An Adenovirus Vector-Mediated Reverse Genetics System for Influenza A Virus Generation[▽]

Makoto Ozawa,^{1,2} Hideo Goto,^{1,2} Taisuke Horimoto,^{1,2} and Yoshihiro Kawaoka^{1,2,3*}

Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108-8639, Japan¹; Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Saitama 332-0012, Japan²; and Department of Pathological Sciences, School of Veterinary Medicine, University of Wisconsin—Madison, Madison, Wisconsin 53706³

Received 14 May 2007/Accepted 18 June 2007

Plasmid-based reverse genetics systems allow the generation of influenza A virus entirely from cloned cDNA. However, since the efficiency of virus generation is dependent on the plasmid transfection efficiency of cells, virus generation is difficult in cells approved for vaccine production that have low transfection efficiencies (e.g., Vero cells). Here we established an alternative reverse genetics system for influenza virus generation by using an adenovirus vector (AdV) which achieves highly efficient gene transfer independent of cell transfection efficiency. This AdV-mediated reverse genetics system will be useful for generating vaccine seed strains and for basic influenza virus studies.

The artificial generation of influenza A virus entirely from cloned cDNA in plasmid-transfected cells, the so-called “plasmid-based reverse genetics system” (1, 13), represents an important advance in influenza virology (12, 15). This technology has advanced both basic and applied research on influenza virus, most notably, the development of vaccine seed strains for highly pathogenic influenza viruses, including the currently circulating H5N1 viruses (5, 17–20).

Since, until recently, at least eight plasmids had to be transfected into a single cell for virus generation, the limiting factor for plasmid-based reverse genetics was the transfection efficiency of the cells. In general, 293T cells, which are readily transfected with plasmids (2), have been used for plasmid-based systems (4, 13). However, 293T cells cannot be used for the development of human vaccine seed strains because they are not validated for such use. African green monkey kidney (Vero) cells, which have been used for the production of rabies virus and poliovirus vaccines (9), are the WHO-recommended cell line for vaccine production (20), but these cells are not readily transfected (6–8). It is therefore difficult to efficiently generate influenza viruses by using plasmid-based systems in these cells, although some success has been achieved (1, 16, 19).

To address these limitations, we established a reverse genetics system that uses adenovirus type 5-based gene transfer, which has been safely administered in numerous clinical trials (21). A replication-incompetent adenovirus vector (AdV) with E1 and E3 deleted that possesses the cDNAs of viral RNA (vRNA) under the control of the human RNA polymerase I (PolI) promoter and the mouse PolI terminator allowed efficient vRNA synthesis and led to a high virus yield in Vero cells. These results suggest that the AdV-mediated system would be

valuable for the production of vaccine seed strains in pandemic situations.

AdV-mediated synthesis of influenza virus RNA. In plasmid-based reverse genetics systems, plasmids possessing the cDNA of viral genes under the control of the human PolI promoter and the mouse PolI terminator have been used for vRNA synthesis (13). Therefore, we cloned the cDNA corresponding to the transcriptional region of pPolI-GFP (Fig. 1A) (14) into pAd/PL-DEST (Invitrogen), which contains the genome sequence of human adenovirus type 5 with E1 and E3 deleted as a viral vector backbone, by means of the Gateway system using LR clonase (Invitrogen). Transfection of the resultant plasmid into 293A cells produced AdV for the synthesis of a reporter vRNA (AdV/PolI-GFP, Fig. 1B).

To test whether AdV/PolI-GFP can produce the reporter vRNA in Vero cells, we transduced this AdV into cells. These cells were simultaneously transfected with four plasmids to express the A/WSN/33(H1N1, WSN) viral polymerase subunits (PB2, PB1, and PA) and NP, which are necessary and sufficient for vRNA transcription and replication and which form the viral ribonucleoprotein complexes (vRNPs) with vRNA. The multiplicity of infection (MOI) used was 50, an MOI at which >99% of the cells express a transduced gene (data not shown). Forty-eight hours later, we detected green fluorescent protein (GFP)-expressing cells (Fig. 2B), whereas no GFP expression was detected in mock-transfected cells (Fig. 2A). AdV/PolI-GFP transduction of Vero cells thus resulted in the synthesis of the reporter vRNA.

