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Construction of Attenuated Influenza Virus Vaccines of 2006～2007*

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Abstract Six gene segments, PB1, PB2,PA, NP, M and NS, were fully synthesized which derived from the master donor virus (MDV), cold-adapted(ca), temperature sensitive(ts), live attenuated influenza virus strain A/Ann Arbor/6/60(MDV-A). Meanwhile, five amino acid substitutions (PB1-391E, 581G, 661T, PB2-265S, NP-34G) were artificially altered by human intervention. HA and NA fragments derived from the 2006～2007 circulating strain A/New Caledonia/20/99 (H1N1). Eight fragments were ligated with modified pAD3000 for rescue plasmid construction. Eight transcription/expression plasmids were named as pMDV-A-PB2, pMDV-A-PB1, pMDV-A-PA, pMDV-A-NP, pMDV-A-M, pMDV-A-NS, pMDV-A-HA, pMDV-A-NA, respectively. The COS-1 cells were co-transfected with eight plasmids representing 6 internal viral backbone of the strain A/AA/6/60 and two plasmids containing the cDNA of the HA and NA segments of the strain A/New Caledonia/20/99 (H1N1), the results showed that cold-adapted, attenuated reassortant influenza A virus was rescued successfully. Titers of a reassorted influenza A virus in embryonated chicken eggs ranged from $1:2^9$ to $1:2^{10}$. The rescue system of six internal genes used as backbone opens the way for further research on gene function and neotype vaccine candidate of cold-adapted, live attenuated human influenza virus.

Key words influenza virus, reverse genetics, eight-plasmid system, reassorted influenza virus

Influenza is a contagious, acute respiratory disease caused by an influenza virus infection. Influenza virus, a member of the family *Orthomyxoviridae*, is a negative-sense RNA virus with a segmented genome. Influenza viruses are divided into types A, B and C. Influenza A viruses are the principal causes of influenza in humans and it imposes a huge burden on the human health and the national economy. Its genetic composition allowed this virus to evolve by reassortment of gene segments from different strains[1]. To date, vaccine is still a principal means for influenza prophylaxis. Vaccines can be characterized as whole virus vaccines, split virus vaccines, surface antigen vaccines and live attenuated virus vaccines. While appropriate formulations of any of these vaccine types is able to produce a systemic immune response, live attenuated virus vaccines are also able to stimulate local mucosal immunity in the respiratory tract.

Commercially available trivalent inactivated influenza vaccines given by intramuscular injection are derived from essentially modified viruses. By exploiting the segmented nature of the influenza A genome, a reassortant virus carrying the circulating virus′s gene segments that encode haemagglutinin and neuraminidase is produced, the major targets of neutralizing antibodies. The remaining six-gene segments are supplied from PR8, a laboratory-adapted avirulent H1N1 strain. The resulting reassortant virus has the antigenic properties of the circulating strain and the safety and high-yield properties of PR8. Trivalent inactivated vaccine used now suffered from limited efficacy in cross-protective immunity and routes of administration due to antigenic shift and

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drift [2]. Recently, the reverse genetics provided a favourable opportunity for live attenuated vaccines. Efforts are currently under way to develop a live attenuated influenza vaccine which based on reverse genetics. Since 1999, Neumann and Hoffmann, *et al*. have developed an eight-plasmid DNA transfection system for the rescue of infectious influenza A virus entirely from cloned cDNA [3,4]. This technology opens the way for the study of neotype influenza vaccine. In 2003, cold-adapted live, attenuated influenza vaccine (FluMist™) is licensed for use in USA[5]. Production of live attenuated vaccines takes advantage of the segmented nature of the influenza virus genome by reassorting a donor strain with a wild-type virus. There are two master donor strains used in the USA to produce cold-adapted virus, one for production of influenza A strain, A/Ann Arbor/6/60 (H2N2), and another for production of influenza B strains, B/Ann Arbor/1/66 [6]. The master strains are cold-adapted, temperature sensitive and attenuated: thus, the virus has more limited replication at the warmer temperatures of the lower respiratory tract, where wild-type strains cause infection *in vivo*. Here, our aim was to produce a cold-adapted recombinant, live attenuated human influenza A virus expressing the HA and NA of A/New Caledonia/20/99 that are related to strains circulating during the 2006～2007 influenza season based on A/AA/6/60 master strain, which was adapted for optimal efficiency of viral rescue from cDNA in the eight-plasmid reverse genetics system. Our results implied that plasmid-based reverse genetics could be used for research of gene functions of influenza viruses and generation of cold-adapted human vaccine candidates.

