenmuller et al., 1997). This approach, although it has been proved to be useful for the study of astrovirus biology, requires the addition of a capping analog during the *in vitro* transcription reaction as a critical modification for the efficient translation of the synthesized RNA when transfected into cells, however, in general the efficiency of the *in vitro* capping reaction is low

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(Fuchs et al., 2016). The efficiency of the system was improved with the utilization of electroporation to introduce the viral capped RNA. This approach allowed identifying cell lines with different efficiencies for infectious particle recovery. Velázquez-Moctezuma et al. reports the successful recovery of infectious particles from Huh7.5.1. cells (Velazquez-Moctezuma et al., 2012).

Here, we report the development of a reliable reverse genetics system for HAstV that generates infectious virus based on two key features (Jeong et al., 2012): a plasmid containing a full-lenght cDNA copy of the HAstV genome under a T7 promoter, that is transfected into cells stably expressing the T7 polymerase, and (Mitchell et al., 1999) an encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) to drive the translation of the T7 polymerase-transcribed viral RNA. Here we demonstrate the efficiency and versatility of this genetic reverse system by generating a recombinant chimeric virus carrying the HAstV serotype 1 ORF1a and ORF1b genome regions and the HAstV serotype 8 ORF 2.

2. Results

2.1. Design of a plasmid expressing a chimeric HAstV genome

In this work, we generated a recombinant chimeric virus carrying the HAstV serotype 1 ORF1a and ORF1b genome regions and the HAstV serotype 8 ORF 2 (Fig. 1). For this, a full-length cDNA clone was assembled via the subcloning strategy described in the Material and Methods section. Briefly, to construct the chimeric HAstV serotype 1/8 infectious clone, ORF1a and ORF1b from HAstV-1 and ORF2 from HAstV-8 strain Yuc-8 were cloned in the vector pT7CFE1-CHis. The final plasmid contains the following elements, considering as nucleotide (nt) 1 the first nt of the first T7 promoter of the construct (Fig. 1C). Position 1–18, sequence for the first T7 promoter that drive RNA transcription starting at nt 19. Next, there is an IRES element from EMCV present at nt positions 70 to 539, and downstream the IRES a second T7 promoter was engineered at positions 545 to 562. The chimeric HAstV 1/8 genome was cloned at nt 563 to 7323. The chimera contains the 5′-non-translated region (NTR) of HAstV-8 at nt 563 to 647 and the HAstV-8 3′-NTR at nt positions 7239 to 7323. The 3′-NTR region of the chimeric RNA also includes an engineered 30 nt-long polyA tail at nt positions 7324–7353. To stop the RNA transcription of the two RNA species transcribed from the two different T7 promoters, the construction also contains a T7 terminator sequence at nt positions 7373 to 7420.

Each of the two T7 transcription promoters would produce a different RNA species; the first, containing the HAstV recombinant genome preceded by the IRES sequence to drive the synthesis of the viral proteins. The second RNA species was designed to work as a template for replication of the viral genomic RNA. The 5′ and 3′ NTRs, corresponding to the HAstV-8, were engineered to ensure the correct potential interaction that may occur between the 5′- and 3′- terminal ends of the genome, since these interactions have been found to be critical for the replication of several RNA viruses (Alvarez et al., 2005; Sandoval-Jaime and Gutierrez-Escolano, 2009). The incorporation of a T7 terminator sequence to end transcription improves the system, since it avoids a restriction step to linearize the plasmid that could interfere with the transfection efficiency (Fig. 1C). The final construction was completely sequenced and ten nucleotide changes were identified as compared to the reported sequences for HAstV serotype 1 strain Oxford (accession number L23513.1) and HAstV serotype 8 (accession number AF260508.1). Four of the mutations changed the amino acid; the nature and position of the changes are compiled in Table 2.

