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ORIGINAL ARTICLE

Construction of an infectious clone of human adenovirus type 41

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Abstract Human adenovirus type 41 (HAdV-41) is well known for its fastidiousness in cell culture. To construct an infectious clone of HAdV-41, a DNA fragment containing the left and right ends of HAdV-41 as well as a kanamycin resistance gene and a pBR322 replication origin was excised from the previously constructed plasmid pAd41-GFP. Using homologous recombination, the plasmid pKAd41 was generated by co-transformation of the *E. coli* BJ5183 strain with this fragment and HAdV-41 genomic DNA. Virus was rescued from pKAd41-transfected 293TE7 cells, a HAdV-41 E1B55K–expressing cell line. The genomic integrity of the rescued virus was verified by restriction analysis and sequencing. Two fibers on the virion were confirmed by western blot. Immunofluorescence showed that more expression of the hexon protein could be found in 293TE7 cells than in 293 cells after HAdV-41 infection. The feature of non-lytic replication was preserved in 293TE7 cells, since very few progeny HAdV-41 viruses were released to the culture medium. These results show that pKAd41 is an effective infectious clone and suggest that the combination of pKAd41 and 293TE7 cells is an ideal system for virological study of HAdV-41.

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Introduction

Human adenovirus F (HAdV-F) possesses several characteristics that distinguish it from most of other HAdVs [22]. HAdV-F has a compressed genome [5] that allows it to encode the same number of, or even more, genes in a genome that is smaller than those of other HAdVs. There are two kinds of fibers protruding out of the penton bases on the HAdV-F virion [11, 26]. The long one can recognize and bind the coxsackie virus and adenovirus receptor (CAR), while the receptor of the short fiber remains undiscovered so far [19]. There is no RGD motif in the penton base of HAdV-F [1]. These structural features may contribute to its exclusive tropism for the gastrointestinal tract.

HAdV-F is the only adenovirus that has been proven to cause diarrhea in human beings, although other HAdVs, such as HAdV-G (HAdV-52) and HAdV-C, can also be found in human feces [1, 10, 16]. Its property of gut tropism has attracted growing interest in reconstructing HAdV-F as a recombinant vector for oral vaccines or gene therapy targeting the gastrointestinal tract [3]. The relationship of the phenotype of HAdV-F to gut tropism has been studied *in vitro*. For example, HAdV-F was found to be resistant to acid and digestive enzymes, and it tends to interact with lipids on the surface of gastric and intestinal mucosa [3, 6, 18]. However, the molecular mechanism behind these phenomena remains largely unknown, partly due to the fastidiousness of this virus.

HAdV-F is very difficult to grow in cell culture systems *in vitro* [22] and is therefore considered a fastidious or non-cultivable adenovirus. This feature severely hampers virological study of HAdV-F as well as its development as a gene transfer vector. HAdV-F includes two types: human adenovirus type 40 (HAdV-40) and HAdV-41. Recently, a HAdV-41 E1B55K–expressing cell line, 293TE7, was established in our lab [28]. Here, we have attempted to construct an infectious clone of HAdV-41 using this cell line, which we hope will facilitate the virological study of HAdV-41 using reverse genetics methods.

Materials and methods

Cells, viruses and plasmids

293A, a subclone of the 293 cell line, was purchased from Invitrogen (Carlsbad, CA, USA). 293TE7, a HAdV-41 E1B55K–expressing 293 cell line, was established in the laboratory recently [28]. Wild-type HAdV-41, which originated from a fecal sample (NIVD103), has been described elsewhere [18]. pAd41-GFP, a plasmid that was constructed previously [18], contains the whole genome of HAdV-41 except the E1 region, which was replaced by a GFP gene.

Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 8% fetal bovine serum (FBS; HyClone, Logan, UT, USA) at 37 °C in a humidified atmosphere supplemented with 5% CO_2, and passaged twice a week. For 293TE7 cells, G418 was regularly added to a final concentration of 400 μg/ml. For virus amplification experiments, confluent 293TE7 cells were split at a ratio of 1:2 or 1:3. Twenty-four to 48 hours later, when the cells had grown to 90% confluence, the culture medium was replaced with fresh DMEM containing 2% FBS. Thereafter, virus suspension was added to and mixed carefully with the medium, and the culture flasks were transferred to and kept in an incubator until harvest time.

