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Development of a recombinant adenovirus vector production system free of replication-competent adenovirus by utilizing a packaging size limit of the viral genome

Takayuki Suzuki [a,b], Tomomi Sasaki [a], Koyori Yano [a], Fuminori Sakurai [a,f], Kenji Kawabata [a,c], Masuo Kondoh [b], Takao Hayakawa [d,e], Kiyohito Yagi [b], Hiroyuki Mizuguchi [a,f,g,*]

[a] Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, Osaka, Japan
[b] Department of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan
[c] Department of Biomedical Innovation, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan
[d] Pharmaceutics and Medical Devices Agency, 3-3-2 Kasumigaseki, Chiyoda-Ku, Tokyo 100-0013, Japan
[e] Pharmaceutical Research and Technology Institute, Kinki University, 3-4-1 Kowakae, Higashi-Osaka, Osaka 577-8502, Japan
[f] Department of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan
[g] The Center for Advanced Medical Engineering and Informatics, Osaka University, Osaka, Japan

ARTICLE INFO

ABSTRACT

In a conventional adenovirus (Ad) vector production method using 293 cells, homologous recombination between Ad vector DNA and 293 cell-derived Ad E1 DNA occurs with low efficiency, resulting in the generation of replication-competent adenovirus (RCA). RCA can induce the spread of replication-incompetent Ad vectors, leading to unexpected tissue damage. In order to overcome this problem, we developed an Ad vector production system free of RCA generation by utilizing the Ad packaging size limit of the viral genome. It is well known that up to approximately 105% (37.7 kb) of the wild-type genome (35.9 kb) can be packaged in the Ad virion. We designed the Ad vector genome by insertion of a transgene expression cassette into the E3 region, such that homologous recombination between the Ad vector DNA and 293 cell-derived Ad E1 DNA would produce an Ad vector genome that exceeds in the size of the packaging limit. In accord with our strategy, no RCA generation was observed during the passages when we used the E1 (3.2 kb)-deleted Ad vectors containing a more than 3.0-kb transgene expression cassette in the E3 region. In contrast, the E1 (3.2 kb)-deleted Ad vectors, which retain 37.7 kb of the viral genome and have an insertion of a 2.1-kb transgene expression cassette in the E3 region, generated RCA, although RCA derived from this Ad vector exceeded the packaging size limit (105.0%). These results suggest that RCA generation can be avoided when the genome size of RCA is more than 108.3% (38.9 kb) of the wild-type Ad genome. This Ad vector production system generates safe, easy, and efficient Ad vector stock for both basic study as well as clinical research.

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1. Introduction

Recombinant adenovirus (Ad) vectors based on human Ad type 5 are widely used for gene transfer studies and clinical gene therapy trials due to their many advantages as gene delivery vehicles. For example, Ad vectors can be grown to high titers and they facilitate efficient transduction into both dividing and non-dividing cells. In addition, relatively large transgene expression cassettes can be inserted into the Ad vector genome. Currently, Ad vectors are used in approximately 24% of all clinical gene therapy protocols in the world.

In conventional replication-incompetent Ad vectors, the E1 (E1A and E1B) region, which is crucial for the self-replication of Ad, is deleted to make Ad vectors replication-incompetent, and a transgene expression cassette is often inserted into the E1-deleted region. Therefore, Ad vectors should be propagated in E1-transcomplementing cell lines, such as 293 cells. 293 cells (Graham et al., 1977), which are a human embryonic kidney cell line, are a principal E1-transcomplementing cell line used worldwide for Ad vector production. Replication-incompetent Ad vectors are efficiently propagated in 293 cells; however, replication-competent adenovirus (RCA) is generated at a low frequency by double crossover recombination between the Ad vector DNA and the integrated Ad E1 region in 293 cells. Generation of RCA is a

A **A conventional Ad vector production system**

Ad vector genome size:
Ad-GFP(E1) = 32.8 kb + 2.1 kb = 34.9 kb
Ad-Luc(E1) = 32.8 kb + 3.0 kb = 35.8 kb
Ad-LacZ(E1) = 32.8 kb + 4.4 kb = 37.2 kb

RCA genome size:
RCA derived from Ad-GFP(E1)
        = 32.8 kb + 3.1 kb = 35.9 kb
RCA derived from Ad-Luc(E1)
        = 32.8 kb + 3.1 kb = 35.9 kb
RCA derived from Ad-LacZ(E1)
        = 32.8 kb + 3.1 kb = 35.9 kb

RCA generation

B **A novel Ad vector production system developed in the present study**

Ad vector genome size:

Ad-Luc(E3) = 32.8 kb + 3.0 kb = 35.8 kb
Ad-LacZ(E3) = 32.8 kb + 4.4 kb = 37.2 kb

RCA genome size:

RCA derived from Ad-Luc(E3)
        = 32.8 kb + 3.0 kb + 3.1 kb = 38.9 kb
RCA derived from Ad-LacZ(E3)
        = 32.8 kb + 4.4 kb + 3.1 kb = 40.3 kb

RCA genome size is more than the packaging size limit.
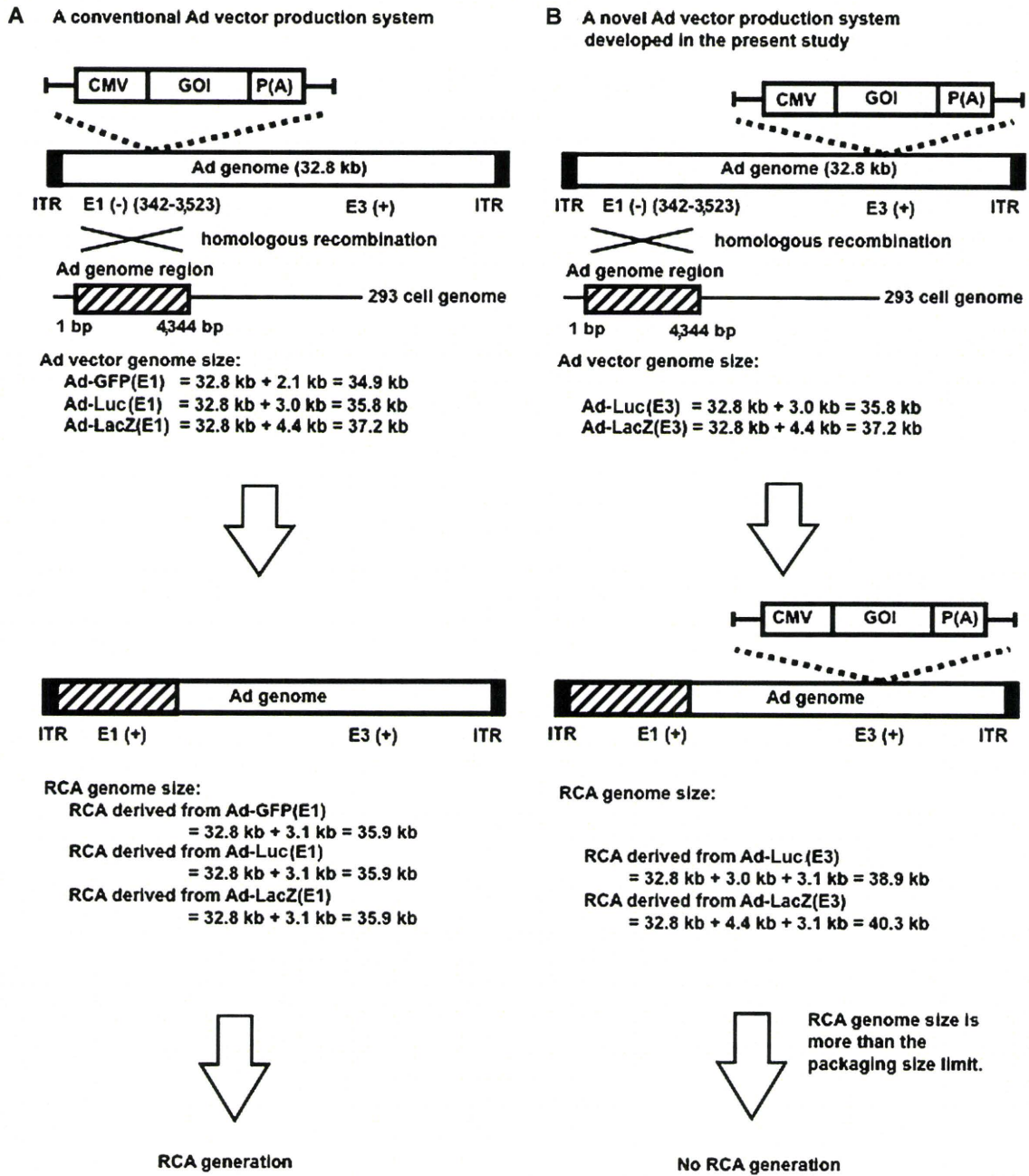
No RCA generation

Fig. 1. Strategy of the RCA-free Ad vector production system utilizing a viral packaging size limit. (A) A conventional Ad vector production system. (B) The novel RCA-free Ad vector production system developed for the present study. Note that Ad-GFP(E3) generated RCA, although RCA derived from Ad-GFP(E3) exceeded the packaging size limit (37.7 kb) (see text). *Abbreviations*: CMV, cytomegalovirus promoter; GOI, gene of interest; P(A), poly A signal; ITR, inverted terminal repeat.

major drawback of the Ad vector production system using 293 cells. 293 cells contain an Ad genome region with the E1A and E1B genes (bp 1-4344) in chromosome 19 at 19q13.2 (Louis et al., 1997). Since common first-generation Ad vectors have a deleted segment from approximately bp 400 to bp 3,500, both ends of the Ad E1 gene in the 293 cell genome are homologous with the Ad vector genome (Fig. 1). Contamination with RCA in Ad vectors is problematic for both basic research and clinical studies due to unintended Ad vector replication and the subsequent risk of an increased host inflammation response and/or tissue damage (Imler et al., 1995; Lochmuller et al., 1994; Hehir et al., 1996; Hermens and Verhaagen, 1997). For clinical use, the U.S. Food and Drug Administration (FDA) currently recommends that Ad vector preparations contain less than 1 RCA in

3×10^{10} particles (United States Food and Drug Administration & Biological Response Modifiers Advisory Committee; 5 April 2001).