2.2. Antigenic and sequence characterization of infectious virus particles generated by cell transfection of pT7HAstV 1/8

To produce infectious recombinant viruses, plasmid pT7HAstV 1/8

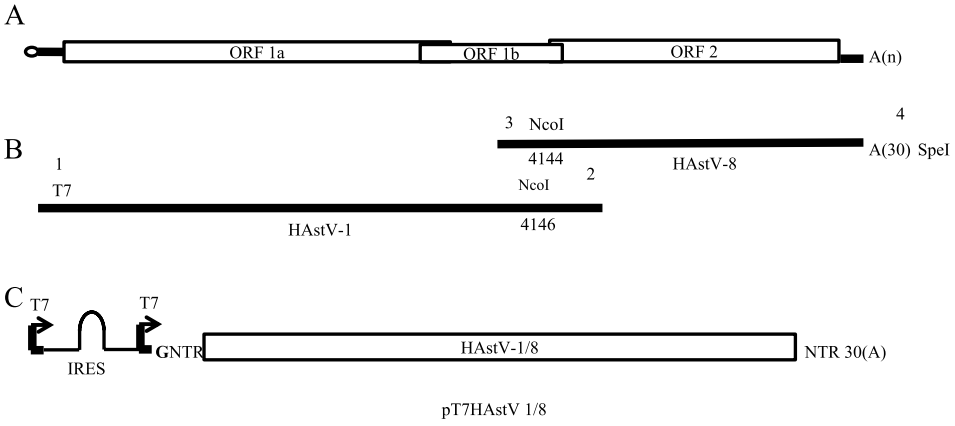

Fig. 1. Construction of the HAstV pT7HAstV 1/8. (A) Representation of the organization of the HAstV genome indicating the open reading frames. (B) PCR fragments used for the construction of the chimeric plasmid indicating the NcoI site present in ORF1b in both strains. (C) Representation of the complete chimeric HAstV 1/8 genome assembled in pT7CFE1-CHis. (D) Features of the plasmid constructed. The nucleotide positions are based on the first nucleotide of the first T7 promoter in the pT7HAstV 1/8 clone.

D

Feature	Nucleotide position	Feature Length
T7 promoter	1-18	18
EMCV IRES	70-539	470
T7 promoter	545-562	18
5´NTR HAstV-8	563-647	85
HAstV-1/8 genome	648-7238	6591
3´NTR HAstV-8	7239-7323	85
Poly A tail	7324-7353	30
T7 terminator	7373-7420	48

Table 1
Sequence of primers used for construction of chimeric pT7HAstV 1/8.

Primer name	Primer Sequence 5′-3′
1 T7 Asv sense	**TAATACGACTCACTATAG**CCAAAAGGGGGGTGGTGATTGG
2 Asv1-8 4333	CTAGCCATCACACTTCTTTGGTCC
3 Asv8 sen NcoI	ATCACTCCATGGGAAGCTCCTATGC
4 Asv8 rev SpeI	TGCTCAGCGGACTAGT(T_{30})GCATCTGATTAAATCAATTTTAAATGG

Nucleotide sequences of the oligonucleotides used in this work. Restriction sites and T7 promoter sequences are underlined and indicated in bold, respectively.

Table 2
Sequence analysis of nucleotide differences between the infectious cDNA clone pT7HAstV 1/8 and both parental serotype 1 and 8 viruses.

Nucleotide position	Parental virus codon	Chimeric virus codon	Amino acid change
5	G	A	NTR
2191	gaT	gaC	Silent D
2335	gtA	gtG	Silent V
2716	gtA	gtT	Silent V
2719	ttT	ttC	Silent F
2936	aGa	aAa	R/K
3213	acA	acG	Silent T
5412	aCt	aAt	T/D
5439	cTT	cCT	L/P
6663	gAc	gGc	D/G

was transfected into HEK293T-T7 cells. At 48 hpt the total cell lysate was collected and the infectious virus particles produced were quantitated by infecting Caco-2 cell monolayers. Infectious foci were easily detected, rendering a titer of 3×10^2 ffu/ml. Upon a first passage of these viruses the titer raised to 7×10^5 ffu/ml.