Construction of a plasmid carrying the HAdV-41 genome

Wild-type HAdV-41 was amplified in 293TE7 cells and purified by one round of cesium chloride density gradient ultracentrifugation. Genomic DNA was extracted from purified wild-type HAdV-41 using a standard method [4]. Plasmid pAd41-GFP was digested with *Bgl* II, treated with alkaline phosphatase, and resolved on a 0.7% agarose gel. A 7611-bp band containing the right end of HAdV-41, the kanamycin resistance gene, the replication origin and part of the left end of HAdV-41 (Fig. 1) was excised from the gel, recovered, and used to co-transform *Escherichia coli* strain BJ5183 with HAdV-41 genomic DNA by electroporation. Dozens of colonies grew up on a kanamycin-containing LB agar plate. Six small and two normal-size colonies were picked and cultured in LB-kanamycin broth. All of the plasmids that were obtained from small colonies had a molecular weight of more than 21 kb when resolved on a 0.7% agarose gel with a lambda/*Hin*d III DNA marker. Three of them were used to transform the *E. coli* TOP10 strain to increase plasmid yield. Plasmids were prepared and analyzed with *Eco*R I. Two of the three plasmids, pKAd41#1 and pKAd41#3, yielded the same restriction map, which was consistent with the predicted molecular weights. pKAd41#1, hereafter referred to as pKAd41, was used in the subsequent experiments.

Rescue, amplification and purification of wild-type HAdV-41

Plasmid pKAd41 was linearized with *Pme* I and used to transfect 293TE7 cells after being mixed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. At day 6 post-transfection, the cells together with the medium (about 4 ml) in the 25-cm^2 flask were subjected to three rounds of freezing and thawing. After centrifugation, half of the supernatant (2 ml) was used as seed virus to infect exponentially growing 293TE7 cells in a 25-cm^2 flask, the medium was replaced with flesh DMEM plus 2% FBS the next day, and the virus suspension was prepared on day 6 postinfection. The infection process was repeated two more times until full cytopathic effect (CPE) was observed. The virus suspension, which was harvested from 293TE7 cells with a full CPE, could be amplified normally in 293TE7 cells at a ratio of 1:20. After two more rounds of amplification, viruses were collected from cells grown on 10 standard 150-mm dishes and subjected into two rounds of cesium chloride ultracentrifugation according to the standard method [4]. Purified viruses were preserved in phosphate-buffered saline (PBS) plus 5% glycerol at −80 °C after dialyzing to remove cesium chloride.

Titration of HAdV-41

The viral particle (vp) unit titer was calculated by measuring the genomic DNA content, where 100 ng of genomic DNA is equivalent to 2.86×10^9 vp, since the 34200-bp genome has a molecular mass of 2.11×10^7. In detail, 15 μl of purified virus was thoroughly mixed with an equal volume of lysis solution (20 mM EDTA, 1% SDS, 0.4 mg/ml proteinase K, pH 7.4) and incubated at 50 °C for 2 hours. The lysate was serially diluted with water and immediately resolved on a 0.6% agarose gel with a

