

# An improved reverse genetics system for influenza A virus generation and its implications for vaccine production

Gabriele Neumann\*, Ken Fujii†, Yoichiro Kino‡, and Yoshihiro Kawaoka\*†§¶

\*Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin–Madison, 2015 Linden Drive, Madison, WI 53706;

†Institute of Medical Sciences, University of Tokyo, Tokyo 108-8639, Japan; ‡The Chemo-Sero-Therapeutic Research Institute, Kikuchi Research Center, Kawabe Kyokushi Kikuchi, Kumamoto 869-1298, Japan; and §Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Saitama 332-0012, Japan

Edited by Peter Palese, Mount Sinai School of Medicine, New York, NY, and approved September 27, 2005 (received for review July 2, 2005)

The generation of vaccines for highly pathogenic avian influenza viruses, including those of the H5N1 subtype, relies on reverse genetics, which allows the production of influenza viruses from cloned cDNA. In the future, reverse genetics will likely be the method of choice for the generation of conventional influenza vaccine strains because gene reassortment by more traditional methods is cumbersome. Established systems for the artificial generation of influenza A viruses require transfection of cells with the eight to 12 plasmids that provide the eight influenza viral RNAs as well as the polymerase and nucleoproteins of the virus. However, cell lines appropriate for human vaccine production (e.g., Vero cells) cannot be transfected with high efficiencies. To overcome these problems, we established a reverse genetics system in which the eight RNA polymerase I transcription cassettes for viral RNA synthesis are combined on one plasmid. Similarly, two cassettes encoding the hemagglutinin and neuraminidase segments and six cassettes encoding the remaining proteins were combined. We also combined three RNA polymerase II transcription cassettes for the expression of the polymerase subunits. By combining these cassettes, we reduced the number of plasmids required for virus generation significantly and produced influenza A virus in Vero cells with higher efficiency than with the traditional 12 plasmid system. This new system is thus suitable for influenza virus vaccine production and may be applicable to other reverse genetics systems that rely on the introduction of several plasmids into eukaryotic cells.

RNA polymerase I

Influenza epidemics and pandemics continue to claim human lives and impact the global economy. In the United States alone, influenza causes an estimated 50,000 deaths annually (1), whereas global pandemics can result in millions of influenza-related deaths. A classic example is the so-called “Spanish influenza,” which killed an estimated 40–50 million people worldwide in 1918–1919 (2). The threat imposed by influenza virus has been further elevated with the recent introductions of avian influenza viruses into the human population. Avian influenza viruses were long thought not to be directly transmissible to humans and cause lethal outcomes. However, this perception changed in 1997, when 18 Hong Kong residents were infected by a wholly avian influenza virus of the H5N1 subtype, that resulted in six deaths (3, 4). Over the next few years, several other cases of direct avian-to-human transmission were reported (5–7), including the ongoing outbreak of highly pathogenic H5N1 influenza viruses in several Asian countries that has claimed 41 lives of 54 infected individuals as of January 26, 2005 (8). The increasing numbers of human H5N1 virus infections, together with a high mortality rate and possible human-to-human transmission, make the development of vaccines to these viruses essential.

In the United States, two influenza vaccines are licensed for human use: an inactivated vaccine and a live-attenuated vaccine virus. The production of influenza virus vaccines relies on reassortment (9), which requires coinfection of cells with a circulating wild-type strain that provides the hemagglutinin (HA) and neuraminidase (NA) segments and either A/PR/8/34 (PR8) virus (an attenuated human virus that provides high-growth properties in eggs) or a live attenuated virus that provides the attenuated phenotype. The selection of the desired “six plus two” reassortants (i.e., those containing the HA and NA gene segments of the circulating wild-type strain in the genetic background of PR8 or live attenuated virus) is time-consuming and cumbersome. Moreover, the need for reassortment and selection, as well as the inability of some reassortant viruses to grow to high titers, have resulted in delays in vaccine production. For influenza A and B viruses, highly efficient reverse genetics systems are now in place that allow the generation of these viruses from cloned cDNA (10–13). In one system (10), eight plasmids encoding the eight influenza viral RNA segments under the control of the RNA polymerase I promoter and terminator sequences are transfected into eukaryotic cells together with four RNA polymerase II-driven plasmids for the expression of the three viral polymerase subunits and the nucleoprotein NP. These four proteins are required to initiate viral replication and transcription. An alternative system has also been developed (13) that relies on eight plasmids in which the viral cDNAs are flanked by an RNA polymerase I promoter on one site and an RNA polymerase II promoter on the other site, which permits the viral RNA (vRNA) and mRNA to be derived from the same template. These systems have allowed six plus two reassortants to be engineered without the need for reassortment and screening procedures.