A few strategies have been developed to overcome this problem. One is the development of special packaging cell lines, which lack the homologous sequences between Ad vector DNA and packaging cell-derived Ad E1 DNA (Fallaux et al., 1998; Murakami et al., 2002; Farson et al., 2006; Xu et al., 2006). Although these cell lines allow for the efficient growth of Ad vectors, they are not yet in widespread use. Moreover, RCA has been detected in Ad vector stocks prepared using one (i.e., PER-6) of these packaging cell lines via nonhomologous recombination (Farson et al., 2006; Murakami et al., 2004). Another approach to resolve the RCA issue is the deletion or degeneration of the pIX gene, which is located downstream of the E1B

gene. Deletion of the pIX gene reduces the occurrence of RCA, compared with that observed by conventional Ad vector preparation (Hehir et al., 1996; Zhu et al., 1999; Robert et al., 2001); however, the pIX deletion reduces the upper limit of the packaging size for the Ad vector genome, and the pIX-deleted Ad vectors become weaker when exposed to stressors (e.g., heat and freeze-and-thaw treatment) than conventional Ad vectors (Ghosh-Choudhury et al., 1987; Furcinitti et al., 1989).

In the present study, in order to develop an alternative strategy for the production of RCA-free Ad vectors, we took advantage of the size limitation imposed on the Ad genome packed into the virion. It is well known that the Ad virion has an upper packaging size limit of up to 105% (37.7 kb) of the wild-type Ad genome (35.9 kb) (Bett et al., 1993). An Ad genome larger than the packaging size limit is not packaged into the Ad virion, or frequently undergoes Ad vector genome rearrangements (Bett et al., 1993). We designed new Ad vector plasmids such that homologous recombination in the E1 region would generate an Ad vector genome larger than the packaging size limit. The original concept of this strategy was used for the production of helper-dependent Ad vectors (Parks et al., 1996; Sandig et al., 2000). To date, however, it has not been demonstrated that generation of RCA can be avoided by the strategy of limiting the packaging size. The proof-of-concept study demonstrated that after 16 passages of the E1 (3.2 kb)-deleted Ad vectors (which retain more than 35.8 kb of the viral genome and have an insertion of more than 3.0 kb of a transgene expression cassette in the E3 region), there was no detectable RCA formation. Importantly, the E1 (3.2 kb)-deleted Ad vectors (which retain 37.7 kb of the viral genome and have an insertion of a 2.1-kb transgene expression cassette in the E3 region) generated RCA, although RCA derived from this Ad vector exceeded the packaging size limit (105.0%). These results suggest that RCA generation can be avoided when the genome size of RCA is more than 108.3% (38.9 kb) of the wild-type Ad genome. In contrast, RCA was detected after 11 passages of conventional Ad vectors in which a transgene expression cassette was included in the E1-deleted region.

2. Materials and methods

2.1. Cells

HeLa cells (a human epithelial carcinoma cell line) were cultured in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. 293 cells (a human embryonic kidney cell line), A549 cells (a human pulmonary epithelial cell line), and SK HEP-1 cells (a human hepatoma cell line) were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and antibiotics.

2.2. Plasmids and Ad vectors

The Ad vectors were constructed by means of an improved *in vitro* ligation method described previously (Mizuguchi and Kay, 1998, 1999; Mizuguchi et al., 2001; Sakurai et al., 2005). An Ad vector plasmid, pAdHM41-E3(+), in which I-CeuI/SwaI/PI-SceI sites were included in the E3 region for insertion of a transgene expression cassette, was constructed as follows. An XbaI/ScaI fragment of pGEM-7Zf(+) (Promega Corp., Madison, USA) was ligated with oligonucleotides 1 (5′-CTAGCCTGCAGGATTTAAATGGCGCGCCGCGATCGCGCGGCCGCAGCT-3′) and 2 (5′-GCGGCCGCGCGATCGCGGCGCGCCATTTAAATCCTGCAGG-3′), resulting in pFS3 (SbfI and AscI recognition sequences are italicized and underlined, respectively). The Ad vector plasmid, pAdHM3 (Mizuguchi and Kay, 1998), was digested with AscI/SbfI after an SalI site of pAdHM3 had been changed

into an AscI site by using AscI phosphorylated linkers (New England Biolabs, Beverly, MA), and the fragment of pAdHM3 was ligated with the AscI/SbfI fragment of pFS3. The resulting plasmid, pAd25-35 kb, was digested with XbaI and ligated with oligonucleotides 3 (5′-CTAGGGAATCGATATCG-3′) and 4 (5′-CTAGCGATATCGATTCC-3′) (ClaI recognition sequences are underlined). The resulting plasmid, pAd25-35kb-ClaI, was digested with ClaI and ligated with oligonucleotides 5 (5′-*CGTAACTATAACGGTCCTAAGGTAGCGA*ATTTAAAT**ATCTATGTCGGGTG CGGAGAAAGAGGTAATGAAATGG**CA-3′) and 6 (5′-CGTG**CCATTTCA TTACCTCTTTCTCCGCACCCGACATAGAT**ATTTAAAT*TCGCTACCTTAGG ACCGTTATAGTTA*-3′) (I-CeuI, SwaI, and PI-SceI recognition sequences are italicized, underlined, and bold-faced, respectively). The resulting plasmid, pAd25-35kb-3, contains the Ad genome (from bp 25,292 to bp 33,289, with an I-CeuI/SwaI/PI-SceI site instead of the XbaI site [bp 28,593] of the Ad genome).

Then, pAdHM41 (Koizumi et al., 2003) was digested with I-CeuI/PI-SceI and ligated with oligonucleotides 7 (5′-ATCG**TCTAGA**ATCGGTGC-3′) and 8 (5′-CGATT**CTAGA**CGATTTAG-3′), resulting in pAdHM41-XbaI (XbaI recognition sequences are underlined). The SphI fragment of pAd25-35kb-3, which contains an I-CeuI/SwaI/PI-SceI site, was ligated with the SphI fragment of pAdHM41-XbaI, which contains the XbaI site in the E1-deleted region, resulting in pAd25-35kb-4. *Escherichia coli* BJ5183 (*recBC* and *sbcBC*) (Qbiogene, Inc., Carlsbad, CA) was co-transformed with SpeI-linearized pAdHM41-XbaI and the PacI/AscI fragment of pAd25-35kb-4 by electroporation. The resulting plasmid, pAdHM41-E3(+), contains a complete E1-deleted Ad genome with an XbaI site in the E1-deleted region (Δ bp 342–3,523), an I-CeuI/SwaI/PI-SceI site (bp 28,593) in the E3 region, a Csp45I site (bp 32,679) in the HI-loop coding region of the fiber knob, and a ClaI site (bp 32,784) in the C-terminal-coding region of the fiber knob. An Ad vector plasmid, pAdHM41-E3(−), in which an I-CeuI/SwaI/PI-SceI site was included in the E3-deleted region (Δ bp 27,865–30,995), was also constructed (Fig. 2).

The shuttle plasmids (pHMCMV-GFP1, pHMCMV-Luc1, or pHMCMV-LacZ1), which contain green fluorescence protein (GFP), *firefly* luciferase (Luc), or the β-galactosidase (LacZ) expression cassette driven by the cytomegalovirus (CMV) promoter, respectively, were digested by I-CeuI/PI-SceI and ligated with the I-CeuI/PI-SceI-digested pAdHM3 or I-CeuI/PI-SceI-digested pAdHM41-E3(+), resulting in pAdHM3-GFP(E1), pAdHM3-Luc(E1), pAdHM3-LacZ(E1), and pAdHM41-E3(+)-GFP(E3), pAdHM41-E3(+)-Luc(E3), or pAdHM41-E3(+)-LacZ(E3), respectively.

For generating Ad vectors (Ad-GFP(E1), Ad-Luc(E1), Ad-LacZ(E1), Ad-GFP(E3), Ad-Luc(E3), and Ad-LacZ(E3)), each vector plasmid was digested with PacI to release the recombinant viral genome, and the Ad vector plasmids were transfected into 293 cells on 60-mm dishes with SuperFect transfection reagent (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions.