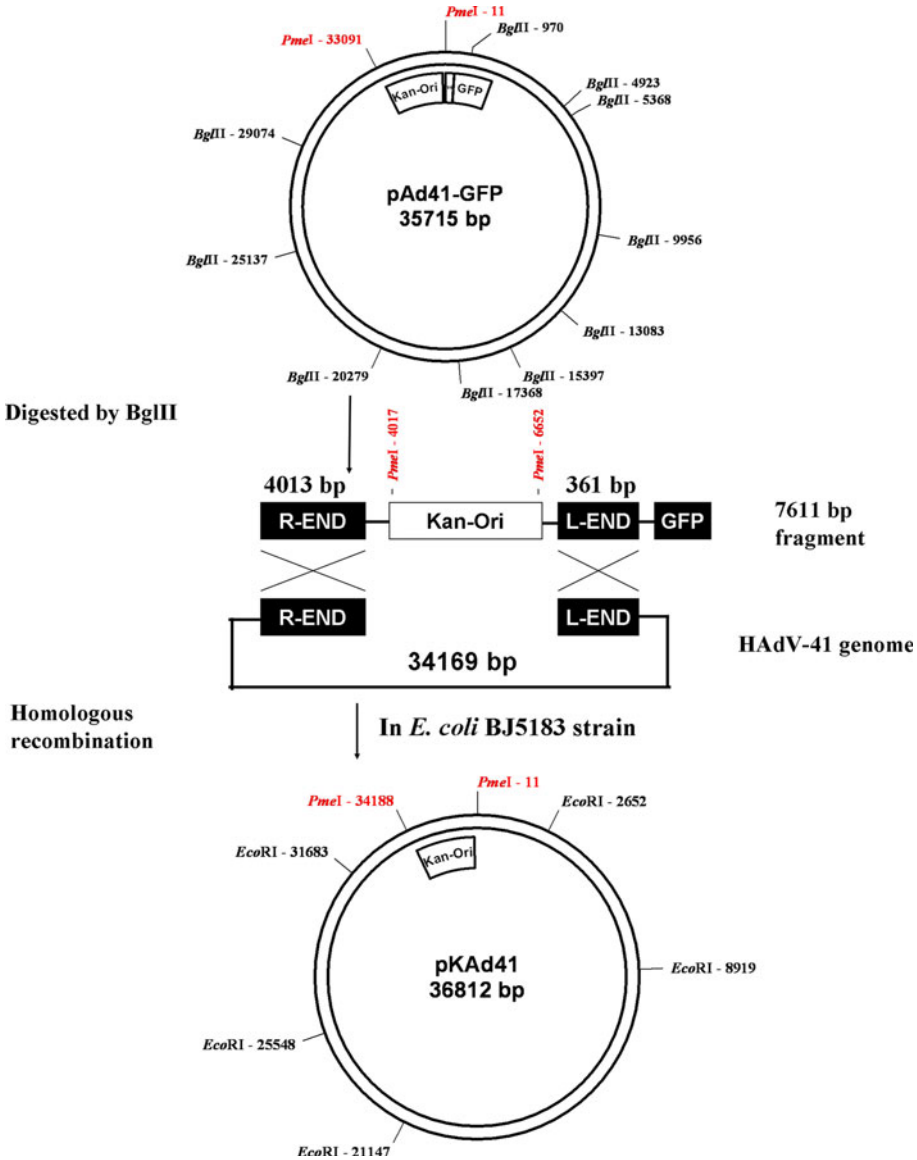

Fig. 1 Schematic diagram of the construction of the human adenovirus type 41 (HAdV-41) infectious clone. Kan-Ori: Kanamycin resistance gene and pBR322 origin of replication; GFP, enhanced green fluorescence protein gene; R-END, right end of the HAdV-41 genome; L-END, left end of the HAdV-41 genome

quantified lambda DNA/*Hin*d III digest as control. The gel was stained for 30 minutes in TBE buffer containing 0.5 μg/ml ethidium bromide and photographed on a UV transilluminator. The image was analyzed, and the DNA bands were quantified using the software Gel-Pro Analyzer 4 (Media Cybernetics, Silver Spring, MD, USA). The multiplicity of infection (MOI) was calculated from particle titers.

Infectious titers (IU/ml) of progeny viruses were determined by limiting dilution assay on 293A cells. Briefly, tenfold serially diluted viruses were used to infect exponentially growing 293A cells in a 96-well plate. At 48 hours postinfection, virus-infected cells were visualized by immunofluorescence (details are described below). The infectious titer was calculated as average number of virus-infected cells per well × dilution factor/volume of virus suspension per well (0.1 ml).

Electron microscopy

A purified virus suspension was diluted 1:2 in PBS containing 0.04% BSA and then dropped onto a piece of parafilm. A grid covered with carbon support formvar film was floated on the diluted virus suspension for 1 minute, stained with sodium phosphotungstate (pH 7.0), exposed to UV radiation for 10 min, and observed under a FEI Tecnai-12 electron microscope [13].

Restriction analysis of the viral genome

293TE7 cells were infected with purified HAdV-41 at an MOI of 100, and viral genomic DNA was extracted by the Hirt method [9] after two days of culture. DNA was digested with restriction endonucleases and analyzed by agarose gel electrophoresis.

Sequencing the E1 and E4 regions of HAdV-41

Six pairs of primers were synthesized and used to amplify the E1 and E4 regions of HAdV-41 (Table 1). The PCR products, which covered the whole E1 and E4 regions, were resolved on an agarose gel, recovered and sequenced.

SDS-PAGE and western blot

2×10^{10} purified HAdV-41 particles were mixed with 2 × SDS loading buffer, boiled for 5 minutes, and centrifuged at 12,000 g for 10 minutes. Twenty μl of supernatant was loaded into each well of a gel for SDS-PAGE. A western blot was done to detect the two fibers in HAdV-41 virions using a mouse anti-adenovirus-fiber monoclonal antibody (mAb) (clone 4D2) (catalog no. ab3233, Abcam, Hongkong). Recombinant HAdV-5 Ad-GFP-D19K was loaded as a positive control [17].

Table 1 Primers for amplification of the E1 and E4 regions of rescued HAdV-41 (refer to GenBank accession no. HM565136)

Name	Sequence	Range and length of PCR product (bp)
1107E1F1	ggtgcgatgt gacgtagagg ta	70–1230; 1161
1107E1R1	ggcgtcgtct acaggaaact ct	
1107E1F2	tactaggtcc gatttcagat gcg	1099–2196; 1098
1107E1R2	atattcaaag ctagttccca gtcc	
1107E1F3	tgggctgatt tggaagatga gtt	2075–3252; 1178
1107E1R3	aaactcacag cgttcccttc cat	
1107E4F1	ccccttccac tttatcttat acacct	31693–32900; 1208
1107E4R1	gagtggctaa acattgccct gaa	
1107E4F2	accatctgtc gcgttgcatc at	32752–33785; 1034
1107E4R2	ttatttcggt ggtggttcct ca	
1107E4F3	aaggtaaagc cacaataagc agc	33629–34101; 473
1107E4R3	aggtgcgatg tgacgtagag gt	

Immunofluorescence assay

Cells were infected with HAdV-41. At the indicated times after infection, the culture medium was removed, and the cells were washed once with PBS, fixed in cold methanol at −20 °C for 15 minutes, rinsed three times with PBS containing 1% bovine serum albumin (BSA-PBS), and incubated with a 1:250-diluted polyclonal antibody against the adenovirus hexon protein (AB1056, Millipore, Billerica, MA, USA) at room temperature for 2 hours. The cells were then washed three times with BSA-PBS and covered with 1:125-diluted FITC-labeled rabbit-anti-goat IgG for another 1 hour. After further washing, FITC-positive cells were observed and photographed under a fluorescence microscope and then counted using the UTHSCSA ImageTool (http://ddsdx.uthscsa.edu/dig/).