A limited number of mammalian cell lines are available for the production of influenza virus vaccines. They include Madin–Darby canine kidney (MDCK) (14–16) and African green monkey kidney (Vero) (17–20) cells. These cell lines cannot be transfected with high efficiencies, which sometimes limits their use in reverse genetics systems for influenza virus vaccine production; however, the generation of influenza virus in Vero cells has been demonstrated (12, 21). To address this limitation, we established a reverse genetics system that reduces the number of plasmids required for virus generation. Rather than providing RNA polymerase I or II transcription cassettes from individual plasmids, we combined up to eight RNA polymerase I transcrip-

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: vRNA, viral RNA; HA, hemagglutinin; NA, neuraminidase; MDCK, Madin–Darby canine kidney; TCID<sub>50</sub>, 50% tissue-culture infectious dose.

¶To whom correspondence should be addressed. E-mail: kawaokay@svm.vetmed.wisc.edu.

© 2005 by The National Academy of Sciences of the USA

tion cassettes for the synthesis of the respective viral RNAs on one plasmid. We, similarly, combined the transcription cassettes for the three viral polymerase subunits on one plasmid. This approach allowed the efficient and robust generation of influenza A virus in Vero cells.

## Materials and Methods

**Cells.** 293T human embryonic kidney cells and African green monkey kidney (Vero) cells were maintained in DMEM supplemented with 10% fetal calf serum. For all experiments, we used Vero CCL-81 cells, which have been previously used to produce an inactivated Japanese encephalitis vaccine and have been screened for lack of tumorigenicity and adventitious infectious agents (22). MDCK cells were maintained in modified Eagle's medium containing 5% newborn calf serum. All cells were maintained at 37°C in 5% CO<sub>2</sub>.

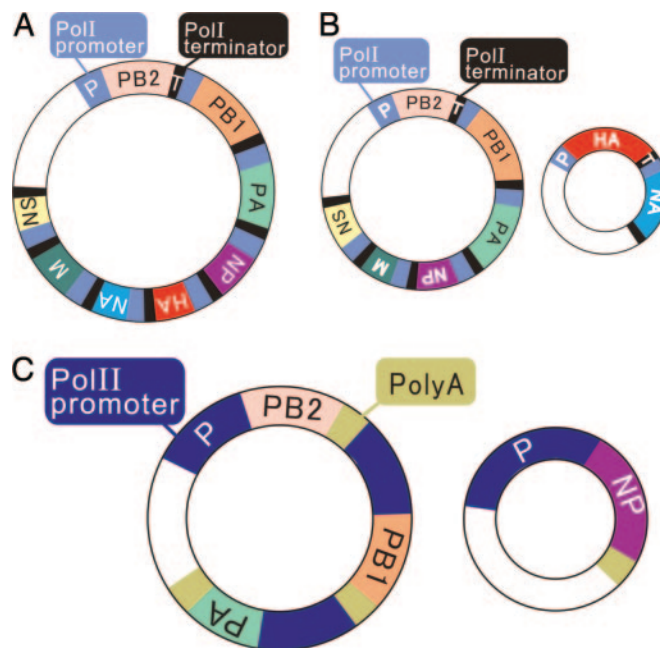
**Construction of Plasmids.** To combine RNA polymerase I transcription cassettes for the synthesis of the influenza viral RNA segments, transcription cassettes comprising the human RNA polymerase I promoter, an influenza viral cDNA in negative-sense orientation, and the mouse RNA polymerase I terminator (10) were amplified by PCR with oligonucleotides that contained recognition sequences for restriction endonucleases that were not present in the viral genome. As templates, we used pPolI-WSN-PB2, -PB1, -PA, -HA, -NP, -NA, -M, -NS (all described in ref. 10), which contained the respective viral cDNA of A/WSN/33 (H1N1) virus positioned between RNA polymerase I promoter and terminator sequences. PCR products were cloned into standard vectors that contained the respective restriction sites and were sequenced to confirm that they lacked unwanted mutations. We also confirmed the functionality of the resulting plasmids by reverse genetics.

A modified pTM1 vector (23) with flanking, unique restriction sites was used to stepwise-combine individual RNA polymerase I transcription cassettes (this vector is described in more detail in *Discussion*). At each cloning step, we confirmed the functionality of the resulting plasmids, which contained two to seven RNA polymerase I transcription units, by reverse genetics. The final plasmid, pPolI-WSN-All (Fig. 1A), contained eight RNA polymerase I transcription cassettes for the synthesis of all eight influenza A/WSN/33 viral RNAs. This plasmid was stably maintained in *Escherichia coli* JM109 cells at room temperature; bacterial cultures were grown in Terrific Broth medium for ≈30 h.