Approximately ten days post-transfection, the cells were harvested, and the crude viral lysates were prepared by four cycles of freezing and thawing. The lysates were designated as passage 0. Each batch of the virus was prepared by transfection of each linearized vector plasmid. The lysates (passage 0) were then amplified on fresh 293 cells. Approximately 48 h later, the cells were harvested, and the crude viral lysates were released as described above; these lysates were designated as passage 1. For all subsequent passages, a 1/20 volume of cell lysates was added to fresh 293 cells. These procedures were repeated until passage 7, 11, or 16. At the last round of amplification before viral purification, cell lysates were added to five 150-mm dishes containing sub-confluent 293 cells. Crude viral lysates of passages 7, 11, and 16 were centrifuged to remove the cell debris, and the samples were then digested for 30 min at 37 °C with DNase I (0.2 mg/ml) and RNase A (0.2 mg/ml) in the presence of 10 mM $MgCl_2$ (Lieber et al., 1999). The viruses were
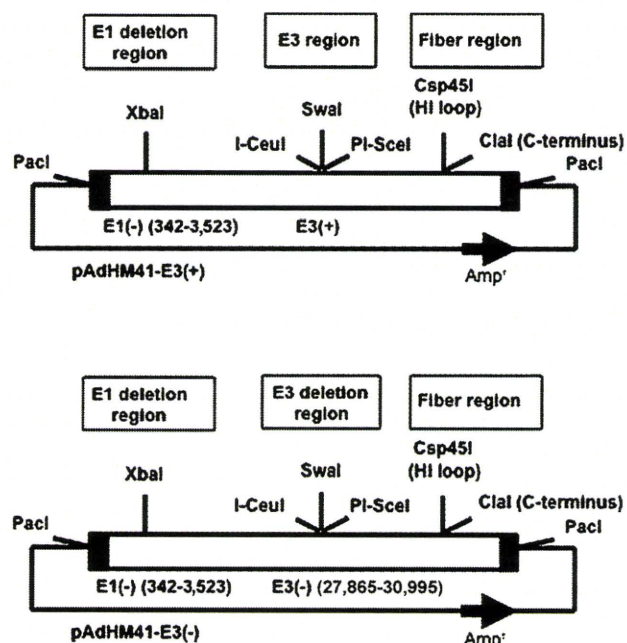
Fig. 2. Ad vector plasmids, pAdHM41-E3(+) and pAdHM41-E3(−). Both pAdHM41-E3(+) and pAdHM41-E3(−) contain a complete E1-deleted Ad genome with an XbaI site in the E1-deleted region (Δ bp 342–3,523), an I-CeuI/SwaI/PI-SceI site (bp 28,593) in the E3 region, a Csp45I site (bp 32,679) in the HI-loop-coding region of the fiber knob, and a ClaI site (bp 32,784) in the C-terminal-coding region of the fiber knob. The I-CeuI/SwaI/PI-SceI site of pAdHM41-E3(+) was introduced into the E3 region (bp 28,593), while that of pAdHM41-E3(−) was introduced into the E3-deleted region (Δ bp 27,865–30,995). PacI sites are located outside of Ad genome. Either vector plasmid can be used depending on the size of the transgene.

purified by two rounds of cesium chloride-gradient ultracentrifugation, dialyzed, and stored at −80 °C. The virus particles (VP) and biological (infectious unit; ifu) titer were determined spectrophotometrically by the method of Maizel et al. (1968) and by using an Adeno-XTM Rapid Titer Kit (Clontech, Inc., Palo Alto, CA), respectively. Purified virus titer was approximately $3–10 \times 10^{11}$ VP/ml in average.

2.3. Detection of RCA

The amount of RCA in the Ad vector stock solution was detected according to our previously developed method (Ishii-Watabe et al., 2003). Briefly, HeLa cells were seeded at 1.5×10^6 cells/dish on 100-mm dishes. On the following day, the cells were transduced with 10^9 VP of Ad vectors (666 VP/cell) for 2 h. Four days later, the cells and supernatants were recovered and centrifuged at 1,200 rpm for 5 min, and the cell pellets were then suspended in PBS(−). After being subjected to four cycles of freezing and thawing, the cells were centrifuged at 2,000 rpm for 10 min to remove the cell debris, and the supernatants were incubated with DNase I (0.2 mg/ml) and RNase A (0.2 mg/ml) in the presence of 10 mM $MgCl_2$ at 37 °C for 30 min in order to digest the nucleic acids derived from the cells. Then, the DNAs derived from the Ad vectors were extracted using SMI TEST EX-R&D (Genome Science Laboratories, Fukushima, Japan) in accordance with the manufacturer's instructions, and the extracted DNAs were dissolved in 20 μl of distilled water. In order to quantify the amount of RCA in 10 μl of DNA sample out of 20 μl of extracted DNA, real-time PCR was performed using the ABI prism 7000 system (Applied Biosystems, Foster City, CA). The sequences of the primers and probe used were as follows: for Ad5dE1-1035F, 5′-TCCGGTCCTTCTAACACACCTC-3′; for Ad5dE1-1105R, 5′-ACGGCAACTGGTTTAATGGG-3′; and

for Ad5dE1-1058TM probe, FAM-TGAGATACACCCGGTGGTCCCGC-TAMRA. These sequences were designed using Primer Express software version 1.0 (Applied Biosystems), and it was confirmed that they amplified the products of desired molecular weight. The PCR conditions were initial denaturation at 95 °C for 10 min, and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. An RCA calibration curve was generated using the DNA extracted from the Adenovirus Reference Material (ATCC VR-1516). The ratio of 1:8.3 represented the titer of infectious units to the VP titer of the Adenovirus Reference Material. Individual batches of Ad vector were examined in triplicate for RCA contamination. We designated as RCA-positive each experiment in which RCA was detected in at least one of the triplicate samples.

2.4. Luciferase assay

Luciferase activity in the cells was measured using a luciferase assay system (PicaGene LT2.0; Toyo Ink, Tokyo, Japan).

2.5. PCR analysis in the E3 region of Ad-GFP(E3)

To examine whether RCA derived from Ad-GFP(E3) underwent genome rearrangement in the GFP expression cassette, PCR analysis was performed using KOD Plus DNA polymerase (Toyobo, Osaka, Japan). The DNAs extracted for an infectivity PCR analysis were used as template. The sequences of the primers used for PCR were as follows: for Ad-E3-F, 5′-CCGAGCTCAGCTACTCCATC-3′; for Ad-E3-R, 5′-AGGCTGGCTCCTTAAAATCC-3′. The primers were located in the Ad genome E3 region outside of the GFP expression cassette (Fig. 5A). The PCR conditions were initial denaturation at 94 °C for 2 min, and then 30 cycles of denaturation at 94 °C for 15 s, annealing at 57 °C for 30 s, and extension at 72 °C for 2 min, followed by a final incubation at 72 °C for 5 min. The PCR products were subjected to gel electrophoresis with 0.7% TAE gels or 2% TBE gels followed by ethidium bromide staining. Only bands corresponding to DNA fragments of about 2.6 kb should be obtained unless genome rearrangement occurred in the GFP expression cassette.

2.6. Statistical analysis

Statistical significance ($P < 0.05$) was determined using Student's *t* test.

3. Results and discussion

In order to develop an Ad vector production system that prohibits RCA formation, we took advantage of the packaging limit of the viral genome. It is well-known that the upper limit for the size of the viral genome is 105% (37.7 kb) of the wild-type Ad genome (35.9 kb) (Bett et al., 1993). In order to render the Ad vector genome larger than the packaging size limit in cases involving the homologous recombination of the E1 region, two types of new Ad vector plasmids, pAdHM41-E3(+) and pAdHM41-E3(−), were constructed. pAdHM41-E3(+) contains the E3 region (although this region is often deleted in conventional Ad vectors in order to increase the capacity for the insertion of foreign genes), whereas the other Ad vector plasmid, pAdHM41-E3(−), has a 3.1-kb deletion in the E3 region (Fig. 2). The E1 region (bp 342–3,523) is deleted in both pAdHM41-E3(+) and pAdHM41-E3(−). The maximum cloning capacities for the transgene in pAdHM41-E3(+) and pAdHM41-E3(−) were approximately 5.0 and 8.1 kb, respectively. The I-CeuI/SwaI/PI-SceI site was introduced into the E3 region (bp 28,593) or the E3-deleted region for the simple and efficient insertion of a transgene expression cassette via an improved *in vitro* ligation method (Mizuguchi and Kay, 1998, 1999). Conventional Ad vectors carry a transgene expression cassette in the E1-deleted
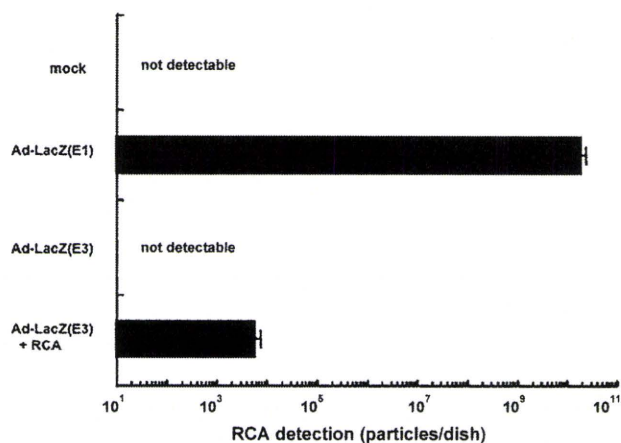
Fig. 3. RCA detection in Ad vectors using the infectivity PCR method. HeLa cells were transduced for 2 h with 10^9 VP of Ad-LacZ(E1) or Ad-LacZ(E3), or 10^9 VP of Ad-LacZ(E3) plus 1 infectious unit of RCA. After a 4-day incubation, the cells were harvested and the viral DNA was extracted. Then, the amount of RCA in the viral DNA was determined by real-time PCR, as described in Section 2. Data are presented as the mean value ± S.E. ($n = 3$). The numbers of RCA-positive samples in Ad-LacZ(E1), Ad-LacZ(E3), and Ad-LacZ(E3) plus RCA were 3, 0, and 3, respectively.

region. However, the new Ad vectors described in the present study carry a transgene expression cassette in a region other than the E1-deleted region (e.g., the E3 region). Both pAdHM41-E3(+) and pAdHM41-E3(−) also contain cloning sites (ClaI or Csp45I sites) in the HI-loop- or the C-terminal-coding region of the fiber knob, respectively, such that the foreign peptide-coding sequence can be inserted and viral tropism can be modified (Mizuguchi et al., 2001; Koizumi et al., 2003). pAdHM41-E3(+) and pAdHM41-E3(−) are used in a manner dependent on the size of the transgene expression cassette.