One-step growth curve

The procedure for establishing a one-step growth curve can be found elsewhere [28]. In brief, exponentially growing 293TE7 cells were infected with HAdV-41 at an MOI of 50 for two hours. At the indicated time points postinfection, progeny viruses associated with cells or in the supernatant were harvested and titrated.

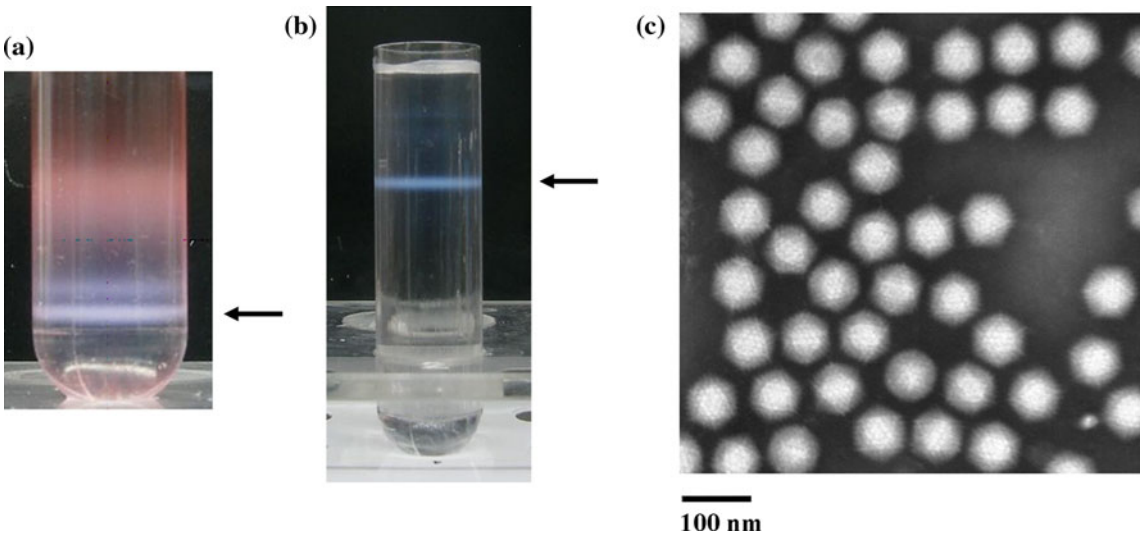

Fig. 2 Rescue, amplification and purification of HAdV-41. The pKAd41 plasmid was linearized with *Pme* I and used to transfect 293TE7 cells. The virus was harvested at day 6 post-transfection and blindly passaged three more times on fresh 293TE7 cells before a complete cytopathic effect (CPE) could be observed. After two more rounds of amplification, viruses harvested from 293TE7 culture in ten 15-cm^2 dishes were subjected to two rounds of CsCl gradient centrifugation (**a** and **b**). Typical morphology of adenovirus seen under the electron microscope (**c**)

Results

Cloning of the HAdV-41 genome and rescue of the virus

The 7611-bp fragment of *Bgl* II-digested pAd41-GFP contained the left end (361 bp) and the right end (4013 bp) of the HAdV-41 genome, which were separated by a kanamycin resistance gene and a pBR322 origin. Plasmid pKAd41 was generated by homologous recombination of the 7611-bp fragment and wild-type HAdV-41 genomes in *E. coli* strain BJ5183 (Fig. 1). pKAd41 contained the full-length genome of HAdV-41, which was confirmed by restriction analysis (data not shown). pKAd41 was partially sequenced. The sequence information for the left end of the genome was combined with that of pAd41-GFP, which was