1 Materials and methods

1.1 Viruses, cells and plasmids

Influenza virus strain A/New Caledonia/20/99 (H1N1) were obtained from China CDC and propagated in 10-day-old pathogen-free embryonated research grade eggs (Center of laboratory animal, Beijing).COS-1 cells and Madin-Dardy canine kidney (MDCK) cells were obtained from Yangzhou University and maintained in essential medium (DMEM, Sigma) containing 10% fetal bovine serum (FBS).The positive plasmids carrying the eight gene segments of the high-growth PR8 (H1N1) virus (pHW191 to pHW198) were the same as those used by Hoffmann, *et al*[7].

1.2 Construction of vector pAD3000

The plasmid pHW2000 was modified by replacing the bovine growth hormone (BGH) polyadenylation signals with a polyadenylation signal sequence derived from Simian virus 40 (SV40) and named pAD3000[8]. Sequence derived from SV40 was amplified with expand high fidelity PCR system (Roche) using designated primer pairs (PolyA-1, PolyA-2). The plasmid pcDNA3.1 was used as a template. A desired 138 bp fragment containing the SV40 polyadenylation signals was excised from the resulting fragment with *Eco*R Ⅴ and *Bst*E Ⅱ, isolated from an agarose gel, and ligated between the unique *Pvu* Ⅱ and *Bst*E Ⅱ sites in pHW2000 using conventional techniques. The vector pHW2000 was modified via a series of reconstruction based on pcDNA3.0. and was a gift by Hoffmann. PolyA-1: 5′ AACAATTGAGATCTCGGTCACCTCAGACATGA-TA AGATACATTGATGAGT 3′; PolyA-2: 5′ TAT-AACTGCAGACTAGTGATATCCTTGTTTATTGC-AGCTTATAATGGTTA 3′

1.3 Virus RNA extraction, PCR amplification, and gene cloning

Influenza virus strain A/AnnArbor/6/60 (H2N2), a cold-adapted (*ca*), temperature sensitive (*ts*), live attenuated was used as the master donor virus (MDV) for virus rescue, in which six internal gene fragments were fully synthesized. Meanwhile, five amino acid substitutions (PB1-391E, 581G, 661T, PB2-265S, NP-34G) (Table1) have been artificially altered by human intervention and six pairs specific primers were designed according to references [3,4,7,9]. A/New Caledonia/20/99 (H1N1) was propagated in 10-day-old embryonated chicken eggs and concentrated by density gradient centrifugation on sucrose. Total RNA was extracted from infected allantoic fluid with the RNeasy kit (Qiagen) in accordance with manufacturer's instructions. Reverse transcription was carried out with the uni12 primer (5′ AGCAAAAGCAGG 3′) and AMV reverse transcriptase (Invitrogen). HA and NA gene were amplified by using the universal primer pairs according to Hoffmann, *et al* [9]. Expand High Fidelity PCR System (RoChe) was used and 8～10 clones were sequenced in order to confirm the authenticity of 5′ and 3′ ends. Finally, we spliced the 8 whole gene sequences containing the non-coding region of 3′ and 5′ ends by BLAST. cDNA fragments with *Bsm*B Ⅰ, *Bsa* Ⅰ and *Aar* Ⅰ of A/Ann Arbor/6/60 and A/New Caledonia/20/99 were cloned between the

two *Bsm*B Ⅰ sites of the vector pAD3000. Eight transcription /expression plasmids were obtained and named as pMDV-A-PB2, pMDV-A-PB1, pMDV-A-PA, pMDV-A-NP, pMDV-A-M, pMDV-A-NS, pMDV-A-HA, pMDV-A-NA, respectively. Sites of the three restriction endonucleases(New England Biolabs) in six internal genes were as followed (Table2). All cloned were confirmed by full-length sequencing.