The CMV promoter-driven GFP (2.1 kb)-, luciferase (3.0 kb)-, or LacZ (4.4 kb)-expressing cassettes were inserted into the E3 region of pAdHM41-E3(+), but not into the E1-deleted region. In 293 cells, if homologous recombination takes place between the E1-deleted region of Ad-GFP(E3), Ad-Luc(E3), or Ad-LacZ(E3) and the E1 gene, the size of the viral genome would be 38.0 kb, 38.9 kb, or 40.3 kb, respectively, i.e., these viral genomes would theoretically lack the capacity to be incorporated into the virion due to having exceeded the packaging size limit (Fig. 1B). On the other hand, in the control groups, the transgene expression cassettes were included in the E1-deleted region of the conventional vector system, pAdHM3 (Mizuguchi and Kay, 1998), which contains the E3 region and the same E1-deleted region as that in pAdHM41-E3(+). Replacement of the E1-deleted, transgene-substituted region with the 293 cell-derived E1 gene by homologous recombination during viral amplification should result in a reduction of the size of the viral genome package below the size limit (35.9 kb), leading to RCA generation (Fig. 1A). Here, the yields of all Ad vectors were comparable (data not shown), and all Ad vectors expressed the target transgene (GFP, luciferase, or LacZ) (data not shown).

As a proof of the principle, we next examined RCA generation after different passages of Ad vectors. The frequency of RCA generation was examined after passages 7, 11, and 16 of the Ad vectors in 293 cells. For the RCA assay, our previously developed infectivity PCR method was used (Ishii-Watabe et al., 2003). One infectious unit of RCA spiked into 10^9 particles of Ad vectors was detected by this method (Ishii-Watabe et al., 2003) (Fig. 3). Moreover, RCA was found in all types of conventional Ad vectors that contained the GFP-, luciferase-, or LacZ-expressing cassette in the E1-deleted region (Ad-GFP(E1), Ad-Luc(E1), and Ad-LacZ(E1)), at least at passages 11 and 16 (Table 1). In contrast, all batches of Ad vectors

Table 1
RCA detection in batches of Ad vector after serial passages using an infectivity PCR method.

Ad vectors	Passage 7	Passage 11	Passage 16
Ad-GFP(E1)	3/3	1/3	3/3
Ad-GFP(E3)	0/3	1/3	3/3
Ad-Luc(E1)	0/3	2/3	1/3
Ad-Luc(E3)	0/5	0/5	0/5
Ad-LacZ(E1)	1/3	2/3	3/3
Ad-LacZ(E3)	0/5	0/4	0/5

Individual batches of Ad vector were examined in triplicate for RCA contamination, as described in Section 2. Batches of Ad vectors in which RCA was detected in at least in one of the triplicate samples were determined to be RCA-positive. The ratio of RCA-positive batches per tested batches ($n = 3$–5) is shown.

containing either the luciferase- or LacZ-expressing cassette in the E3 region (Ad-Luc(E3) and Ad-LacZ(E3)) completely lacked RCA generation, even after passage 16. Ad-GFP(E3) was less susceptible to the formation of RCA compared to Ad-GFP(E1) at passage 7, but generated RCA, even though homologous recombination between the E1-deleted region of Ad-GFP(E3) and the E1 gene in 293 cells yielded 38.0 kb of viral genome, i.e., the viral genome product exceeded the previously reported packaging limit. Since Ad-Luc(E3) and Ad-LacZ(E3) contain 35.8 kb and 37.2 kb of the viral genome, they would produce 38.9 kb and 40.3 kb of the viral genome, respectively, which exceed the packaging size limit (37.7 kb) by more than 1.2 kb, assuming that the E1-deleted region became E1-positive due to homologous recombination. These results indicate that if the Ad genome exceeds the packaging size limit by more than 1.2 kb (the Ad genome exceeds 108.3% (38.9 kb) of the wild-type Ad genome), it would not be incorporated into the virion. Moreover, in order to prevent RCA formation simply, the Ad vector genome should be designed to contain the transgene in regions other than the E1-deleted region (i.e., the E3 region, the end of the fiber-coding region, etc.), and the viral genome should be at least 35.8 kb. If the vec-
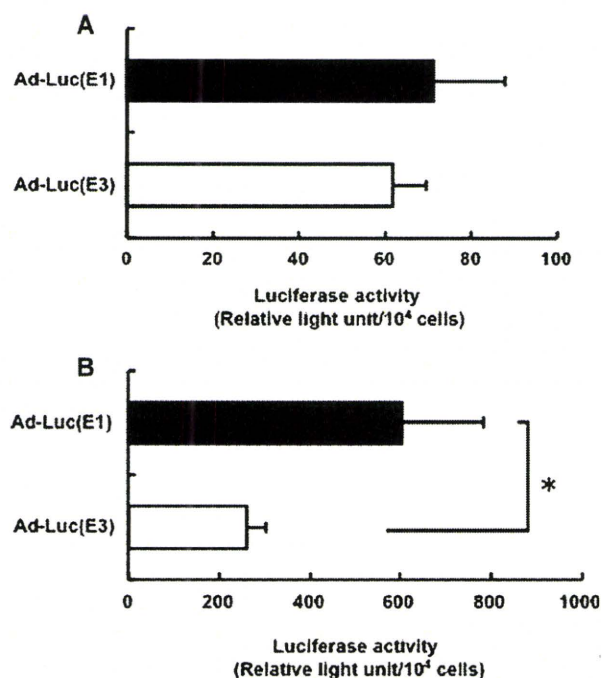

Fig. 4. Comparison of luciferase activity in the cells transduced by Ad-Luc(E1) or Ad-Luc(E3). A549 (A) and SK HEP-1 (B) cells were transduced with 5 ifu/cell of Ad-Luc(E1) or Ad-Luc(E3). After 48 h of culture, the luciferase activity in the cells was measured by a luminescence assay. Data are presented as the mean value ± S.D. ($n = 4$). *$p < 0.05$.
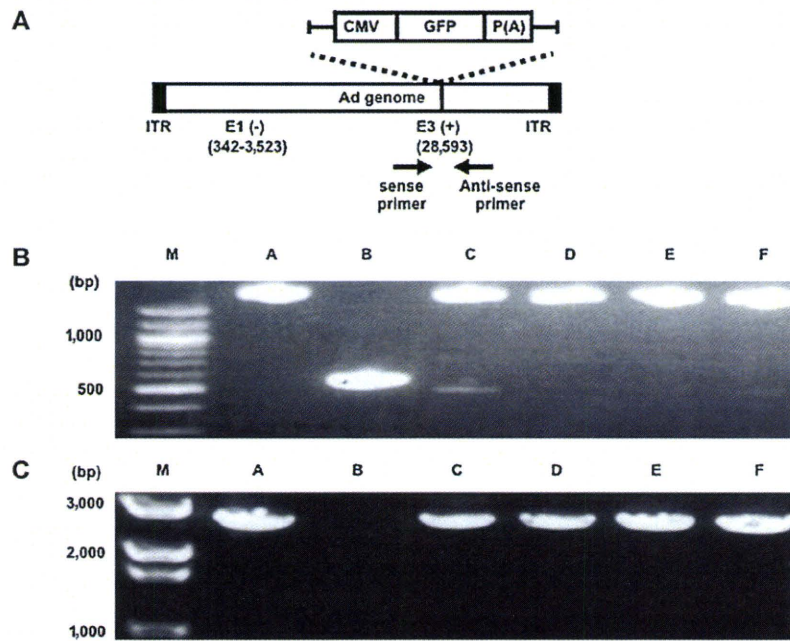
Fig. 5. PCR analysis of genome rearrangement in the E3 region of Ad-GFP(E3). (A) The locations of the PCR primers. The primers were located in the E3 region of the Ad genome outside of the GFP expression cassette. (B and C) PCR analysis. The DNAs extracted for an infectivity PCR analysis were used as a template. PCR fragments were subjected to gel electrophoresis with 2% TBE gels (B) and 0.7% TAE gels (C). Lane M, molecular weight marker; lane A, plasmid, pAdHM41-E3(+)-GFP; lane B, plasmid, pAdHM41-E3(+); lane C, Ad-GEP(E3) in passage 11; lane D–F, Ad-GFP(E3) in passage 16 (the viral batches were different in lanes D–F). Note that a faint band corresponding to a DNA fragment of approximately 0.5 kb was obtained in lane F.

tor genome is smaller than 35.8 kb, a "stuffer" sequence should be introduced in order to increase the viral genome size and prevent RCA formation.

pAdHM41-E3(+) contains the E3 region, which encodes several types of Ad gene, including the Ad death protein (ADP) gene (the ADP ORF is located from bp 29,491 to 29,772). Ad vectors containing the ADP gene allow for more efficient propagation than Ad vectors lacking ADP gene (Tollefson et al., 1996a,b). The cloning site for the transgene in pAdHM41-E3(+) is located at bp 28,593, and this location would not prevent ADP expression. Thus, an Ad vector derived from pAdHM41-E3(+) should be easily and efficiently propagated. When the transgene expression cassette is larger than 5.0 kb, the vector plasmid pAdHM41-E3(+) cannot be used, because it will produce a desired virus whose size is in excess of the packaging size limit. In this case, the vector plasmid pAdHM41-E3(−) should be used instead.