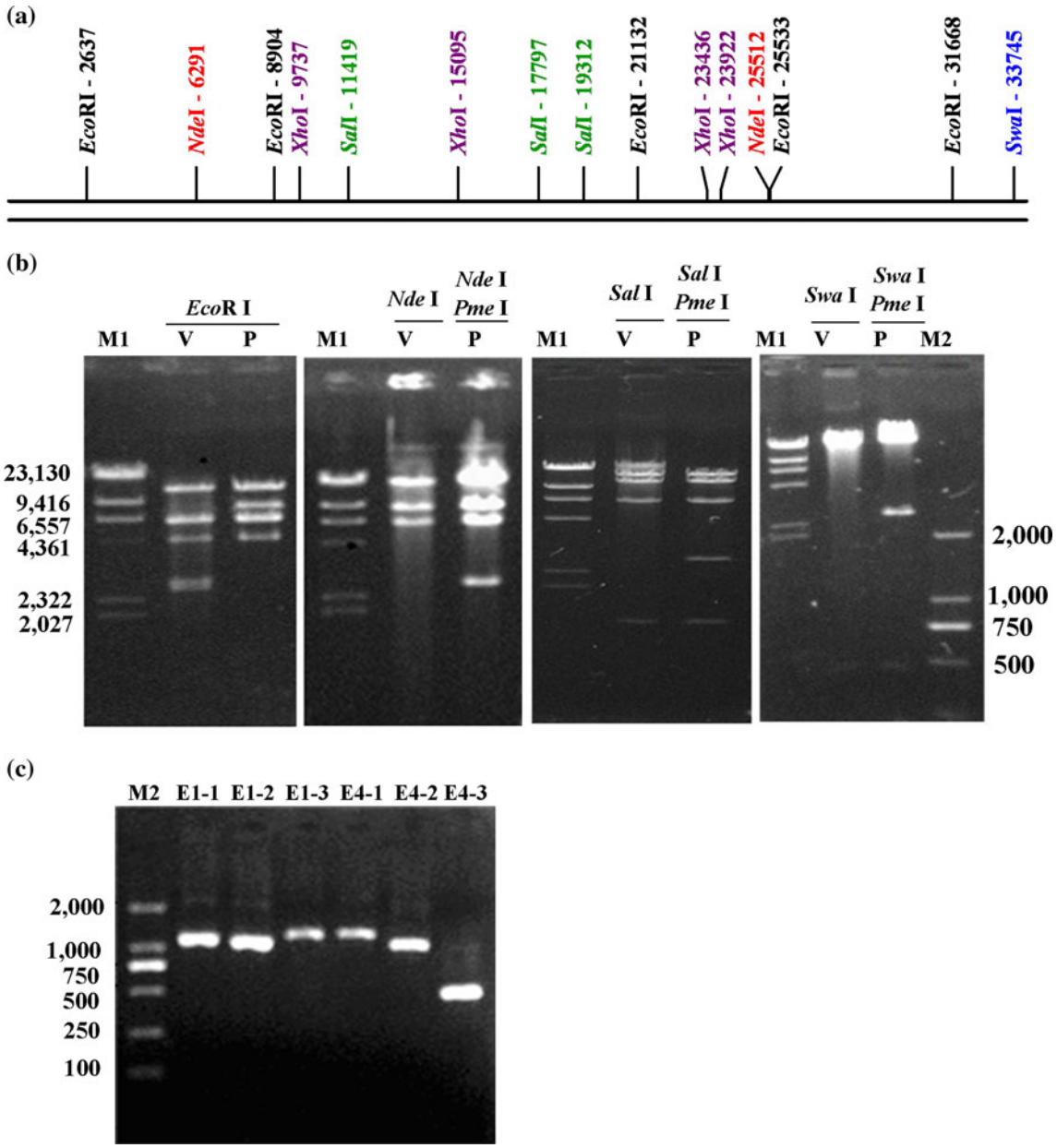

Fig. 3 Analysis of the integrity of the HAdV-41 genome. (**a**) Restriction map of HAdV-41. (**b**) The viral genome was extracted from purified HAdV-41, digested with restriction enzymes, and resolved in a 0.7% agarose gel. The pKAd41 plasmid was used as a control. The predicted molecular weights of digested fragments of the viral genome are 12,228, 6267, 6135, 4401, 2637, and 2501 bp for *Eco*R I; 19,221, 8657, and 6291 bp for *Nde* I; 14,857, 11,419, 6378, and 1515 bp for *Sal* I; and 33,745 and 424 bp for *Swa* I. (**c**) PCR was performed to amplify sequences of the E1 and E4 regions as described in "Materials and methods". The products were resolved in a 0.7% agarose gel, recovered and subjected to sequencing. The predicted molecular weights of the PCR products of E1-1, -2, -3, E4-1, -2, -3 were 1161, 1098, 1178, 1208, 1034, and 473 bp, respectively. M1: lambda/Hind III DNA marker; M2: DL2000 DNA marker (Takara, Dalian, China)

sequenced previously [18], to generate the complete genome sequence (GenBank accession no. HM565136.3).

pAd41-GFP was linearized with a restriction enzyme to release HAdV-41 genomic DNA, which was then used to transfect the packaging cell line 293TE7. Extensive CPE was observed after three rounds of blind passage of frozen and thawed infected cells. After further amplification, the virus suspension was applied to a cesium chloride gradient. Two clear bands were formed in the cesium chloride gradient after ultracentrifugation (Fig. 2a). The lower band was denser and thicker than the upper one and was thought to contain active viruses. The lower band was collected, mixed with cesium chloride solution, and subjected to buoyant density gradient ultracentrifugation. One clear band could be seen after the second round of purification (Fig. 2b). When observed under an electron microscope, the purified virions had a typical icosahedral appearance (Fig. 2c).