Using the same strategy, we generated two plasmids, pTM-PolI-WSN-HA-NA and pTM-PolI-WSN-PB2-PB1-PA-NP-M-NS (Fig. 1B), that contained RNA polymerase I transcription cassettes for the HA and NA segments and the remaining six viral segments (i.e., PB2, PB1, PA, NP, M, and NS), respectively.

Plasmids pCAWSPB2, pCAWSPB1, and pCAWSPA comprised the chicken  $\beta$ -actin promoter, the coding sequence of the A/WSN/33 PB2, PB1, or PA protein, and a polyadenylation signal. These RNA polymerase II transcription units were flanked by recognition sequences for unique restriction endonucleases, either by PCR or by inserting short DNA linkers. PCR-amplified transcription cassettes were sequenced in their entirety before the three RNA polymerase II transcription cassettes were combined by using the unique restriction sites. The functionality of the resulting plasmid (pC-PolII-WSN-PB2-PB1-PA) (Fig. 1C) was verified by reverse genetics.

**Generation of Virus from Plasmids.** We transfected 293T cells ( $1 \times 10^6$ ) or Vero CCL-81 cells ( $5 \times 10^5$ ) by using Trans IT-LT1 (Mirus, Madison, WI) according to the manufacturer's instructions. Briefly, transfection reagent (2  $\mu$ l of Trans IT-LT1 per  $\mu$ g of DNA for the transfection of 293T cells; 4  $\mu$ l of Trans IT-LT1 per  $\mu$ g of DNA for the transfection of Vero cells) was diluted in 100  $\mu$ l of OptiMEM (GIBCO/BRL), incubated for 5 min at



**Fig. 1.** Schematic diagrams of plasmids possessing multiple influenza viral genes. (A) pTM-PolI-WSN-All for the transcription of all eight influenza viral RNAs from one template. Transcription units comprising the human RNA polymerase I promoter (blue, Pol I promoter), a cDNA encoding an influenza viral segment in negative-sense orientation (shown in different colors), and the mouse RNA polymerase I terminator (black, Pol I terminator) were combined on one plasmid by using unique recognition sites for restriction endonucleases. (B) pTM-PolI-WSN-PB2-PB1-PA-NP-M-NS and pTM-PolI-WSN-HA-NA for the transcription of six and two influenza viral RNAs, respectively, from one plasmid. These plasmids and the terminology were generated as outlined for pTM-PolI-WSN-All. (C) pC-PolII-WSN-PB2-PB1-PA for the transcription of PB2, PB1, and PA mRNAs from one plasmid. Transcription units comprising an RNA polymerase II promoter (dark blue, Pol II promoter) (i.e., the chicken  $\beta$ -actin promoter), the coding region for the respective viral protein (shown in different colors), and a polyadenylation sequence (gold, PolyA) were combined on one plasmid by using unique recognition sites for restriction endonucleases.

room temperature, and added to premixed DNAs. For all transfection experiments, we used 0.1  $\mu$ g of each of the single-unit plasmids for the synthesis of viral RNAs (i.e., pPolI-WSN-PB2, -PB1, -PA, -HA, -NP, -NA, -M, -NS; described in ref. 10), 1  $\mu$ g of plasmids containing more than one RNA polymerase I transcription unit (i.e., pTM-PolI-WSN-All, pTM-PolI-WSN-HA-NA, or pTM-PolI-WSN-PB2-PB1-PA-NP-M-NS), and 1  $\mu$ g of each of the protein expression plasmids. At times after transfection indicated in Tables 1 and 2, we determined the 50% tissue-culture infectious dose (TCID<sub>50</sub>) in MDCK cells.

## Results

### Plasmids Containing Multiple RNA Polymerase I or II Transcription Cassettes.

To allow influenza virus generation from <8–12 plasmids (10, 12, 13), we combined RNA polymerase I transcription cassettes for vRNA synthesis, or RNA polymerase II transcription cassettes for mRNA synthesis on one plasmid. As a model, we used the A/WSN/33 (WSN) virus, for which parameters and efficiencies of viral generation are well established. Briefly, RNA polymerase I transcription cassettes comprising the human RNA polymerase I promoter, an influenza viral cDNA in negative-sense orientation, and the mouse RNA polymerase I terminator were amplified by PCR, cloned and sequenced, and then joined stepwise by the use of unique restriction sites. As a vector backbone, we used a modified pTM1