To examine whether the expression levels of Ad vectors with the transgene insertion in the E3 region are the same as those of the vectors with insertion in the (deleted) E1 region, luciferase activities in A549 or SK HEP-1 cells transduced with Ad-Luc(E1) were compared with those of the same cell types transduced with Ad-Luc(E3) (Fig. 4). Luciferase activities in A549 cells transduced with Ad-Luc(E3) were comparable to those of A549 cells transduced with Ad-Luc(E1), while in SK HEP-1 cells, Ad-Luc(E1) was more efficient than Ad-Luc(E3). Several genes are located upstream or downstream of the cloning region (bp 28,593) in the E3 region. The influence of the transgene location (E1 or E3) on the efficiency of transgene expression might differ depending on the cell type. When the cloning site for the transgene in the E3 region is different from the location at bp 28,593 of the Ad genome, the efficiency of transgene expression might be changed. In the case of Ad vectors generated from pAdHM41-E3(−), the efficiency of transgene expression would also be changed because almost the entire E3 region was deleted in pAdHM41-E3(−).

Bett et al. (1993) reported that approximately 105% of the wild-type genome could be packaged into an Ad virion without

affecting the viral growth rate or titer. The genome size of the RCA derived from Ad-GFP(E3) is approximately 0.3 kb above the packaging size limit when the E1-deleted region is changed into an E1-positive. Thus, our finding that Ad-GFP(E3) generated RCA was unexpected (Table 1). In cases of small overages of the size limit, a viral genome could be packaged by force, possibly accompanied by genome rearrangement, which might occur in a region unessential for virus replication, such as a transgene region. To examine whether genome rearrangement of Ad-GFP(E3) occurred, PCR analysis in the E3 region was performed (Fig. 5). In the case of a virus which did not undergo genome rearrangement, approximately 2.6 kb DNA fragments containing a GFP expression cassette should be obtained. Two out of four Ad-GFP(E3) batches tested showed approximately 0.5 kb DNA fragments in addition to the 2.6 kb DNA fragments (Fig. 5B, lanes C and F), probably due to the loss of the GFP expression cassette. These viral stocks would contain both the Ad vector with correct GFP expression cassette and the vector with deletion of GFP expression cassette. These results suggested that at least a part of RCA derived from Ad-GFP(E3) underwent genome rearrangement in the GFP expression cassette.

The original concept to prevent RCA generation by utilizing the Ad packaging size limit of the viral genome was used for the production of helper-dependent Ad vectors (Parks et al., 1996; Sandig et al., 2000). However, the precise size limitation to prevent RCA generation was not examined. This is the first study that clearly demonstrated the prevention of RCA generation by this strategy and the minimum viral genome size required for the preparation of RCA-free Ad vector stocks.

Several types of special packaging cell lines lacking the homologous sequences between Ad-vector DNA and packaging cell-derived Ad E1 DNA have been developed (Fallaux et al., 1998; Murakami et al., 2002; Farson et al., 2006; Xu et al., 2006). However, these cell lines are not widely used. The advantages of the method described in the present study include the use of a conventional packaging cell line (293 cells), and the relatively easy construction of an Ad vector containing the trans-

gene expression cassette in a region other than the E1-deleted region.

The sensitivity of the infectivity PCR method (Ishii-Watabe et al., 2003) used in the present study is so high that one infectious unit of RCA, spiked into 10^9 particles of Ad vector, was detected. Individual batches of the Ad vector were examined in triplicate for RCA contamination. We designated as RCA-positive each experiment in which RCA was detected in at least one of the triplicate samples. Five experiments using Ad-LacZ(E3) and Ad-Luc(E3) are shown in Table 1. Thus, the results of Ad-LacZ(E3) and Ad-Luc(E3) in passage 16 in Table 1 show that there was no RCA in the experiments using 15 dishes, which means there was no RCA in 1.5×10^{10} particles of virus stock of Ad-LacZ(E3) and Ad-Luc(E3). In order to determine whether or not virus stock contains less than one RCA in 3.0×10^{10} particles (i.e., the U.S. FDA recommendation for RCA contamination for the clinical use of Ad vector) (United States Food and Drug Administration & Biological Response Modifiers Advisory Committee; 5 April 2001), experiments using 30 dishes would be needed to conducted to expand upon the results of the present study, in which 10^9 particles of Ad vectors were tested using only one dish.

4. Conclusions

In summary, we have developed a novel RCA-free Ad vector production system by utilizing a size limit for packaging the viral genome into a virion. In the present study, the use of this method of limiting the genome packaging size resulted in an absence of RCA formation in all batches of Ad vectors containing a transgene expression cassette in the E3 region that had a genome size of at least 35.8 kb. The present system is simple, easy to use, and suitable for large-scale Ad vector preparation, which suggests its potential usefulness for a wide range of clinical settings.

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Adenovirus vector-mediated assay system for Hepatitis C virus replication

Takeshi Yoshida[1], Masuo Kondoh[1,*], Manabu Ojima[1], Hiroyuki Mizuguchi[2], Yoshiaki Yamagishi[1], Naoya Sakamoto[3] and Kiyohito Yagi[1,*]

[1]Laboratory of Bio-Functional Molecular Chemistry, [2]Department of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka and [3]Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan

ABSTRACT

The efficient delivery of the hepatitis C virus (HCV) RNA sub-genomic replicon into cells is useful for basic and pharmaceutical studies. The adenovirus (Ad) vector is a convenient and efficient tool for the transduction of foreign genes into cells *in vitro* and *in vivo*. However, an Ad vector expressing the HCV replicon has never been developed. In the present study, we developed Ad vector containing an RNA polymerase (pol) I-dependent expression cassette and a tetracycline-controllable RNA pol I-dependent expression system. We prepared a hybrid promoter from the tetracycline-responsive element and the RNA pol I promoter. Ad vector particles coding the hybrid promoter-driven HCV replicon could be amplified, and interferon, an inhibitor of HCV replication, reduced HCV replication in cells transduced with the Ad vector coding HCV replicon. This is the first report of the development of an Ad vector-mediated HCV replicon system.

INTRODUCTION

Hepatitis C virus (HCV) is a member of *Flaviviridae* that contains a 9.6 kb positive-sense RNA genome. A total of 170-million people worldwide are infected with HCV, leading to chronic hepatic inflammation, hepatic fibrosis, hepatic cirrhosis and hepatocellular carcinoma (1). Chronic infection with HCV is a major cause of hepatocellular carcinoma (1). Interferon (IFN) therapy is the gold standard method for HCV patients, but it is effective in only 50% of patients and its use has been limited because of severe side effects (2–4). Additional pharmaceutical therapies are needed to overcome HCV. However, the tropism of HCV is limited to chimpanzees and

humans, and the mechanism of HCV infection and replication is not fully understood. The HCV genome encodes a polyprotein precursor of about 3000 amino acids that is cleaved into at least 10 proteins: core, envelope 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B (5). An HCV subgenome replicon (called HCV replicon in the present study) consisting of a reporter gene and HCV NS genes has allowed various studies of HCV replication and the development of anti-HCV agents (6–8). The delivery of the HCV genome or HCV replicon is a powerful tool for basic and pharmaceutical research, and the transduction of *in vitro* translated HCV RNA genome is often performed by electroporation. However, a convenient and efficient method to transfer the 9.6-kb HCV RNA genome or the 8–9-kb HCV replicon has never been fully developed.

Transcribed RNAs are classified into rRNAs, mRNAs and short RNAs (tRNAs) in mammalian cells. RNA polymerases differ among the transcribed RNA species: RNA polymerase (pol) I for rRNAs, RNA pol II for mRNA and RNA pol III for short RNAs. RNA pol I transcribes RNA without a 5′-cap structure or a 3′-poly-A tail, and a plasmid vector encoding RNA pol I promoter and terminator has been applied to the development of RNA virus-expression system. For instance, influenza viruses, arenavirus and uukuniemi viruses are generated using RNA pol I-driven expression plasmid vectors coding each segment of negative-sense RNA (9–12). Recombinant adenovirus (Ad) vectors have been widely used to deliver foreign genes to a variety of cell types and tissues *in vitro* and *in vivo* in basic research and clinical therapy. Ad vector can be easily prepared, grown to a high titer, and used to efficiently transfer genes into dividing and non-dividing cells. Furthermore, several types of Ad vectors have been developed to expand their tropism and to increase the size of encoded genes (13,14). Ad vector encoding RNA pol I-driven expression of influenza virus

*To whom correspondence should be addressed. Tel: +81 6 6879 8196; Fax: +81 6 6879 8199; Email: masuo@phs.osaka-u.ac.jp
Correspondence may also be addressed to Kiyohito Yagi. Tel/Fax: +81 6 6879 8195; Email: yagi@phs.osaka-u.ac.jp

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

RNA has been developed for the generation of vaccine seed strains and for basic influenza virus studies (15). These findings indicate that the RNA pol I Ad vector system can be a promising tool for basic and pharmaceutical studies on HCV. However, the development of an RNA pol I-driven vector system expressing the HCV RNA genome has never been reported.