From 2×10^8 cells, 3 ml of purified viruses was ultimately obtained, with a particle titer of 1.1×10^{12} vp/ml. This means that one packaging cell could produce nearly 16,000 virions, a yield similar to that of HAdV-5 produced in 293 cells. Since the infectious titer was 8.3×10^9 IU/ml, the ratio of particles to infectious titer was approximately 133, which was about 10 times lower than that of HAdV-5.

Sequence analysis of HAdV-41 genomic DNA

After six passages of HAdV-41 in 293TE7 cells, its genomic DNA was extracted and analyzed using restriction enzymes. The theoretical restriction map is shown in Fig. 3a. The results of restriction digestion were consistent with the predicted fragments (Fig. 3b). To identify point mutations, small deletions, and insertion mutations, PCR was performed to amplify the whole E1 and E4 regions. PCR products were resolved on an agarose gel (Fig. 3c), and no unexpected bands were observed. PCR products were then sequenced, and no mutations were found (data not shown). These results demonstrate that the genome of HAdV-41 is genetically stable when propagated in 293TE7 cells.

Detection of fibers in HAdV-41

One feature of HAdV-41 is that the virion contains two fibers. A western blot detected two bands when using an antibody against the fiber proteins (Fig. 4), and the molecular weights of these bands were consistent with the predicted ones (41.4 kDa for the short fiber; 60.6 kDa for the long fiber), which further confirmed that the rescued virus was HAdV-41.

Expression of the hexon protein in HAdV-41-infected 293TE7 cells

293 or 293TE7 cells were transiently infected with HAdV-41 at an MOI of 50, and hexon-protein-expressing cells were visualized by immunofluorescence staining. No hexon-positive cells could be detected at 0 or 12 hours post-infection (hpi). Hexon-positive cells could be observed starting at 24 hpi. At 24 hpi, the numbers of fluorescent cells were very similar in these two cell lines, and then the percentage of hexon-expressing 293TE7 cells increased more dramatically (Fig. 5a). At 48 hpi, hexon-expressing cells in HAdV-41-infected 293TE7 were 2.5 times higher than in HAdV-41-infected 293 cells (Fig. 5b). These results illustrate that the late genes of HAdV-41 can be expressed more efficiently in 293TE7 cells and suggest a possible reason why HAdV-41 could be rescued and amplified in this cell line.

Non-lytic replication of HAdV-41 in 293TE7 cells

Viruses associated with the cells or in the culture medium were collected at serial time points postinfection and titrated. As shown in Fig. 6, progeny viruses in cells could start to be detected at 24 hpi. Virus production increased quickly to a high level at 48 hpi and remained quite stable after that. Although viruses could be found in the culture medium at 48 hpi, the titer was several orders of magnitude lower than that in cells. As dead cells appeared due to gradual exhaustion of nutrients in the medium, the amount of virus in the supernatant increased. However, this amount

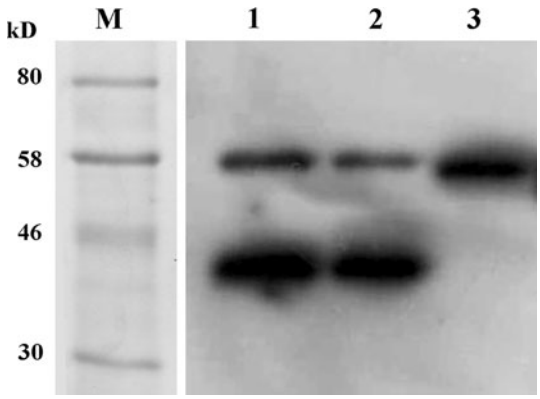

Fig. 4 Detection of rescued HAdV-41fibers by western blot. Purified viruses were lysed with 2 × SDS loading buffer, resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a PVDF membrane. Bands of fibers were visualized by western blot using an anti-fiber antibody (clone 4D2) (catalog no. ab3233, Abcam, Hongkong). The predicted molecular weight was 60.6 kDa for the long fiber of HAdV-41 and 41.4 kDa for the short one. M, protein marker (catalog no. P7708S; NEB, Ipswich, MA, USA); 1, wild-type HAdV-41 from sample isolate NIVD103; 2, rescued HAdV-41 from pKAd41 plasmid; 3, HAdV-5 control