Table 1 Substitution were introduced in a polynucleotide and amino acid position

RNA segment	Base (amino acid) position	"wt" A/AA/6/60	rMDV-A
PB2	821(265)	A(Asn)	G(Ser)
PB1	1195(391)	A(Lys)	G(Glu)
	1766(581)	A(Glu)	G(Gly)
	2005(661)	G(Ala)	A(Thr)
NP	146(34)	A(Asp)	G(Gly)

Table 2 Sites of the three restriction endonucleases in six internal genes

Restriction endonucleases	PB2	PB1	PA	NP	M	NS
Aar Ⅰ	—	—	—	—	—	—
Bsa Ⅰ	1535，1724	2136	—	—	510	444，650
*Bsm*B Ⅰ	1187，2207	—	—	—	—	—

1.4 Virus rescue from cloned cDNA

The rescue of infection virus from cloned cDNA was done under GMP conditions. COS-1 cells were used for transfection at a confluency of approximately 70%～80%. Plasmid DNA transfection was performed using PolyFect transfection reagent (Qiagen) by mixing 0.2 μg of each of the eight recombinant plasmids (PB2, PB1, PA, HA, NP, NA, M, NS)with 10 μl of PolyFect diluted in 100 μl DMEM. The DNAs and transfection reagent mixture were incubated at room temperature for 5～10 min followed by addition of 500 μl DMEM. The transfection mixture was then added dropwise to the COS-1 cells. The transfection cells were incubated at 33℃ for 48 h, 1 ml of DMEM containing 1 mg/L trypsin (PIERCE) was added to the dish and incubated for an additional 12～24 h. The recovered virus was then amplified in confluent MDCK cells or directly amplified in embryonated chick eggs. The amplified viruses were stored at –80℃. 200 μl of the clarified supernatant was injected into the allantoic cavity of individual 10-day-old embryonated chicken eggs. After 72 h incubation at 33℃, each egg was candled to determine embryo viability before chilling at 4℃. Allantoic fluid was harvested from each egg and was tested for haemagglutination activity. HA assays were performed by mixing 50 μl of serially 2-fold diluted culture supernatants with 50 μl of 1% chick red blood cells in 96-well plates.

1.5 Identification and analysis of recombinant virus

1.5.1 RT-PCR. RT-PCR was performed to map the genotypes of the recovery viruses. Virus RNA was isolated from the infected cell culture supernatant or allantoic fluid from the second passage using the RNeasy mini kit and the six internal gene segments were amplified by RT-PCR using specific primers of each MDV-A gene segment.

1.5.2 Electronic microscope. The allantoic fluid from the second passage was purified and concentrated by density gradient centrifugation on sucrose. Then, morphology of the rescued virus and wild-type virus strain were observed by electronic microscope.

1.5.3 Cold-adapted phenotype. Cold-adapted properties of the rescued virus were determined by plaque assay (PFU) on MDCK cells at 33, 37 and 39℃. The wild-type virus strain A/New Caledonia/20/99 (H1N1) was conducted as control. MDCK cells in 6-well plates were infected with 400 μl of 10-fold serially diluted virus and adsorbed at room temperature for 60 min.The inoculants were removed and replaced with 1×DMEM containing 0.8% agarose and 1 mg/L TPCK-trypsin. The infected cells were incubated at 33, 37 or 39℃ in 5% CO_2 incubators. After three day's

incubation, the monolayers were stained with crystal violet and the plaques were visualized.

1.5.4 The cytoplasmic effect. The allantoic fluid from the second passage was used to infected fresh MDCK cells. MDCK cells in 12-well plate were infected with 0.2 ml of the allantoic fluid for 40～60 min at room temperature, the mixture was then removed and replaced with 2 ml DMEM containing 1 mg/L TPCK-trypsin. The infected cells were incubated at 33℃ for 3～4 days in DMEM with/without 1 mg/L TPCK-trypsin. The cytoplasmic effect of the recombinant virus on infected MDCK cells was observed using a microscope.