In the present study, we developed an RNA pol I-driven vector system to monitor HCV replication using an HCV replicon in which structural genes were replaced by the luciferase gene. We prepared an Ad vector containing a tetracycline (tet)-regulated RNA pol I-expression cassette consisting of an RNA pol I-driven responsive vector and a *trans*-activator vector, and we successfully developed an Ad vector-mediated HCV replication system.

MATERIALS AND METHODS

Cell culture

Huh7.5.1 1bFeo [genotype 1b HCV replicon cell line, (8)] were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and G418 (Nacalai Tesque, Kyoto, Japan) at $500 \mu g/ml$. Huh7 and 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS. The cells were maintained in a 5% CO_2 atmosphere at $37°C$.

Preparation of RNA pol I-driven plasmid vectors

An RNA pol I expression-cassette was subcloned as follows: pHH21 (kindly provided by Dr Kawaoka, Tokyo, Japan) containing RNA pol I expression cassette was digested with *Afl*III, blunted by the Klenow fragment of DNA polymerase, ligated with EcoRI linker and digested with EcoRI/NheI, resulting in a fragment of the RNA pol I expression-cassette. The RNA pol I cassette was inserted into the EcoRI-XbaI site of pHM5 (16), generating pPol I. A fusion gene of enhanced green fluorescent protein and firefly luciferase (EGFPLuc, Clontech, Mountain View, CA, USA) was inserted into pPol I, resulting in pP$_I$WT-EL.

The subgenomic HCV sequence and the replication-incompetent subgenomic HCV sequence deleting GDD motif (MLVNGDDLVV) in NS5B were amplified by polymerase chain reaction (PCR) using pRepFeo as a template (8). The PCR fragments were inserted into pPol I, generating pPol I-1bFeo and pPol I-1bFeoΔGDD. The Feo fragment in pPol I-1bFeo or pPol I-1bFeoΔGDD was replaced with firefly luciferase, generating pPol I-HCV or pPol I-ΔGDD coding firefly luciferase reporter, HCV NS3, NS4A, NS5A and NS5B or mutated NS5B, respectively. A plasmid expressing β-galactosidase, pCMVβ, was purchased from Marker Gene Inc. (Eugene, OR, USA).

Preparation of tet-controllable RNA pol I-driven plasmid vectors

To develop the tet-controllable RNA pol I promoter expression system, the minimal cytomegalovirus promoter was replaced by fragments of RNA pol I promoters (from −235 to −1, from −311 to −1 or from −412 to −1) in pHM5-TREL2 (17), generating pP$_I$235, pP$_I$311 or pP$_I$412. These RNA pol I plasmid vectors were used for optimization of the tetracycline responsive element (TRE)/RNA pol I chimeric promoter. pHM5-tTA, pHM5-rtTA and pHM5-TREL2 were used in tet-regulated experiments (17).

Preparation of Ad vector expressing HCV replicon

The subgenomic HCV replicon fragments cloned from pPol I-HCV or pPol I-ΔGDD were inserted into pP$_I$235, and then the firefly luciferase was replaced by the renilla luciferase to form pP$_I$235-HCV or pP$_I$235-ΔGDD. Ad vectors were constructed by an improved *in vitro* ligation method (18). Briefly, pP$_I$235-EL, pP$_I$235-HCV and pP$_I$235-ΔGDD were digested with I-CeuI and PI-SceI, and then ligated with I-CeuI/PI-SceI-digested pAdHM4 and pAdHM36, respectively. The resulting plasmids were digested with PacI and transfected into 293 cells with SuperFect (Qiagen, Valencia, CA, USA). AdP$_I$235-EL, AdP$_I$235-HCV and AdP$_I$235-ΔGDD were purified by $CsCl_2$ gradient centrifugation and dialyzed with a solution containing 10 mM Tris (pH 7.5), 1 mM $MgCl_2$ and 10% glycerol. The multiplicity of infection (MOI) of Ad vectors was measured using an Adeno-X rapid titer kit (Clontech). Ad-tTA vectors were prepared as previously described (17).

Expression of plasmid-based HCV replicon

Huh7 cells were transfected with $0.8 \mu g$ of pPol I-HCV. After 24 h of incubation, the cells were lysed in LCβ (Toyo Ink, Tokyo, Japan). The cell lysates were frozen-thawed and centrifuged at 32 000 g for 5 min. The luciferase activity in the resulting supernatant was measured using a commercially available kit (PicaGene; Toyo Ink).

Inhibition assays of HCV replication in plasmid- or Ad-based RNA pol I HCV system

Huh7 cells were transfected with $0.8 \mu g$ of pPol I-HCV and $0.2 \mu g$ of pCMVβ or infected with AdP$_I$235-HCV (10 MOI) and Ad-tTA (50 MOI). After 24 or 1.5 h of transfection, the cells were treated with recombinant human interferon-α8 (IFN-α8) at the indicated concentration. After an additional 72 h of incubation, the cells were lysed in LCβ. Luciferase activity and β-galactosidase activity in the lysates was measured with PicaGene and a Luminescent β-gal Kit (Takara Bio Inc., Shiga, Japan), respectively. The cell viability was measured with a WST-8 kit according to the manufacturer's instruction (Nacalai Tesque, Kyoto, Japan).

Evaluation of tetracycline-controllable promoters in plasmid vector

Huh7 cells were co-transfected with $0.1 \mu g$ of reporter plasmid (pP$_I$235-EL, pP$_I$311-EL, pP$_I$412-EL or pP$_I$WT-EL), $0.8 \mu g$ of tet-responsive *trans*-activator plasmid (pHM5-rtTA in the tet-on system or pHM5-tTA in the tet-off system) and $0.1 \mu g$ of pCMVβ. After 2.5 h, the cells were treated with doxycycline (Dox) at the indicated

concentration for 48 h. Then, luciferase and β-galactosidase activities in the lysates were measured.

Expression of Ad vector containing tetracycline-controllable promoter system

Huh7 cells were transfected with a reporter Ad vector (AdP$_I$235-EL or AdP$_I$235-HCV at MOI of 5 or 10) and a *trans*-activator vector (Ad-tTA at MOI of 10 and 50). After an additional 48 h of incubation, luciferase activity in the cell lysates was measured.

Western blotting

Huh7 cells were co-infected with AdP$_I$235-HCV at 10 MOI and Ad-tTA at 50 MOI. The cells were lysed in RIPA buffer [50 mM Tris–HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA] containing a cocktail of protease inhibitors (Sigma, St Louis, MO, USA). The cell lysates (30 μg of protein) were subjected to sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS–PAGE), followed by western blotting onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking in 5% skim milk, the filter was incubated with mouse anti-NS5A (Meridian Life Science, Sacao, ME, USA) or anti-β-actin Ab (Sigma). Then, the peroxidase-labeled secondary antibodies were added. The immunoreactive bands were visualized by chemilumines-cence reagents (GE Healthcare, Buckinghamshire, UK).

Evaluation of NS5B-dependent replication

Huh7 cells were transfected with AdP$_I$235-HCV or AdP$_I$235-ΔGDD at 3 MOI and Ad-tTA at 15 MOI. After 24 h, the cells were treated with 10 μg/ml of Dox for 48 h. Then, luciferase activities in the lysates were measured.

Detection of a fragment of the HCV negative strand RNA

Huh7 cells were co-infected with AdP$_I$235-HCV or AdP$_I$235-ΔGDD at 3 MOI and Ad-tTA at 15 MOI. After 24 h, the cells were treated with 10 μg/ml of Dox for 48 h. The total RNAs were purified with High Pure RNA Isolation kit (Roche, Mannheim, Germany). The RNAs were reverse-transcribed to cDNA using a commer-cial available kit [TaKaRa RNA PCR Kit (AMV) Ver. 3.0] and a primer for the HCV negative strand RNA (5′-GCCAGCCCCCGATTGGGG-3′) or a primer for GAPDH (5′-TCTACATGGCAACTGTGA-3′), respect-ively. The transcription products of NS3 and GAPDH were amplified by PCR using paired primers (5′-ATGG CGCCTATTACGGCC-3′ and 5′-TGGTCTACATTAG TGTAC-3′) and (5′-GGTGGTCTCCTCTGACTTCAA CA-3′ and 5′-GTGGTCGTTGAGGGCAATG-3′), re-spectively. The putative sizes of the PCR products were 242 bp for NS3 and 89 bp for GAPDH. The PCR products were separated on 2% agarose gel.

RESULTS

RNA pol I-driven plasmid vector

First, we constructed an RNA pol I-driven plasmid coding an HCV replicon in which structural coding genes were replaced by the luciferase gene (Figure 1A). To investigate the expression of the HCV replicon from the RNA pol I plasmid vector, we transfected the plasmid vector into Huh7 cells. As shown in Figure 1B, the luciferase activity was observed in the RNA pol I vector-transfected cells (670 ± 39.1 RLU). IFN is the most popular agent used to inhibit HCV replication. To examine whether the RNA pol I plasmid vector functions as an assay system for anti-HCV activity, we investigated the effect of IFN on the expression of the HCV replicon in the RNA pol I plasmid-transfected Huh7 cells. IFN dose-dependently reduced the replication of the HCV genome (Figure 1C), reaching 29.2% of the control at 5 pg/ml. IFN treatment did not cause any cytotoxicity (Figure 1D). These data suggest that the RNA pol I plasmid coding the HCV replicon works as an assay system for HCV replication.