Table 1. Efficiency of virus generation in 293T cells

Components/results	Experimental samples													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
vRNA synthesis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8-Unit plasmid	+	+	+	-	-	-	-	-	-	-	-	-	-	-
6-Unit plasmid	-	-	-	+	-	+	+	+	-	-	-	-	-	-
2-Unit plasmid	-	-	-	-	+	+	+	+	-	-	-	-	-	-
8 × 1-Unit plasmid	-	-	-	-	-	-	-	-	+	+	-	-	+	-
Protein synthesis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pCAWS-PB2	-	+	-	-	-	-	+	-	+	-	+	-	-	-
pCAWS-PB1	-	+	-	-	-	-	+	-	+	-	+	-	-	-
pCAWS-PA	-	+	-	-	-	-	+	-	+	-	+	-	-	-
pCAWS-NP	-	+	+	-	-	-	+	+	+	+	+	+	+	-
pC-PolII-WSN-PB2-PB1-PA	-	-	+	-	-	-	-	+	-	+	-	+	-	-
Total no. of plasmids	1	5	3	1	1	2	6	4	12	10	4	2	8	0
Exp. 1, TCID <sub>50</sub> /ml	3.2 × 10 <sup>6</sup>	2 × 10 <sup>7</sup>	4.6 × 10 <sup>7</sup>	0	0	3.7 × 10 <sup>4</sup>	5.6 × 10 <sup>7</sup>	6.3 × 10 <sup>7</sup>	6.3 × 10 <sup>6</sup>	6.3 × 10 <sup>6</sup>	0	0	0	0
Exp. 2, TCID <sub>50</sub> /ml	3.7 × 10 <sup>7</sup>	2.1 × 10 <sup>8</sup>	1.5 × 10 <sup>8</sup>	0	0	1.6 × 10 <sup>5</sup>	6.3 × 10 <sup>7</sup>	6.3 × 10 <sup>7</sup>	6.3 × 10 <sup>7</sup>	6.3 × 10 <sup>7</sup>	0	0	0	0
Exp. 3, TCID <sub>50</sub> /ml	6.3 × 10 <sup>4</sup>	2.7 × 10 <sup>8</sup>	3.2 × 10 <sup>7</sup>	0	0	3.2 × 10 <sup>5</sup>	1.6 × 10 <sup>8</sup>	1.3 × 10 <sup>8</sup>	6.3 × 10 <sup>7</sup>	1.3 × 10 <sup>8</sup>	0	0	0	0

293T cells were transfected with the indicated plasmids (+). Forty-eight hours later, virus titers in the supernatant were determined by plaque assays in MDCK cells. Shown are the results of three independent experiments. Eight-unit plasmid, pTM-PolI-WSN-All; 6-unit plasmid, pTM-PolI-WSN-PB2-PB1-PA-NP-M-NS; 2-unit plasmid, pTM-PolI-WSN-HA-NA; 8 × 1-unit plasmid, combination of pPolI-WSN-PB2, -PB1, -PA, -HA, -NP, -NA, -M, -NS. All TCID<sub>50</sub>/ml values reflect data collected 48 h after transfection.

vector (23) that stably supported an Ebola viral cDNA of 20 kb (24) and is therefore suitable for the insertion of large DNA fragments. The generation of pTM-PolI-WSN-All (≈22.5 kb in length) (Fig. 1A), which contains eight individual RNA polymerase I transcription units, did not present major obstacles; however, growth of *E. coli* JM109 bacteria at room temperature was required to prevent recombination of this plasmid.

For the annual generation of influenza virus vaccines, only two viral RNA segments, i.e., those encoding the HA and NA surface glycoproteins, need to be replaced. For this reason, we generated a plasmid in which the transcription units for the HA and NA segments were combined (pTM-PolI-WSN-HA-NA) (Fig. 1B), whereas a second plasmid combined the transcription units encoding the internal proteins (pTM-PolI-WSN-PB2-PB1-PA-NP-M-NS) (Fig. 1B). Both of these plasmids were stable during amplification in *E. coli* JM109 bacteria at 37°C.

To further reduce the number of plasmids required for virus generations, we combined the three RNA polymerase II transcription units for the WSN PB2, PB1, and PA proteins on one vector backbone, with the same strategy that allowed us to join the RNA polymerase I transcription units for vRNA synthesis. The resulting plasmid was stable in *E. coli* JM109 bacteria at 37°C and was designated pC-PolII-WSN-PB2-PB1-PA (Fig. 1C). Of note, we were unable to construct a plasmid combining the RNA polymerase II transcription unit for the polymerase proteins and for the nucleoprotein NP.