To antigenically characterize the identity of the HAstV-1/8 chimeric virus, Caco-2 cell monolayers were infected with either the recovered virus from transfected cells, or with the wild-type HAstV-1 and HAstV-8 strains. At 16 hpi the cells were fixed and stained with antibodies that specifically recognize the spike domain of either serotype 1 or serotype 8 viruses. The pattern of intracellular distribution of the capsid proteins for parental and chimeric viruses was similar, but the recombinant HAstV-1/8 was only recognized by the HAstV spike8 antibody, confirming that the recombinant virus contained the HAstV-8 capsid protein (Fig. 2). To determine the chimeric identity of the recovered virus and to discard the possibility of a possible contamination with parental HAstV-8, viral RNA from the recovered virus was extracted from infected cells after two passages, and its nucleotide sequence was determined.

A fragment of 1538 nt was amplified from the extracted viral RNA by RT-PCR using the HAstV-1 specific oligo Forward 5′-AAACTACAA AGGGCCCCAGAAGACC-3′ and the HAstV-8 specific oligo Reverse 5′-CTAGCCATCACACTTCTTTGGTCC-3′. The product was sequenced and aligned to the reported parental sequences. The presence of the HAstV-1/8 switch-point in the recombinant viral genome, at nt position 4131, was verified (Fig. 3), confirming the isolation and passage of the recombinant 1/8 virus.

2.3. Analysis of virus yield per cell

We next compared the yield of infectious chimeric and wild-type viruses produced per infected cell. For this, Caco-2 cells were infected at different multiplicities of infection and the titer of the virus produced was determined at 16 hpi. The number of progeny virus per infected cell was not significantly different for the different virus strains: 158 ffu/cell ± 86 for HAstV-1, 418 ffu/cell ± 144 for HAstV-8 and 319 ffu/cell ± 42 for HAstV-1/8. This finding suggests that the chimeric virus replicates with efficiency similar to that of both parental wild-type viruses (Fig. 4).

3. Discussion

Reverse genetic systems are a powerful tool to study the replication cycle of viruses. Since the first demonstration that genomic RNA from positive-sense RNA viruses was able to generate viral progeny upon transfection into permissive cells. (Racaniello and Baltimore, 1981).

The first reverse genetics systems reported for astrovirus are based on *in vitro* transcription of a full-length cDNA copy of the virus genome, and incorporation during transcription of a cap structure at the 5′end of the RNA to promote protein translation. The presence of a 5′-cap structure has been shown to efficiently substitute for the viral VPg in reverse genetics systems for both astrovirus (Geigenmuller et al., 1997) and calicivirus (Sosnovtsev and Green, 1995). The first attempt to develop a DNA-based reverse genetics system for astrovirus used an RNA pol II-dependent plasmid that was transcribed in the nucleus of transfected cells. Transfection of 293T cells with DNA plasmids under the CAG (hybrid promoter resulting from the human cytomegalovirus and chicken β-actin) and hGAPDH (human glyceraldehyde 3-phosphate dehydrogenase) promoters. This system allowed for a low level replication and translation of the viral genome, and an inefficient production of recombinant viral particles (8 ± 0 and 3 ± 2.83 infectious units per ml) (Chapellier et al., 2015). The better efficiency of the system described in this work as compared to the previously reported DNA-based reverse genetics system is most probably due to the fact that in our system the viral RNA is synthesized in the cytoplasm from a T7 promoter, mimicking the environment where a natural infection occurs, and also from the fact that translation of the RNA is driven from an IRES, ensuring the efficient synthesis of proteins (Sandoval-Jaime et al., 2015). On the other hand, the efficiency of the *in vitro* transcription RNA system reported by Geigenmuller et al. (1997) is higher than our DNA-based system, determined after one round of virus amplification, since the yield of infectious virus in their system was $7.5.1 \times 10^8$ per ml (Geigenmuller et al., 1997), as compared to ours of 7×10^5 ffu/ml.