To provide the vRNP components entirely from AdVs, we made four additional AdVs for the expression of the polymerase subunits and NP (AdV/CMV-PB2, -PB1, -PA, and -NP) by cloning the cDNAs corresponding to the open reading frames of each WSN viral protein into pAd/CMV/V5-DEST (Invitrogen). Cotransduction of these AdVs into Vero cells with AdV/PolI-GFP (MOI = 50) resulted in highly efficient GFP expression 48 h posttransduction (Fig. 2C). These results show that AdV transduction achieves functional vRNP formation at a

* Corresponding author. Mailing address: Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. Phone: 81-03-5449-5310. Fax: 81-03-5449-5408. E-mail: kawaoka@ims.u-tokyo.ac.jp.

[▽] Published ahead of print on 27 June 2007.

FIG. 1. Schematic diagrams of the transcription cassettes of pPolI and AdVs for reporter vRNA synthesis. In pPolI-GFP (14), the 3' noncoding region of NP vRNA (3' NCR), the GFP open reading frame in the negative sense, and the 5' noncoding region of NP vRNA (5' NCR) were inserted between the PolI promoter (P_{PolI}) and the PolI terminator (T_{PolI}). In cells transfected with pPolI-GFP, the reporter vRNA containing the GFP gene is synthesized by cellular PolI (A). AdV/PolI-GFP possessed the same transcription cassette of pPolI-GFP for reporter vRNA synthesis (B). The vRNA transcriptional region in AdV/CMV-PolI-GFP was flanked by the human cytomegalovirus immediate-early promoter (P_{CMV}) and the herpes simplex virus thymidine kinase polyadenylation signal (TK pA). In cells transduced with AdV/CMV-PolI-GFP, the reporter vRNA and mRNA containing the GFP genes are synthesized by cellular PolI and PolII, respectively. The backbone of the adenovirus clones (Ad) was the genome of adenovirus type 5 with E1 and E3 deleted. The transcriptional initiation site and orientation of the GFP gene are indicated by the white arrow. All of the recombinant replication-incompetent AdVs used in this study were produced by the ViraPower Adenoviral Expression System (Invitrogen) according to the manufacturer's instructions.

much higher efficiency than does plasmid transfection in Vero cells.

To determine the optimal ratio of AdVs for protein expression to vRNA synthesis, AdV/PolI-GFP was transduced into Vero cells at different MOIs together with AdV/CMV-PB2, -PB1, -PA, and -NP (MOI = 50). The results showed that fivefold fewer AdVs for vRNA synthesis than for viral protein expression are sufficient for efficient functional vRNP formation (data not shown).

Influenza virus generation entirely from AdVs. To generate infectious influenza virus entirely from AdVs, we cloned PolI transcription cassettes for all eight WSN vRNAs (13) into pAd/PL-DEST and made eight AdVs for the synthesis of each vRNA segment. Vero cells were cotransduced with a total of 12 AdVs, 8 AdVs for vRNA synthesis (MOI = 10) and 4 AdVs for viral protein expression (MOI = 50). To compare the efficiencies of virus generation, two methods of plasmid-based reverse genetics were used, the 12-plasmid system (11) and the 3-plasmid system (10). At 72 h after AdV transduction or plasmid transfection, culture supernatants were harvested and

subjected to plaque assay on MDCK cells to determine the amounts of virus generated. Influenza virus was detected in the supernatant of cells transduced with 12 AdVs (Fig. 3), demonstrating the capacity of this AdV-mediated reverse genetics system for influenza virus generation. The virus yield from the 12-AdV transduced cells was approximately 1,000-fold higher than that from the 12-plasmid transfected cells and comparable to that from the 3-plasmid transfected cells (Fig. 3).