**Virus Generation in 293T Cells from Plasmids Containing Multiple Transcription Cassettes.** To test the functionality of plasmids containing multiple transcription cassettes, we first transfected 293T cells with pTM-PolI-WSN-All (for the transcription of all eight vRNAs) (Table 1, experimental samples 2 and 3) or pTM-PolI-WSN-HA-NA and pTM-PolI-WSN-PB2-PB1-PA-NP-M-NS (for the transcription of two and six vRNAs) (Table 1, experimental samples 7 and 8). Cells were cotransfected with four plasmids for the expression of NP and the polymerase subunits from separate plasmids (Table 1, experimental samples 2 and 7), or with two plasmids that express NP or PB2, PB1, and PA, respectively (Table 1, experimental samples 3 and 8). We successfully generated viruses from these plasmids, demonstrating that RNA polymerase I or RNA polymerase II transcription

units can be combined, thus reducing the number of plasmids required for the artificial generation of influenza virus. At 48 h after transfection, the efficiency of virus generation ranged from 2 × 10<sup>7</sup> to 2.7 × 10<sup>8</sup> TCID<sub>50</sub>/ml (mean, 1.1 × 10<sup>8</sup> TCID<sub>50</sub>/ml) (Table 1, experimental samples 2, 3, 7, and 8). These efficiencies were slightly higher ( $P = 0.17$ ) than those obtained for control experiments in which cells were transfected with eight separate plasmids for the transcription of the influenza vRNAs and four or two plasmids for the synthesis of NP and the three polymerase subunits (yielding 6.3 × 10<sup>6</sup> to 1.3 × 10<sup>8</sup> TCID<sub>50</sub>/ml; mean, 5.5 × 10<sup>7</sup> TCID<sub>50</sub>/ml) (Table 1, experimental samples 9 and 10).

We also carried out a number of control experiments, including mock transfections (Table 1, experimental sample 14), cells transfected with protein expression plasmids only (Table 1, experimental samples 11 and 12), cells transfected with eight plasmids for vRNA synthesis only (Table 1, experimental sample 13), or cells transfected with plasmids for the synthesis of two or six vRNAs (Table 1, experimental samples 4 and 5, respectively). None of these controls yielded viruses. However, we consistently detected appreciable virus titers in cells transfected with pTM-PolI-WSN-All (Table 1, experimental sample 1) or with a combination of pTM-PolI-WSN-HA-NA and pTM-PolI-PB2-PB1-PA-NP-M-NS (Table 1, experimental sample 6). These plasmids were designed for the transcription of negative-sense viral RNAs, and synthesis of NP and the three polymerase proteins was not expected. Thus, virus generation with these plasmids alone was not expected either (for possible explanations, see *Discussion*).

**Virus Generation in Vero Cells from Plasmids Containing Multiple Transcription Cassettes.** Next, we tested the efficiency of virus generation in Vero cells, which are difficult to transfect to high efficiencies. At 48 h after transfection, virus generation from 12 plasmids was negligible in two experiments and low in one experiment (Table 2, experimental sample 9), whereas, at 72 h after transfection, virus was detected in all three experiments. The use of only one or two plasmids for the synthesis of vRNAs increased the efficiency of virus generation at 72 h after transfection, especially in combination with pC-PolII-WSN-PB2-PB1-PA, yielding up to 2.5 × 10<sup>6</sup> TCID<sub>50</sub>/ml [Table 2, experimental sample 9 vs. experimental sample 3 ( $P = 0.0017$ ) and experi-



**Table 2. Efficiency of virus generation in Vero cells**

Components/results	Experimental samples													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
vRNA synthesis	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8-unit plasmid	+	+	+	–	–	–	–	–	–	–	–	–	–	–
6-unit plasmid	–	–	–	+	–	+	+	+	–	–	–	–	–	–
2-unit plasmid	–	–	–	–	+	+	+	+	–	–	–	–	–	–
8 × 1-Unit plasmid	–	–	–	–	–	–	–	–	+	+	–	–	+	–
Protein synthesis	–	–	–	–	–	–	–	–	–	–	–	–	–	–
pCAWS-PB2	–	+	–	–	–	–	+	–	+	–	+	–	–	–
pCAWS-PB1	–	+	–	–	–	–	+	–	+	–	+	–	–	–
pCAWS-PA	–	+	–	–	–	–	+	–	+	–	+	–	–	–
pCAWS-NP	–	+	+	–	–	–	+	+	+	+	+	+	–	–
pC-PolII-WSN-PB2-PB1-PA	–	–	+	–	–	–	–	+	–	+	–	+	–	–
Total no. of plasmids	1	5	3	1	1	2	6	4	12	10	4	2	8	0
Exp. 1 (48 h p.t.), TCID <sub>50</sub> /ml	<50	1.7 × 10 <sup>4</sup>	3.7 × 10 <sup>4</sup>	0	0	0	<10	3.2 × 10 <sup>4</sup>	<10	<50	0	0	0	0
Exp. 2 (48 h p.t.), TCID <sub>50</sub> /ml	0	<50	3.7 × 10 <sup>4</sup>	0	0	0	6.2 × 10 <sup>2</sup>	4.4 × 10 <sup>4</sup>	<10	1.5 × 10 <sup>3</sup>	0	0	0	0
Exp. 3 (48 h p.t.), TCID <sub>50</sub> /ml	0	3 × 10 <sup>2</sup>	5.1 × 10 <sup>4</sup>	0	0	0	1.6 × 10 <sup>2</sup>	2 × 10 <sup>4</sup>	2 × 10 <sup>3</sup>	2.5 × 10 <sup>3</sup>	0	0	0	0
Exp. 1 (72 h p.t.), TCID <sub>50</sub> /ml	3.2 × 10 <sup>4</sup>	6.3 × 10 <sup>5</sup>	2.5 × 10 <sup>6</sup>	0	0	0	5.3 × 10 <sup>3</sup>	3.9 × 10 <sup>5</sup>	2.5 × 10 <sup>3</sup>	6.3 × 10 <sup>4</sup>	0	0	0	0
Exp. 2 (72 h p.t.), TCID <sub>50</sub> /ml	<10	3.1 × 10 <sup>3</sup>	1.6 × 10 <sup>6</sup>	0	0	0	7.6 × 10 <sup>4</sup>	6.3 × 10 <sup>5</sup>	6.3 × 10 <sup>3</sup>	2.1 × 10 <sup>5</sup>	0	0	0	0
Exp. 3 (72 h p.t.), TCID <sub>50</sub> /ml	50	3.2 × 10 <sup>4</sup>	2 × 10 <sup>6</sup>	0	0	50	2 × 10 <sup>4</sup>	5.1 × 10 <sup>5</sup>	1.6 × 10 <sup>5</sup>	2.5 × 10 <sup>5</sup>	0	0	0	0