It is important for virus recovery that the transcribed RNA has an authentic viral genome 5′-end. For this reason, and since we introduced an IRES sequence to initiate translation, we engineered a second T7 promoter between the IRES and the first nucleotide of the HAstV genome to avoid the presence of the IRES sequence in our viral templates and avoid 5′-terminal variations that may prevent encapsidation of the genomic RNA. Every RNA transcript from the second T7 promoter results in a G residue as a first nucleotide in the RNA genomic template, suggesting that the virus replication machinery might be able to remove the presence of that extra base, in the primary transcript. This is consistent with previous observations in norovirus, where the virus replication signals are able to edit a fragment of 500 nucleotides fused to the 5′-end of the transcript to render the correct 5′-end of the genomic RNA (Sandoval-Jaime et al., 2015). The characterization of 5′-end of the primary transcription product of plasmid pT7HAstV 1/8, and the search for a possible editing system that might fix the 5′-end in the astrovirus genome is a future goal of this work that should allow us to better understand the details of the reverse genetics system described.

Our system is dependent on the presence of the T7 RNA polymerase in the transfected cells, and uses the HEK293T-T7 cell that stably produces the T7 polymerase to recover the virus; although BHK cells modified to stably express the T7 polymerase have been also reported

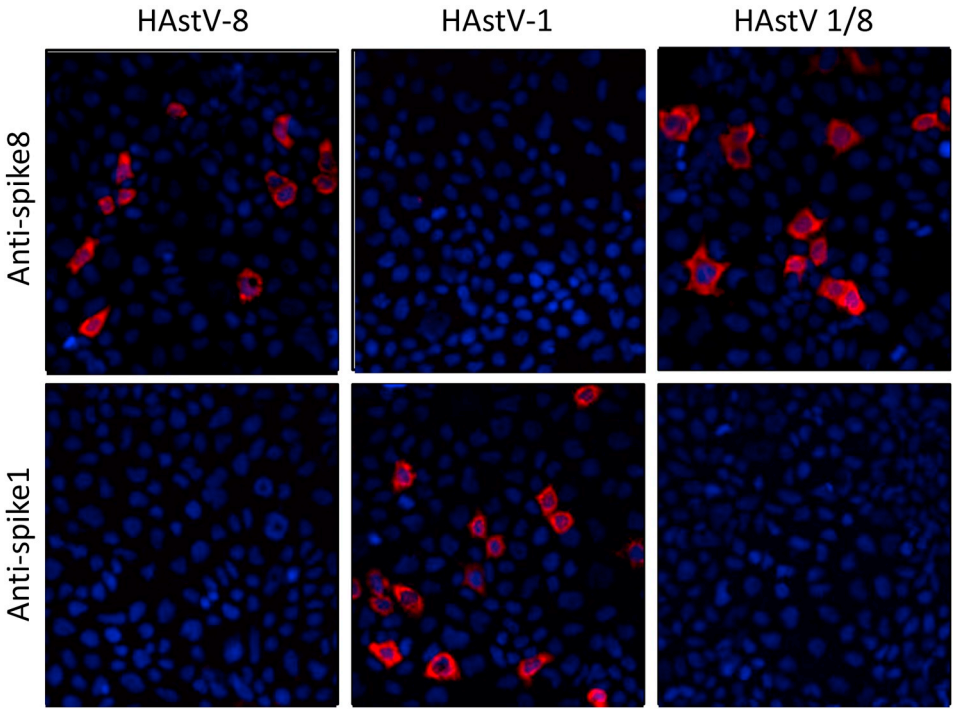

Fig. 2. Immunofluorescence detection of the capsid spike domain of parental and chimeric viruses. Caco-2 cells were infected with the serotype 1 or 8 viruses, or with the chimeric recovered virus and subjected to immunofluorescence staining with anti-spike8 and anti-spike1 polyclonal rabbit antibodies. The HAstV-infected cells are stained in red (Alexa Fluor 488) and the cell nuclei are stained in blue (DAPI).

(Buchholz et al., 1999), these cells have been shown to be non-optimal for HAstV replication (Brinker et al., 2000). Previous reports suggest that the recovery in HEK293 cells was low compared with Caco-2 and Huh7.5.1 (Velazquez-Moctezuma et al., 2012), however, this cell line has been widely used for their excellent transfection efficiency and reliable growth in culture; of interest, these cells have been reported to be susceptible to astrovirus infection, although the progeny virus does not exit the cell (Mendez et al., 2004), although the viruses can be released by disrupting the cell by freeze and thaw cycles, and further amplified in Caco-2 cells after treatment with trypsin.