Influenza virus generation from eight AdVs based on the PolI-PolII bidirectional transcription system. To reduce the number of AdVs required for virus generation, we tested whether the PolI-PolII bidirectional transcription approach, which allows the simultaneous synthesis of vRNA and mRNA from one template (3), would be applicable to our AdV-mediated reverse genetics system. By cloning the transcriptional region in pPolI-GFP into pAd/CMV/V5-DEST, we made AdV/CMV-PolI-GFP (Fig. 1C). Vero cells transduced only with this AdV (MOI = 50) expressed GFP at a relatively low level at 48 h posttransduction (Fig. 2D). Cotransduction with AdV/CMV-PB2, -PB1, -PA, and -NP enhanced the GFP ex-

FIG. 2. GFP expression in Vero cells transduced with AdVs for reporter vRNA synthesis. Vero cells were transduced with AdV/PolI-GFP (A to C) and AdV/CMV-PolI-GFP (D and E). Simultaneously, the cells were transfected with plasmids (B) or transduced with AdVs (C and E) for the expression of the polymerase subunits (PB2, PB1, and PA) and NP. Forty-eight hours later, GFP expression was examined by fluorescence microscopy. In each experiment, each AdV was transduced at an MOI of 50. The image in panel D was taken with a 10-fold longer exposure time than those in the other panels. Scale bars, 200 μ m.

pression level in individual cells (Fig. 2E). These results indicate that AdV/CMV-PolI-GFP transduction induces the synthesis of both the reporter vRNA and mRNA.

To generate infectious influenza virus from eight AdVs, we cloned PolI transcription cassettes for all eight WSN vRNAs into pAd/CMV/V5-DEST and made eight AdVs containing the bidirectional transcription cassette for each vRNA segment. Vero cells were cotransduced with these AdVs (MOI = 50). The virus yields were determined at 72 h posttransduction by plaque assay on MDCK cells. The amount of virus generated in Vero cells with the 8 AdVs was approximately 10,000-, 10-, and 10-fold higher than those obtained with the 12-plas-

mid ($P = 0.032$), 3-plasmid ($P = 0.045$), and 12-AdV ($P = 0.035$) systems, respectively (Fig. 3).

Here, we demonstrate that the limitation of transfection efficiency of target cells is overcome by using AdV as a gene transfer vehicle. Influenza virus RNA was efficiently transcribed (Fig. 2C and E), and influenza virus was generated with high efficiency in Vero cells transduced with AdV possessing the PolI promoter and terminator (Fig. 3). Moreover, the eight-AdV transduction system, based on the PolI-PolIII bidirectional transcription system (4), achieved a statistically significant increase in virus yield compared to the other systems, including the recently established three-plasmid transfection system (10). Given the relative ease of preparation, the eight-AdV transduction system appears ideal for the efficient generation of influenza vaccine seed strains. This AdV-mediated reverse genetics system could also contribute to basic studies of influenza virus.

We thank Susan Watson for editing the manuscript.

This work was supported by CREST (Japan Science and Technology Agency) and by grants-in-aid from the Ministries of Education, Culture, Sports, Science, and Technology and of Health, Labor, and Welfare of Japan and by National Institute of Allergy and Infectious Diseases, Public Health Service, research grants.

FIG. 3. Comparison of the virus generation efficiency of plasmid transfection systems and AdV transduction systems. Vero cells were transfected with 12 plasmids (11) or 3 plasmids (pTM-PolI-WSN-All, pC-PolI-WSN-PB2-PB1-PA, and pCAWS-NP) (10) or transduced with 12 AdVs (AdV/PolI-PB2, -PB1, -PA, -HA, -NP, -NA, -M, and -NS and AdV/CMV-PB2, -PB1, -PA, and -NP) or 8 AdVs (AdV/CMV-PolI-PB2, -PB1, -PA, -HA, -NP, -NA, -M, and -NS). Seventy-two hours later, virus titers in culture supernatant were determined by plaque assay on MDCK cells. The virus titer detection limit of our system was 5 PFU/ml. The results of three independent experiments (Exp.) are shown.

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