1.5.5 Stability testing in eggs. In order to test the stability of the vaccine virus on propagation, four consecutive passages of the virus were made in embryonated chicken eggs. The virus was adjusted to 10^{-3} dilution with PBS, and 0.2 ml of the solution was injected into the allantoic cavities of four 10-day-old embryonated chicken eggs and then reinjected into another four eggs.

2 Results

2.1 Construction of bi-directional transcription/expression vector pAD3000

Using pcDNA3.1 plasmid as a template, SV40 polyA signal sequence was amplified by PCR and the full-length was 138 bp . The resulting plasmid, pAD3000 (Figure 1), was sequenced and found to contain the SV40 polyadenylation site in the correct orientation.

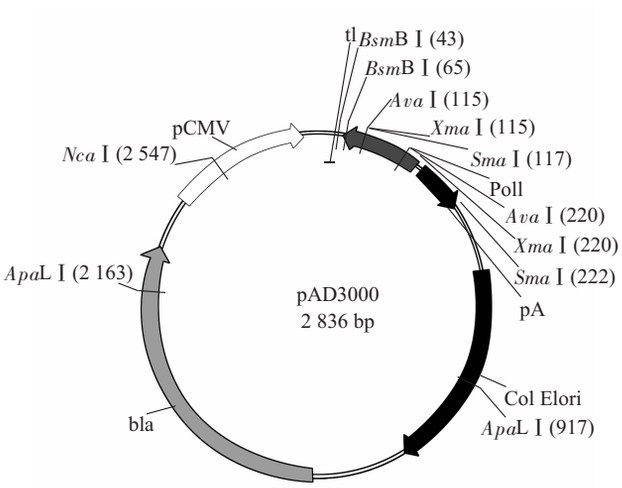

Fig. 1 Illustration of pAD3000 plasmid

2.2 Analysis of the eight recombinant plasmids

Each of the 8 recombinant genome segments cloned in pAD3000 was showed to be functionally expressed in a reassortant experiment by co-transfection a single gene segment from MDV-A together with the complementary seven segments from control A/PR/8/34 strain. All eight single genome segment plasmids in combination with complementary control segments generated infectious reassortant virus, which caused cytopathic effects in infected MDCK cells, showing that all eight plasmids encode functional proteins. Infectious influenza viruses were produced from these transfection reactions as follows (Table 3). Ninety-six hours after the transfection, the virus titer of different 7+1 combination in the embryonated chicken eggs were between 1∶32 and 1∶4096 infectious viruses per milliliter.

Table 3 Analysis of the MDV-A plasmids by single gene reassortant experiment

vgs	Recovery of 7+1 reassortants by plasmids							
	PB2	PB1	PA	NP	M	NS	HA	NA
1	pMDV-A-PB2	pHW191-PB2	pHW191-PB2	pHW191-PB2	pHW191-PB2	pHW191-PB2	pHW191-PB2	pHW191-PB2
2	pHW192-PB1	pMDV-A-PB1	pHW192-PB1	pHW192-PB1	pHW192-PB1	pHW192-PB1	pHW192-PB1	pHW192-PB1
3	pHW193-PA	pHW193-PA	pMDV-A-PA	pHW193-PA	pHW193-PA	pHW193-PA	pHW193-PA	pHW193-PA
4	pHW195-NP	pHW195-NP	pHW195-NP	pMDV-A-NP	pHW195-NP	pHW195-NP	pHW195-NP	pHW195-NP
5	pHW197-M	pHW197-M	pHW197-M	pHW197-M	pMDV-A-M	pHW197-M	pHW197-M	pHW197-M
6	pHW198-NS	pHW198-NS	pHW198-NS	pHW198-NS	pHW198-NS	pMDV-A-NS	pHW198-NS	pHW198-NS
7	pHW194-HA	pHW194-HA	pHW194-HA	pHW194-HA	pHW194-HA	pHW194-HA	pMDV-A-HA	pHW194-HA
8	HW196-NA	pHW196-NA	pHW196-NA	pHW196-NA	pHW196-NA	pHW196-NA	pHW196-NA	pMDV-A-NA
CPE	+	+	+	+	+	+	+	+
HA	1∶32	1∶1 024	1∶4 096	1∶1 024	1∶2 048	1∶1 024	1∶1 024	1∶1 024

Vgs: Virus gene segment.