Many animal species, including humans, have been identified as hosts for astroviruses (AstVs) (Chu et al., 2010; Rivera et al., 2010; Shimizu et al., 1990; Woode et al., 1985). Currently, there are four species in the *Mammastrovirus genus* that are recognized to infect humans: *Mammastrovirus* species 1 that includes classic serotype 1 to 8 HAstVs; species 6, with Melbourne AstV (MLB) as the reference strain; and VA astroviruses divided in *Mammastrovirus* species 8 (VA2 and VA4) and 9 (VA1 and VA3), with Virgina AstV (VA1) as the reference strain (Vu et al., 2017; Bosch SG et al., 2011). The high diversity of host species that can be infected by astroviruses provides the opportunity for recombination events to occur. Natural recombination events have been reported between HAstV serotypes 3 and 5 identified in two different samples from Houston and Mexico City (Walter et al., 2001). Recombinant strains between HAstV-2 and HAstV-3 were reported in Kenya from children with gastroenteritis (Wolfaardt et al., 2011).

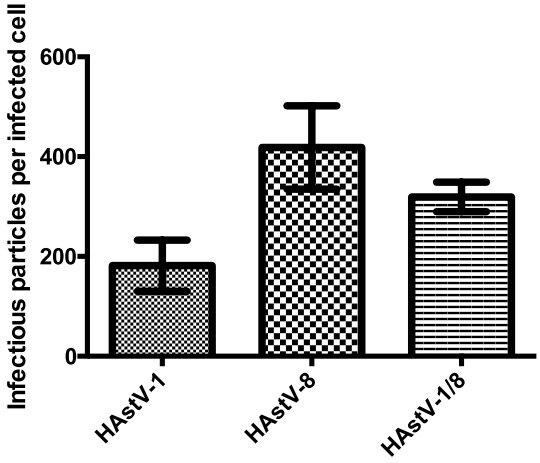

Fig. 4. Comparison of number of infectious virus particles produced by individual cells infected with either the parental serotype 1 and 8 virus or with the recovered chimeric virus. The number of infectious viral particles produced per cell was determined as indicated under Material and Methods. Statistical analysis of the virus obtained was performed by Wilcox matched-pairs signed rank test. Differences between groups were considered to be significant at a P value of < 0.05. Bars represent the standard error mean of two experimental replicas carried out in duplicate. No statistically significant differences was found for the different viruses.

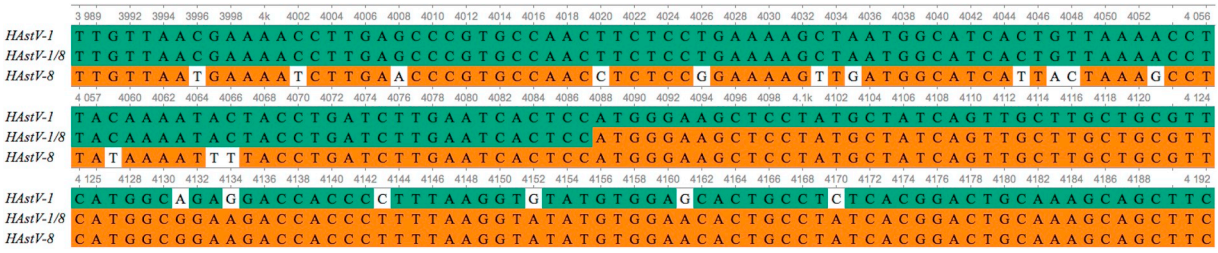

Fig. 3. Comparison of the nucleotide sequence of parental serotypes 1 and 8 virus strains and that of the chimeric HAstV-1/8 strain. The nucleotide coincidences between the sequence of the 1/8 and the parental viruses are highlighted in green for HAstV-1 and in orange for HAstV-8. The change in color indicates the designed recombination site. Alignment performed with MEGA7 (Kumar et al., 2016).