RNA pol I-driven Ad5 vector

The Ad vector is the most efficient gene transfer vector for a variety of mammalian cells *in vitro* and *in vivo* (13,14,19,20). There are more than 51 serotypes of Ad. The Ad type 5 (Ad5) vector has been frequently used in basic research and clinical studies (21). Ad5 vectors are 100- and 1000-fold more efficient at mediating gene trans-duction than cationic lipids, an effective non-viral vector (22). A reverse genetics system for the generation of influ-enza virus using RNA pol I-driven Ad5 vector produced 1000-fold the virus titer of the RNA pol I plasmid system (15). These findings indicate that the Ad5 vector may have advantages for the preparation of an HCV replicon system. We prepared RNA pol I-driven Ad5 vectors and confirmed the expression of a reporter gene from the Ad5 vectors coding luciferase (Supplementary Figure S1). However, we did not succeed in preparing Ad5 vector particles coding the HCV replicon. Indeed, there have been no previous reports of the preparation of Ad5 vector expressing the HCV RNA genome.

We think that two problems must be solved in order to develop Ad5 vectors coding the HCV RNA genome. These problems are the influence of the HCV replicon on the preparation of Ad5 particles and the packaging limit of Ad5 vectors.

Preparation of the TRE/RNA pol I chimeric promoter

The tet-regulated system comprises a regulator vector that expresses tet-controlled *trans*-activators and a response vector consisting of TRE within the promoter that controls expression of the gene of interest. The tet-controlled *trans*-activators are classified into tTA and rtTA that binds to the TRE promoter and activates ex-pression from the TRE promoter in the absence and presence of Dox, respectively (23,24). We speculated that a tet-regulated vector system would minimize the influence of the HCV replicon on the preparation of Ad vector

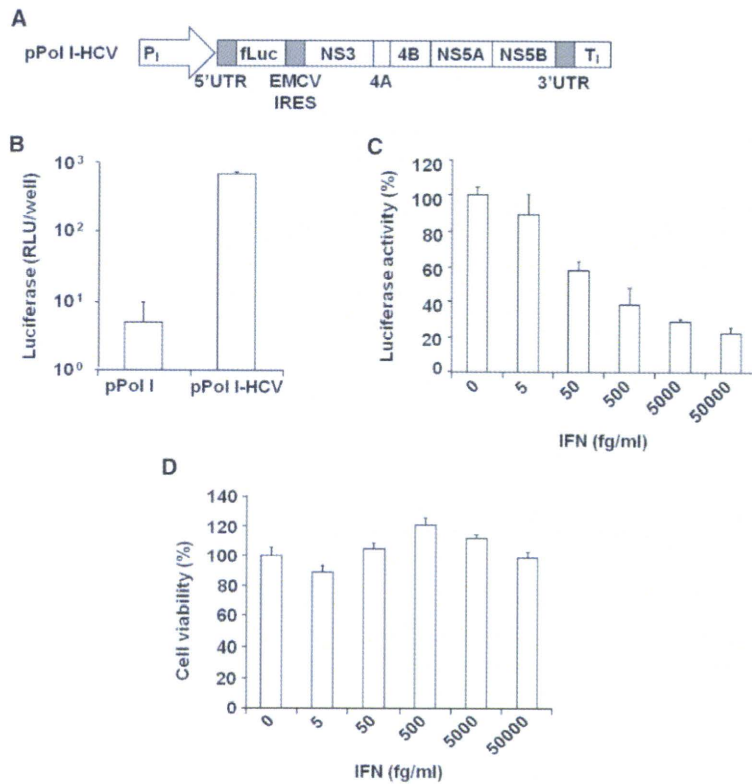**Figure 1.** Preparation of plasmid expressing subgenomic HCV RNA driven by RNA pol I promoter. (**A**) Schematic construct of sub-genomic HCV genome-expression cassette. The HCV sub-genome gene was driven by the RNA pol I promoter (P_I) and terminator (T_I). (**B**) Transgene expression in Huh7 cells. Cells were transfected with pPol I-HCV. After 24 h of transfection, the luciferase activities were measured. Data are mean ± SD ($n = 3$). (**C** and **D**) Effect of IFN on HCV replication in RNA pol I vector-transfected cells. Huh7 cells were transfected with pPol I-HCV. After 2.5 h of transfection, the cells were treated with IFN at the indicated concentration. After an additional 72 h of incubation, the luciferase activity (C) and the cell viability (D) were measured. The luciferase activity (%) was calculated as a percentage of that in the vehicle-treated cells. Data are mean ± SD ($n = 3$).

particles. First, we optimized the chimeric promoter of TRE and the RNA pol I promoter. As shown in Figure 2A, the RNA pol I promoter is a 412-bp fragment containing an upstream control element (UCE) and the binding site of a transcription factor (Core). We constructed three chimeric promoter-driven plasmid vectors and checked the expression profiles using luciferase as a reporter gene. The chimeric vector was co-transfected into Huh7 cells with response vectors coding tTA or rtTA (23,24). As shown in Figure 2B and C, co-transfection with tTA exhibited a higher expression level than that of rtTA. The P_I235 promoter had the lowest luciferase expression in the absence of response vectors (Supplementary Figure S2). We used tTA and the P_I235 promoter in further studies. To investigate whether the chimeric RNA pol I promoter works in the Ad vector, we prepared Ad5 vector coding the chimeric RNA pol I-driven luciferase gene. Ad-TREP$_I235$ (MOI of 10) was co-transduced with Ad-tTA at MOI of 10 and 50. As shown in Figure 2D, the luciferase expression was increased in an Ad-tTA concentration-dependent manner.

Expression of the HCV replicon from Ad vector

The packaging limit of a foreign gene in the conventional Ad5 vector has been estimated to be 8.1–8.2 kb (25). The HCV replicon is ~8.9 kb and contains a 1.7-kb firefly luciferase gene and sequence derived from the HCV genome. Thus, another reason for no previous reports regarding the preparation of Ad5 vector coding the HCV replicon appears to be the packaging limit of the Ad5 vector. Mizuguchi and Hayakawa found that Ad5/35 vector containing chimeric fibers of Ad5 and Ad35 increased the size limit of foreign genes to 8.8 kb (26). We were successful in preparing Ad5/35 vector particles (9.53×10^8 IFU/ml) coding the TRE/RNA pol I chimeric promoter-driven HCV replicon containing the 1.0-kb renilla luciferase gene and sequence derived from the HCV genome (Figure 3A). To investigate the expression of the HCV replicon, Huh7 cells were transfected with the Ad vector coding the HCV replicon and Ad-tTA at MOI of 10 and 50, respectively. As shown in Figure 3B, western blot analysis showed that NS5A was expressed in Huh7 cells transfected with the vectors in the absence of Dox.
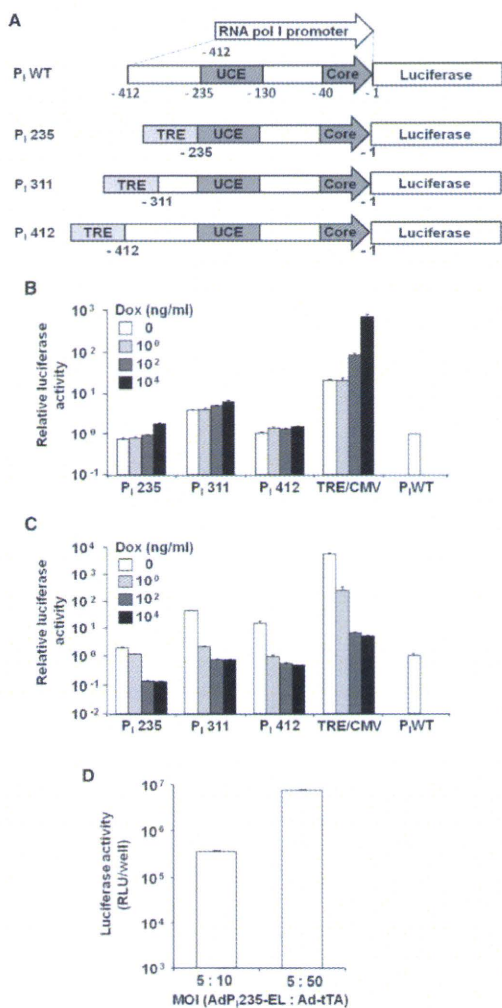
Figure 2. Development of tet-controllable RNA pol I promoter. (**A**) Construct of the chimeric RNA pol I promoter. The 412-bp human RNA pol I promoter contains Core (from −40 to −1), the binding site of the transcription factor, and UCE (from −235 to −130). TRE is connected to the full or partial RNA pol I promoter at the indicated sites, resulting in P_I235, P_I311 and P_I412. (**B and C**) Promoter activities of the chimeric promoter in Huh7 cells. Huh7 cells were co-transfected with the chimeric RNA pol I plasmid coding EGFPLuc, pCMVβ and transactivator plasmid [rtTA (**B**) or tTA (**C**)]. After 2.5 h of transfection, the cells were treated with Dox at the indicated dose. After an additional 48 h of incubation, the luciferase and β-galactosidase activities were measured. The luciferase activity was normalized by the β-galactosidase activity and expressed relative to that of pP_IWT-EL-transfected cells. Data are mean ± SD ($n = 3$). (**D**) Transgene activity of Ad vector coding the chimeric promoter construct. Huh7 cells were co-infected with AdP_I235-EL and Ad-tTA. The MOI ratio of AdP_I235-EL to Ad-tTA was 5:10 or 5:50. After an additional 48 h of incubation, the luciferase activity was measured. Data are the mean ± SD ($n = 3$).