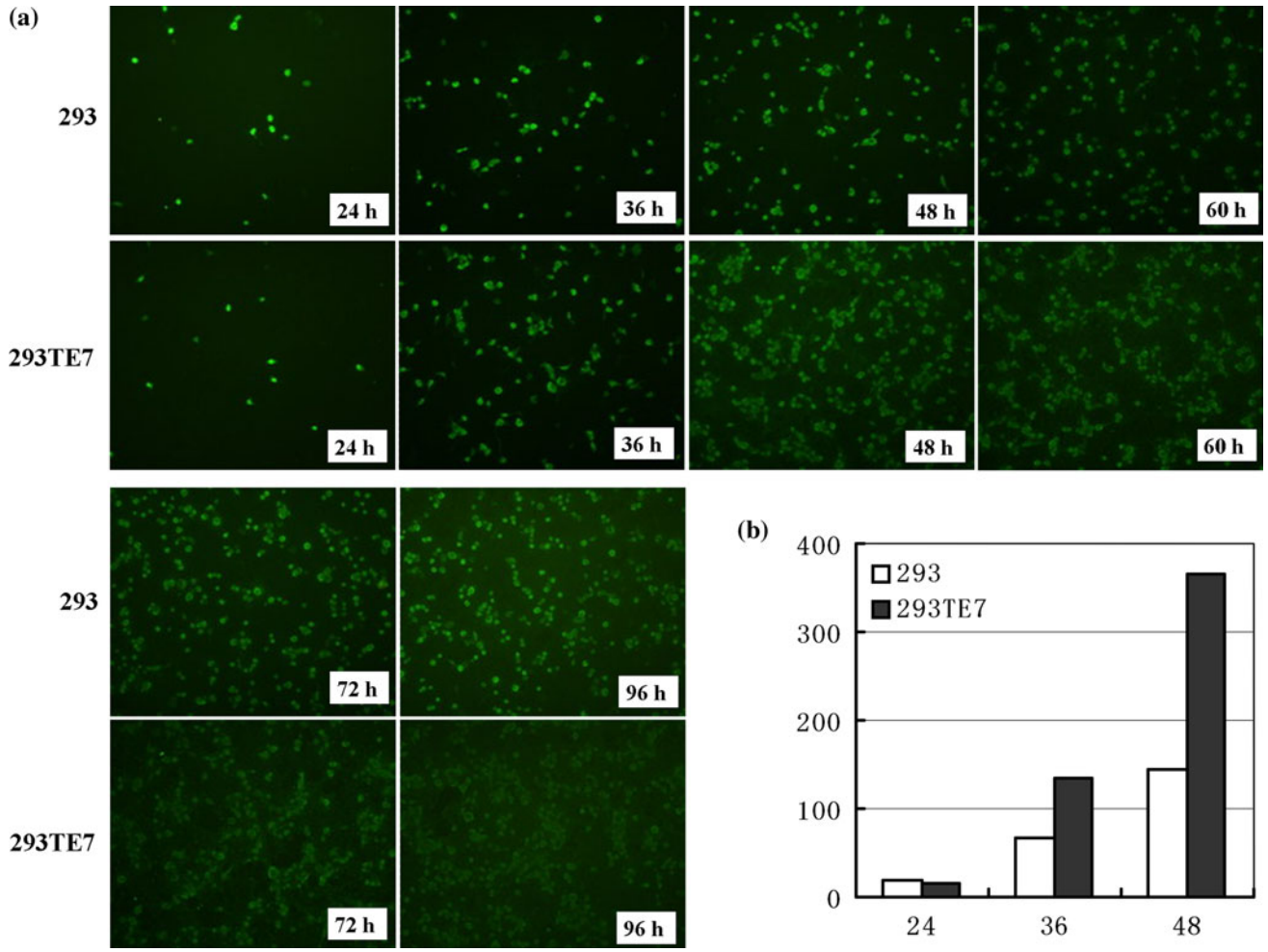

Fig. 5 Expression of hexon protein in HAdV-41-infected 293 and 293TE7 cells. 293 or 293TE7 cells were pulse-infected with HAdV-41 for 2 hours at an MOI of 50. (**a**) At the indicated times postinfection, hexon-expressing cells were visualized by immunofluorescence with anti-hexon antibody (AB1056, Millipore, Billerica, MA, USA). (**b**) At 24, 36 and 48 hours, fluorescence-positive cells were counted using the software UTHSCSA ImageTool (http://ddsdx.uthscsa.edu/dig/)

was still more than 20 times lower than that associated with cells even at eight days postinfection. This result shows that mature progeny viruses were not easily released from the cells.

Discussion

HAdV-41 has attracted great interest because of its potential to be reconstructed as a gene transfer vector for oral vaccines or gastrointestinal gene therapy, which is hindered by its poor growth in cell culture. Many efforts have been made to determine the mechanism of this growth defect *in vitro*. The possible reasons include insufficient expression of early and late virus genes, decreased replication and packaging of the viral genome, difficulty in achieving shutoff of host proteins, or a block in virus release [2, 22]. Although the reason for the fastidiousness of HAdV-F remains elusive, our previous work demonstrated that restricted expression of early genes might be one of the key reasons [18, 28].