Recombination events have also been reported between astroviruses isolated from different animal species; Hata et al. provided evidence of recombination between human and cat AstV strains, present in environmental waters (Hata et al., 2018). Different reports have described that a common recombination site for astroviruses corresponds to a highly conserved junction region between ORF1b and ORF2 (Wolfaardt et al., 2011; Finkbeiner et al., 2008). In the present work, we report the first artificially recombinant HAstV produced. Our findings prompt future search for recombination events that might occur between a broad range of AstVs.

The full nucleotide sequence of our construction showed changes as compared to the pAVIC clone originally reported (Geigenmuller et al., 1997) at nt positions 2191, 2335, 2716, 2719, 2936, and 3213. Three of these differences, at nt positions 5412, 5439, and 6663, were non-synonymous, introducing an amino acid change; these amino acid changes in the capsid viral precursor do not seem to affect virus replication.

In summary, in this work we developed a robust reverse genetics system for HAstV that consists in transfection of a single DNA plasmid carrying a full-length cDNA copy of the viral genome. This system eliminates the need for *in vitro* transcription and inefficient cap incorporation steps, and should facilitate the advancement of our knowledge about astrovirus genome replication and biology.

4. Materials and methods

4.1. Viruses and cells

The colon adenocarcinoma Caco-2 cell line was obtained from the American Type Culture Collection; the cells were grown in Advanced D-MEM (Adv-DMEM [Dulbecco's modified Eagle's medium]) (Invitrogen, Carlsbad, CA) supplemented with 2 mM glutamine and 5% fetal bovine serum (FBS) in a 10% CO_2 atmosphere. HEK293T cells stably expressing T7 polymerase (HEK293T-T7), were kindly provided by Stanislav Sosnovtsev and Kim Green from the National Institutes of Health in Bethesda, MD (Sandoval-Jaime et al., 2015). HEK293T-T7 cells were cultured in Adv-DMEM supplemented with 2 mM glutamine, 5% FBS, and puromycin (4 μg/ml) in a 5% CO_2 atmosphere. The HAstV serotype 8 (strain Yuc-8) was previously described (Mendez-Toss et al., 2000).

4.2. Construction of the chimeric HAstV-1/8 clone

For the construction of the chimeric HAstV-1/8, the genome of a previously characterized HAstV-8 (Mendez-Toss et al., 2000) was used. The HAstV-8 strain was passaged once in Caco-2 cells. The viral RNA genome was isolated from infected cell lysates using the PureLink RNA Mini Kit (Ambion, Carlsbad, CA USA) and reverse transcribed using M-MuLV reverse transcriptase (New England Biolabs Ipswich, MA USA). The cDNA corresponding to ORF 2 was amplified by PCR using PfuUltraII Hotstart (Agilent Technologies Santa Clara, CA USA). The HAstV-8 fragment containing the ORF2 of HAstV-8 was digested with NcoI and SpeI (Fig. 1B). The vector pT7CFE1-CHis (Thermo Fisher, Pierce), also digested with NcoI and SpeI, was ligated with the HAstV-8 ORF2 DNA amplicon, generating the plasmid pT7CFE-HAstV-8 ORF2. To obtain the ORF1a and ORF1b of serotype 1, a full genome-length cDNA clone of the reference strain HAstV-1 (pAVIC) (Geigenmuller et al., 1997) was used as a template using the oligos listed in Table 1. The resulting PCR product containing HAstV-1 ORF1a and ORF1b was digested with NcoI and ligated into the pT7CFE-HAstV-8 ORF2. The backbone clone was digested with MscI and NcoI enzymes and the fragment HAstV-1 containing ORF1a and ORF1b was digested with NcoI. Vector and insert were ligated to generate the pT7CFE-HAstV chimeric 1/8 plasmid (pT7HAstV 1/8) (Fig. 1C). The final plasmid was subjected to nucleotide sequencing to verify the construction, as detailed in (Fig. 1D).

4.3. RNA extraction and sequencing

Viral RNA was extracted from the clarified cell extracts, after freezing and thawing he cells three times, using the PureLink RNA Mini Kit (Ambion). Reverse transcription coupled to PCR (RT-PCR) was performed using the M-MuLV reverse transcriptase. cDNA corresponding to the full-length genome of the HAstV chimera 1/8 was amplified by PCR using PfuUltraII Hotstart and the primers listed in Table 1.