Vero cells were transfected with the indicated plasmids (+). At 48 h or 72 h after transfection, virus titers in the supernatant were determined by plaque assays in MDCK cells. Shown are the results of three independent experiments. Eight-unit plasmid, pTM-PolI-WSN-All; 6-unit plasmid, pTM-PolI-WSN-PB2-PB1-PA-NP-M-NS; 2-unit plasmid, pTM-PolI-WSN-HA-NA; 8 × 1-unit plasmid, combination of pPolI-WSN-PB2, -PB1, -PA, -HA, -NP, -NA, -M, -NS. p.t., posttransfection.

mental sample 9 vs. experimental sample 8 ( $P = 0.0063$ ). We consistently found that expression of the three polymerase proteins from plasmid pC-PolII-WSN-PB2-PB1-PA resulted in more efficient virus generation as compared to providing these proteins from separate plasmids [Table 2, compare experimental samples 2 and 3 ( $P = 0.0054$ ), experimental samples 7 and 8 ( $P = 0.028$ ), and experimental samples 9 and 10 ( $P = 0.2$ )]. We again detected virus from plasmid pTM-PolI-WSN-All only (Table 2, experimental sample 1) or from plasmids pTM-PolI-WSN-PB2-PB1-PA-NP-M-NS and pTM-PolI-WSN-HA-NA (Table 2, experimental sample 6); however, virus generation was not observed consistently, and the resulting virus titers were low, likely because of the lower transfection efficiency of the Vero cells. Taken together, these results show that plasmids containing multiple RNA polymerase I or II transcription units can be highly efficient at generating virus in Vero cells.

**Discussion**

The generation of vaccine viruses can now be achieved by reverse genetics. In fact, this approach is the only efficient way to produce vaccine strains to highly pathogenic avian influenza viruses. These viruses are lethal to humans and embryonated eggs (25); therefore, attenuation [for example, by altering their HA cleavage site sequence (26, 27)] is critical to ensure growth to high titers in embryonated eggs while protecting vaccine production staff against exposure to aerosolized virus. For human use, the production of vaccine strains will require cell lines that are certified for lack of tumorigenicity and adventitious infectious agents. One such cell line is a Vero cell line, which is currently used for the production of rabies and polio vaccines (28). Using a “12-plasmid” approach, Fodor *et al.* (12) reported the generation of 10–20 plaque-forming units from 10<sup>7</sup> Vero

cells on day 4 after transfection. Wood and Robertson (29) generated an H5N1 reference vaccine strain in Vero cells by reverse genetics but did not report the rescue efficiency, whereas A/PR/8/34 (H1N1)- or A/PR/8/34-based viruses were generated in Vero cells with an efficiency of <10<sup>3</sup> plaque-forming units/ml (30). By combining RNA polymerase I and/or II transcription units and thus achieving virus rescue from fewer plasmids, we were able to produce ≈10<sup>5</sup> to 10<sup>6</sup> TCID<sub>50</sub>/ml from 5 × 10<sup>5</sup> Vero cells on day 3 after transfection. We, thereby, achieved more efficient virus generation in Vero cells with these systems as compared with the 12-plasmid approach (Table 2, compare experimental sample 3 or 8 with experimental sample 9). This robust and highly efficient reverse genetics system could, therefore, be an asset for the rapid preparation of vaccine strains in pandemic situations.