Luciferase was also expressed (Figure 3C). Dox dose-dependently attenuated expression of luciferase (Supplementary Figure S3). To discriminate between translation of the RNA pol I-transribed HCV RNA derived from the vector DNA and translation of HCV RNA derived from autonomous HCV replication in the transcribed cells, we prepared replication-incompetent HCV replicon deleting GDD motif in NS5B. Luciferase expression was attenuated in the cells transfected with the GDD-deleted Ad vector (AdP₁235-ΔGDD) (Figure 3D). A fragment of the HCV negative strand RNA, an essential replication intermediate, amplified by RT-PCR has been detected in the cells transfected with AdP₁235-HCV but not AdP₁235-ΔGDD (Figure 3E). Autonomous replication of the HCV RNA may occur in this system. To evaluate whether the Ad vector systems could be used to evaluate inhibitors of HCV replication, we investigated the effect of IFN on luciferase expression from HCV replicon in the Ad vector. As shown in Figure 3E, treatment of cells with 5 pg/ml of IFN reduced luciferase expression (33.3% of vehicle-treated cells). Cell viability was not affected by IFN treatment (Figure 3F). These findings indicate that the tet-controllable RNA pol I Ad vector may be useful for evaluation of anti-HCV activity.

DISCUSSION

HCV is an RNA virus containing the positive strand of a 9.6-kb RNA genome. A technique to transfer all or part of the HCV RNA genome to cells could be widely applicable for basic studies on HCV and pharmaceutical therapy against HCV. However, efficient and convenient methods to transduce the HCV RNA genome have never been fully developed. Electroporation of *in vitro* translated HCV RNA genome into cells is the most popular method. In the present study, we used a tet-controllable expression system to successfully develop an Ad vector system expressing the HCV RNA genome.

To our knowledge, development of Ad vector expressing HCV sub-genome or genome has never been succeeded. The NS3 protease is essential for processing most of the NS proteins from the HCV polyprotein (27–30). The cleavage site of the NS3 protease is estimated to be between the P1 and P1′ position of an acidic amino acid at the P6 position, a Cys or Thr residue at the P1 position, and a Ser or Ala residue at the P1′ position (31). E1A, pIIIa, pol and V proteins of Ad have the cleavage site of the NS3 protease. The lack of previous success in generating Ad vectors coding the HCV genome and sub-genome might be partly due to the degradation of Ad components by the NS3 protease during the preparation of Ad particles. In the tet-regulated system, when Ad vectors coding foreign genes driven by the TRE hybrid promoter are co-transfected with tTA or rtTA vector, the foreign gene can be expressed. Expression of the foreign gene could be suppressed during amplification of Ad vector particles in 293 cells, resulting in the preparation of Ad vector particles. The critical factor in the HCV replicon must be determined in a future study.
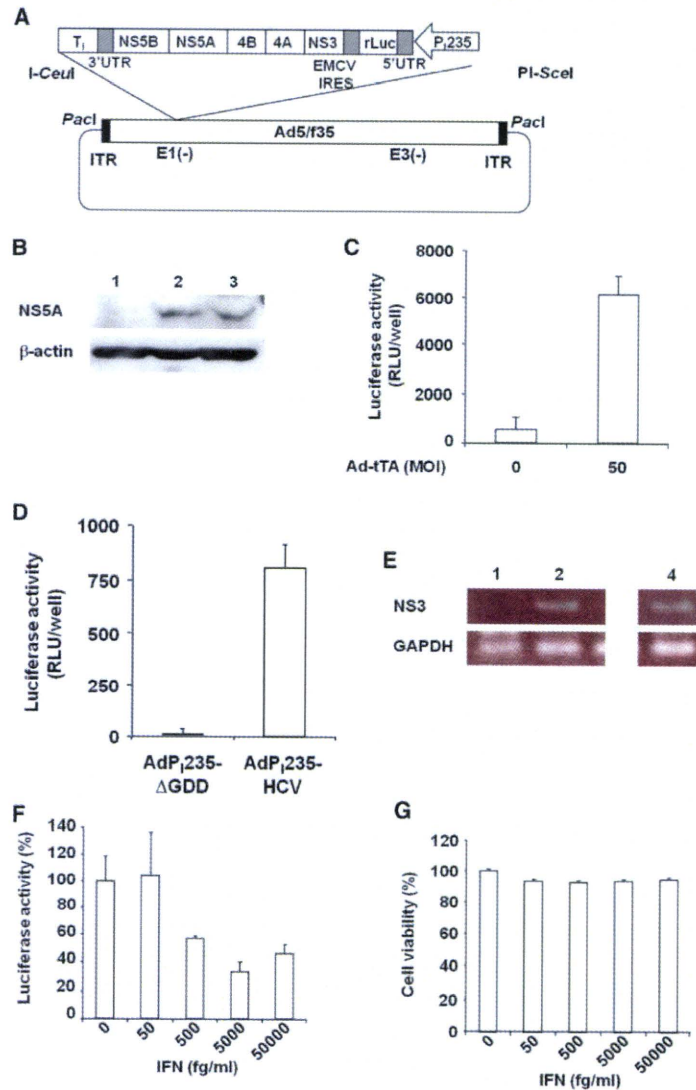
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Figure 3. Preparation of Ad vector to monitor HCV replication. (**A**) Construct of Ad vector. The Ad vector contained the chimeric RNA pol I promoter (P_I235) and the HCV replicon to monitor HCV replication as the luciferase expression. (**B**) Expression of HCV NS5A protein in Huh7 cells transfected with Ad-P_I235-HCV. The cells were transfected with Ad-P_I235-HCV (10 MOI) and Ad-tTA (50 MOI). After 72 h of incubation, the cells were harvested, and the lysates (30 μg) were subjected to SDS–PAGE, followed by immunoblotting with antibody against NS5A. Huh7 cells and Huh7.5.1 1bFeo cells were used as the negative and positive controls, respectively. Lane 1, Huh7 cells; lane 2, Huh7 cells infected with Ad-P_I235-HCV; lane 3, Huh7.5.1 1b Feo cells. (**C**) Expression of luciferase in the Ad vector-transfected cells. Huh7 cells were co-infected with Ad-P_I235-HCV (10 MOI) and 0 or 50 MOI of Ad-tTA. After an additional 48 h of incubation, the luciferase activity was measured. Data represent the mean ± SD (n = 3). (**D**) Involvement of NS5B in expression of luciferase in the Ad vector-transfected cells. Huh7 cells were infected with AdP_I235-HCV or AdP_I235-ΔGDD (3 MOI) and Ad-tTA (15 MOI). After 24 h, the cells were treated with 10 μg/ml of Dox for 48 h. Then, the luciferase activity was measured. Data represent the mean ± SD (n = 3). (**E**) Expression of minus-stranded HCV RNA in the Ad vector-transfected cells. Huh7 cells were co-infected with AdP_I235-HCV or AdP_I235-ΔGDD at 3 MOI and Ad-tTA at 15 MOI. After 24 h, the cells were treated with 10 μg/ml of Dox for 48 h. Then RT-PCR analysis was performed for detection of minus-stranded HCV NS3 and GAPDH. The PCR products were separated on 2% agarose gel. Huh7 cells and Huh7.5.1 1b Feo cells were used as the negative and positive controls, respectively. Lane 1, Huh7 cells; lane 2, Huh7.5.1 1b Feo cells; lane 3, Huh7 cells infected with AdP_I235-ΔGDD; lane 4, Huh7 cells infected with AdP_I235-HCV. (**F** and **G**) Effect of IFN on the replication of HCV replicon. Huh7 cells were infected with Ad-P_I235-HCV (10 MOI) and Ad-rtTA (50 MOI). After 1.5 h of infection, the cells were treated with IFN at the indicated concentration for 72 h. Then, the luciferase activity (D) and the cell viability (E) were measured. Data represent the percentage of vehicle-treated cells. Data are the mean ± SD (n = 3).

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Nucleic Acids Research, 2011 **7**

Transgenes delivered by a conventional Ad5 vector are limited to a size of 8.1–8.2 kb (32), and the size of HCV replicon is ~8.2 kb (containing a 1.0-kb luciferase gene and a 7.2-kb fragment of HCV genome) (8). The lack of a successful preparation of Ad5 vector may be partly due to limitation of packaging transgene. Mizuguchi and Hayakawa prepared a chimeric Ad vector containing type 5 and type 35 fiber proteins, which is a package 8.8 kb of foreign gene (26). CD46 is a receptor for Ad type 35 (Ad35), and CD46 is ubiquitously expressed in human cells (33,34). The Ad5/35 chimera vector can transduce various human cells more effectively than Ad5 vectors, indicating that the Ad5/35 vector may be a better system than Ad5 (26,35). In this study, we successfully prepared an Ad5/35 vector coding a tet-regulated RNA pol I-driven HCV replicon, and we found that the Ad5/35 vectors could be applied to evaluation of anti-HCV activity.

In conclusion, to the best of our knowledge, this is the first report to establish a novel strategy for the preparation of Ad vector expressing the HCV genome by using a tet-controllable expression system. Replication-incompetent HCV particles will be a promising candidate for vaccine therapy for HCV. As mentioned above, the packaging size (8.8 kb) of Ad5/35 vector used in the present study is smaller than that of the HCV RNA genome (9.6 kb), and, therefore, the preparation of inactive HCV particles using Ad5/35 vector is impossible. Helper-dependent Ad vector (HDAd), in which all viral coding sequences are deleted, can deliver a large capacity of ~37 kb to cells (36). Tet-controllable RNA pol I HDAd vector might contribute to the development of vaccine therapy for HCV.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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