293TE7 is a HAdV-41 E1B55K–expressing 293 cell line that was established for propagation of wild-type or recombinant HAdV-41. Here, we have investigated the feasibility of using it as a model to study HAdV-41 by reverse genetics methods. The HAdV-41 genome was cloned into a plasmid (pKAd41) by homologous recombination in bacteria [8]. Wild-type HAdV-41 could be rescued and amplified in 293TE7 cells. This was confirmed by restriction analysis of the viral genome and western blot to detect the two fibers on virions. Enhanced hexon expression could be seen in HAdV-41-infected 293TE7 cells, suggesting that increased viral early protein (E1B55K) could enhance the expression of viral late proteins, therefore resulting in improved virus growth. Most of the progeny viruses were cell-associated, and few viruses

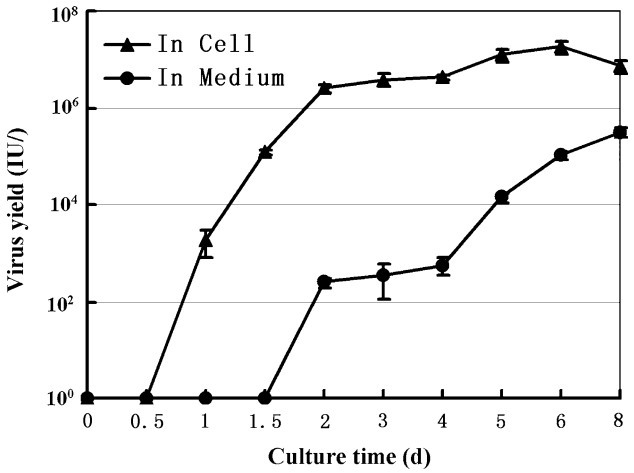

Fig. 6 One-step growth curve of HAdV-41 in 293TE7 cells. 293TE7 cells were seeded in 12-well plates, infected with HAdV-41 at an MOI of 50 for 2 hours. Cells and culture supernatants were collected at the indicated times postinfection. The progeny viruses associated with cells or in supernatants were titrated on 293 cells

could be found in the culture medium, suggesting a non-lytic replication of HAdV-41 in 293TE7, and this explains why plaques had not been observed.

As mentioned in the introduction section, HAdV-41 is an interesting type of human adenoviruses that deserves further study. Recently, several cell lines have been developed to amplify HAdV-F, including 293-SV5/V (293 cells transduced with the V protein gene of the paramyxovirus simian virus 5) [20], 293-CMV (constitutively expressing cytomegalovirus IE1 protein) [12], 293-ORF6, and 2V6.11 (inducibly expressing the HAdV-5 E4orf6 gene) [15, 25]. Some of these cell lines were established to isolate viruses from patients or the environment. Whether they could be used to rescue HAdV-F from its genomic DNA has not been tested. 293-ORF6 has proved to be an excellent cell line for HAdV-41 rescue and propagation. However, inducible expression of HAdV-5 E4orf6 would lead to cell death, whether there were viruses replicating in it or not. This might complicate research on interactions between the virus and its host cell.

The infectious clone of HAdV-41 (pKAd41) and 293TE7 cell line constitute an ideal system to study the virology of HAdV-41. Wild-type HAdV-41 could be rescued from pKAd41-transfected 293TE7 cells, making it useful for studying HAdV-41 using reverse genetics methods. The viral genes can be mutated, modified or deleted in the pKAd41 plasmid using normal molecular biological methods. Reconstructed pKAd41 can then be used to transfect 293TE7 cells to rescue the corresponding virus. Virus-host cell interactions can be studied at this phase, and the phenotype of the modified virus can be further investigated later. Actually, no exogenous gene except HAdV-41 E1B55K has been introduced into 293TE7 cells, while E1B55K is one of HAdV-41 genes. This means no exogenous gene would complicate the HAdV-41-293TE7 system. It is good to control interference factors and to keep the system simple, which will facilitate the analysis of experimental results.

Another advantage of this system is that rescued virus possesses a relatively stable genome. It has been reported that HAdV-41 is genetically unstable when growing in 293-ORF6 cells. Restriction digestion and sequencing were employed to analyze the genomic DNA of HAdV-41 amplified in 293TE7 cells. No obvious structural changes in the whole genome or point mutations in the E1 and E4 regions have been found. A stable genome makes it easier to analyze gene functions by reverse genetics and also makes it possible to evaluate gene functions by testing modified viruses on cell lines other than 293TE7 or in animal models.

The non-lytic property of HAdV-41 was confirmed here, suggesting that 293TE7-HAdV-41 is a good model for studying the release of adenovirus. Most human adenoviruses can lyse host cells, be released, spread to neighboring cells, and eventually form plaques. Using HAdV-5 or HAdV-2 models, many gene products, such as ADP, E4orf4, E4orf6 and i-leader, have been found to participate in this process [7, 14, 21, 23, 24]. HAdV-41 does not have an ADP gene [27]. However, it remains unclear why HAdV-41 cannot be released from host cells. Studying and modifying the non-lytic property of HAdV-41 is very important, since it is the major obstacle to growing plentiful recombinant HAdV-41 for gene therapy and vaccine development. 293TE7 and pKAd41 provide an ideal platform for such studies.

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