Influenza virus generation relies on the expression of the polymerase and NP proteins. We found that the combination of the polymerase subunits on one plasmid enhanced the efficiency of virus generation. This finding may be explained by the reduction in the number of plasmids used for virus rescue, or the combination of the three transcription units may more closely reflect the equimolar ratios of polymerase subunits found in infected cells.

The combination of identical promoter and terminator units on one plasmid is thought to cause recombination. However, here we demonstrated that eight RNA polymerase I or three RNA polymerase II promoter and terminator sequences can be combined on one vector backbone. Hoffmann *et al.* (13) demonstrated that a combination of RNA polymerase I and II promoters allows vRNA and mRNA synthesis from one template. One could therefore design a plasmid that contains four RNA polymerase I/II transcription units for the synthesis of

PB2, PB1, PA, and NP vRNAs and mRNAs, and four RNA polymerase I transcription units for the synthesis of NA, HA, M, and NS vRNAs. Such a construct should allow for the efficient generation of influenza virus from one plasmid. Moreover, our success in combining transcription units on one plasmid may provide the incentive for others to apply this strategy to other reverse genetics systems that rely on the cotransfection of cells with several plasmids or to design vectors for the simultaneous expression of several proteins from one plasmid.

Surprisingly, we observed virus generation from a single plasmid, pTM-PolI-WSN-All. The expression of influenza viral proteins from this plasmid suggests protein synthesis from a (cryptic) RNA polymerase II promoter present in the vector or in the RNA polymerase I promoter or terminator region. To determine whether the RNA polymerase I promoter sequence harbors a promoter in the opposite direction that could potentially drive protein expression from the upstream transcription cassette, we cloned an inverted RNA polymerase I promoter in front of a reporter gene; however, we did not detect appreciable levels of reporter gene expression from this plasmid (data not shown). The generation of influenza virus relies on the expression of four different proteins (PB2, PB1, PA, and NP) and would therefore require several read-through events. Alternatively, protein expression may have resulted from another mechanism, such as internal initiation of translation. We used a

modified pTM1 (23) vector that contains the fl single-stranded DNA origin of replication, the ampicillin resistance gene, a multiple cloning site, and the T7 RNA polymerase transcriptional terminator. The strong T7 RNA polymerase promoter and parts of the encephalomyocarditis virus untranslated region and thymidine kinase sequences that are present in the original pTM1 cloning vector had been eliminated from this modified version. Protein synthesis of the polymerase and NP proteins from this vector was therefore not expected. Nonetheless, the generation of influenza virus from plasmids designed to produce only negative-strand RNAs is intriguing and deserves further study.

In summary, here we present an improved system for the generation of influenza viruses that allows the easy and reproducible production of vaccine viruses in Vero cells. Application of this system may be especially advantageous in situations of outbreaks of highly pathogenic avian influenza viruses.

We thank Krisna Wells and Martha McGregor for excellent technical assistance and Susan Watson for editing the manuscript. This work was supported by Public Health Service research grants from the National Institute of Allergy and Infectious Diseases, by Grants-in-Aid for Scientific Research on Priority Areas from the Ministries of Education, Culture, Sports, Science, and Technology of Japan, and by the Core Research for Evolutional Science and Technology.