4.4. Recovery of the recombinant HAstV 1/8 virus

HEK293T-T7 cells (2×10^5 cells/well) were plated in 12-well plates in Adv-DMEM supplemented with 2 mM glutamine, 10% FBS, and incubated for 24 h at 37°C. Cells at 80% of confluence were transfected with the pT7HAstV 1/8 using Lipofectamine 3000 reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. After transfection, the cells were incubated for 48 h at 37°C, and then lysed by three freeze and thaw cycles. The lysates were clarified, and the virus present in the clarified lysates was activated with 200 μg/ml of trypsin for 1 h at 37 °C. For amplification of the HAstV chimeric 1/8 genome, 500 μl of the clarified transfection supernatant was used to infect a 100 cm^2 cell culture dish seeded with 7×10^6 CaCo-2 cells in FBS-free MEM. After 1 h of adsorption, the monolayer was washed and FBS-free MEM supplemented with 0.5 μg/ml of trypsin was added and incubated for 48 h at 37 °C.

4.5. Analysis of virus yields per infected cell

To compare the yield of recovered chimeric virus with wild-type HAstV-1 and HAstV-8, Caco-2 cells were infected with increasing multiplicities of infection (MOI) and incubated for 16 h at 37 °C. After this time the cells were harvested and lysed by three freeze-thaw cycles, and the titer of the virus produced was determined by an immunoperoxidase assay (Mendez et al., 2004). To determine the number of viral particles produced per cell, an infection was performed in parallel using the same MOIs and in this case the infection was allowed to proceed for 16h, time at which the cells were fixed and immunostained to determine the number of infected cells. To calculate the virus yield per cell, the viral titers obtained were divided by the number of infected cells observed at each corresponding MOI. We compared the resulting number of viruses produced per cell by the different viruses using the Wilcox matched-pairs signed rank test. Differences between groups were considered to be significant at a P value of < 0.05. Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA).

4.6. Virus infectivity assay

Viral titers were determined by an immunoperoxidase assay, as described previously (Mendez et al., 2004). Briefly, monolayers of virus-infected Caco-2 cells were fixed with ice-cold 100% methanol for 15 min at room temperature. The cells were permeabilized by incubating with PBS containing 0.5% Triton-X, and the infected cells were detected using a rabbit hyperimmune serum directed to the capsid protein (Mendez et al., 2004), followed by incubation with a secondary goat anti-rabbit polyclonal antibody conjugated with peroxidase. The secondary antibody was detected using 3-amino-9-ethyl-carbazole (Sigma Chemical Co., St. Louis, MO) as a substrate.

4.7. Indirect immunofluorescence assay (IFA)

Caco-2 cells seeded on glass coverslips in 48-well plates (1.35×10^5 cells/coverslip), were infected with either the chimeric virus or the wild-type serotype 1 or 8 viruses at an MOI of 0.5, and incubated for 16 h at 37 °C. The cells were then fixed with 2%

paraformaldehyde (Sigma) in PBS for 20 min at room temperature, and permeabilized with 0.5% Triton X-100 (Sigma) in blocking solution (1% BSA/PBS, 50 mM NH_4Cl) for 15 min. Fixed and permeabilized monolayers were incubated overnight with either anti-spike1 or anti-spike8 polyclonal rabbit antibodies diluted 1:1000 in blocking solution (Espinosa et al., 2019). The cells were washed thoroughly with 50 mM NH4Cl NH_4Cl in PBS, and then incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen) at a dilution of 1:1000 for 1 h at room temperature. The nuclei were stained with 30 nM DAPI (4′,6-diamidino-2-fenilindol, Invitrogen TM, USA). The coverslips were mounted with Citifluor AF1 (Electron Microscopy Sciences, Hatfield, USA), and the images were obtained using an epifluorescence microscope (Zeiss, Axioskop 2, Alemania) coupled to a digital camera (Photometrics Cool Snap HQ).

Conflicts of interest

The authors declare that there are no conflicts of interest.

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