- Thompson, W. W., Shay, D. K., Weintraub, E., Brammer, L., Cox, N., Anderson, L. J. & Fukuda, K. (2003) *J. Am. Med. Assoc.* **289**, 179–186.
- Potter, C. W. (1998) in *Textbook of Influenza*, eds. Nicholson, K. G., Webster, R. G. & Hay, A. J. (Blackwell Scientific, Malden, U.K.), pp. 3–18.
- Subbarao, K., Klimov, A., Katz, J., Regnery, H., Lim, W., Hall, H., Perdue, M., Swayne, D., Bender, C., Huang, J., et al. (1998) *Science* **279**, 393–396.
- Claas, E. C., Osterhaus, A. D., Van Beek, R., de Jong, J. C., Rimmelzwaan, G. F., Senne, D. A., Krauss, S., Shortridge, K. F. & Webster, R. G. (1998) *Lancet* **351**, 472–477.
- Peiris, J. S., Yu, W. C., Leung, C. W., Cheung, C. Y., Ng, W. F., Nicholls, J. M., Ng, T. K., Chan, K. H., Lai, S. T., Lim, W. L., et al. (2004) *Lancet* **363**, 617–619.
- Fouchier, R. A., Schneeberger, P. M., Rozendaal, F. W., Broekman, J. M., Kemink, S. A., Munster, V., Kuiken, T., Rimmelzwaan, G. F., Schutten, M., Van Doornum, G. J., et al. (2004) *Proc. Natl. Acad. Sci. USA* **101**, 1356–1361.
- Koopmans, M., Wilbrink, B., Conyn, M., Natrop, G., van der Nat, H., Vennema, H., Meijer, A., van Steenbergen, J., Fouchier, R., Osterhaus, A., et al. (2004) *Lancet* **363**, 587–593.
- World Health Organization (2005) *Cumulative Number of Confirmed Human Cases of Avian Influenza A (H5N1)* (WHO, Geneva). Available at [www.who.int/csr/disease/avian\\_influenza/country/en](http://www.who.int/csr/disease/avian_influenza/country/en). Accessed January 28, 2005.
- Gerdil, C. (2003) *Vaccine* **21**, 1776–1779.
- Neumann, G., Watanabe, T., Ito, H., Watanabe, S., Goto, H., Gao, P., Hughes, M., Perez, D. R., Donis, R., Hoffmann, E., et al. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 9345–9350.
- Hoffmann, E., Mahmood, K., Yang, C. F., Webster, R. G., Greenberg, H. B. & Kemble, G. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 11411–11416.
- Fodor, E., Devenish, L., Engelhardt, O. G., Palese, P., Brownlee, G. G. & Garcia-Sastre, A. (1999) *J. Virol.* **73**, 9679–9682.
- Hoffmann, E., Neumann, G., Kawaoka, Y., Hobom, G. & Webster, R. G. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 6108–6113.
- Brands, R., Visser, J., Medema, J., Palache, A. M. & van Scharrenburg, G. J. (1999) *Dev. Biol. Stand.* **98**, 93–100.
- Palache, A. M., Scheepers, H. S., de Regt, V., van Ewijk, P., Baljet, M., Brands, R. & van Scharrenburg, G. J. (1999) *Dev. Biol. Stand.* **98**, 115–125.
- Halperin, S. A., Smith, B., Mabrouk, T., Germain, M., Trepanier, P., Hassell, T., Treanor, J., Gauthier, R. & Mills, E. L. (2002) *Vaccine* **20**, 1240–1247.
- Kistner, O., Barrett, P. N., Mundt, W., Reiter, M., Schober-Bendixen, S. & Dorner, F. (1998) *Vaccine* **16**, 960–968.
- Kistner, O., Barrett, P. N., Mundt, W., Reiter, M., Schober-Bendixen, S., Eder, G. & Dorner, F. (1999) *Dev. Biol. Stand.* **98**, 101–110.
- Kistner, O., Barrett, P. N., Mundt, W., Reiter, M., Schober-Bendixen, S., Eder, G. & Dorner, F. (1999) *Wien. Klin. Wochenschr.* **111**, 207–214.
- Bruhl, P., Kerschbaum, A., Kistner, O., Barrett, N., Dorner, F. & Gerencer, M. (2000) *Vaccine* **19**, 1149–1158.
- Nicolson, C., Major, D., Wood, J. M. & Robertson, J. S. (2005) *Vaccine* **23**, 2943–2952.
- Sugawara, K., Nishiyama, K., Ishikawa, Y., Abe, M., Sonoda, K., Komatsu, K., Horikawa, Y., Takeda, K., Honda, T., Kuzuhara, S., et al. (2002) *Biologicals* **30**, 303–314.
- Moss, B., Elroy-Stein, O., Mizukami, T., Alexander, W. A. & Fuerst, T. R. (1990) *Nature* **348**, 91–92.
- Neumann, G., Feldmann, H., Watanabe, S., Lukashevich, I. & Kawaoka, Y. (2002) *J. Virol.* **76**, 406–410.
- Shortridge, K. F., Zhou, N. N., Guan, Y., Gao, P., Ito, T., Kawaoka, Y., Kodihalli, S., Krauss, S., Markwell, D., Murti, K. G., et al. (1998) *Virology* **252**, 331–342.
- Horimoto, T. & Kawaoka, Y. (1994) *J. Virol.* **68**, 3120–3128.
- Subbarao, K., Chen, H., Swayne, D., Mingay, L., Fodor, E., Brownlee, G., Xu, X., Lu, X., Katz, J., Cox, N., et al. (2003) *Virology* **305**, 192–200.
- Montagnon, B. J., Vincent-Falquet, J. C. & Saluzzo, J. F. (1999) *Dev. Biol. Stand.* **98**, 137–140.
- Wood, J. M. & Robertson, J. S. (2004) *Nat. Rev. Microbiol.* **2**, 842–847.
- Ozaki, H., Govorkova, E. A., Li, C., Xiong, X., Webster, R. G. & Webby, R. J. (2004) *J. Virol.* **78**, 1851–1857.