2.3 Generation of infectious recombinant MDV-A and reassorted influenza virus

Three days post-transfection, culture supernatants with eight recombinant plasmids incorporating the 6 MDV-A internal genes, and HA and NA derived from A/PR/8/34 (6∶2 reassortant, PR8/rMDV-A), or A/New Caledonia/20/99 (H1N1) (6∶2 reassortant, rMDV-A), were used to infect fresh MDCK cells. Then, the infected cells were incubated at 33℃ for 3～4 days in DMEM with 1 mg/L TPCK-trypsin. The cytoplasmic effect of the recombinant virus on infected MDCK cells was observed using a microscope. Expression of viral hemagglutinin was monitored using a standard hemagglutination assay (HA). HA titers were detected to be 1∶512～1∶1 024 approximately from the amplified 6∶2 reassortant virus and transfected recombinant MDV-A. The transfection reaction using the 8 A/PR/8/34 plasmids was used as a positive control. These transfection reactions were as follows (Table 4).

Table 4 Recovery of rMDV-A and PR8/rMDV-A

Plasmids used for recovery of A/PR/8/34, rMDV-A and PR8/rMDV-A			
Virus gene segment	A/PR/8/34(H1N1)	r MDV-A(H1N1)	PR8/rMDV-A
1	pHW191-PB2	pMDV-A-PB2	pMDV-A-PB2
2	pHW192-PB1	pMDV-A-PB1	pMDV-A-PB1
3	pHW193-PA	pMDV-A-PA	pMDV-A-PA
4	pHW195-NP	pMDV-A-NP	pMDV-A-NP
5	pHW197-M	pMDV-A-M	pMDV-A-M
6	pHW198-NS	pMDV-A-NS	pMDV-A-NS
7	pHW194-HA	pMAD-A-HA	pHW194-HA
8	pHW196-NA	pMAD-A-NA	pHW196-NA
CPE	+	+	+
HA	1∶1 024	1∶512～1∶1 024	1∶512～1∶1 024

2.4 RT-PCR analysis

The full-length sequence of the recombinant virus after passage into embryonated chicken eggs was identical to that of the input plasmids. Six desired fragments were amplified by RT-PCR from the second passage, they were 2.3 kb, 2.3 kb, 2.2 kb, 1.5 kb,1.0 kb and 0.9 kb, respectively (Figure 2). However, we couldn′t obtain positive recombinant virus and amplified HA and NS from the supernatant of seven plasmids exclude contamination of plasmids.

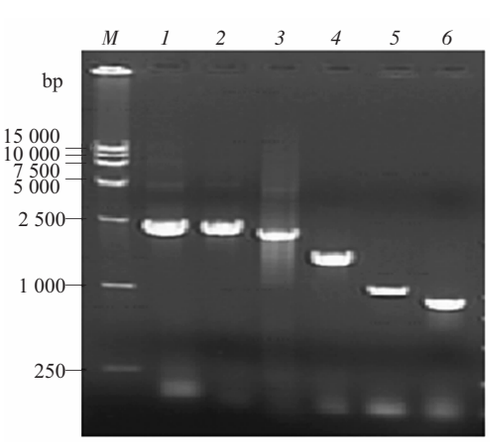

Fig. 2 Six internal gene segments of rMDV-A amplified by PCR

M: DL15000 marker; L*1*～*6*: PB2, PB1, PA, NP, M, NS.

2.5 Electronic microscope

Influenza viral particle with capsule could be observed in two samples. The rescued virus had similar morphology to wild-type virus strain by electronic microscope (Figure 3). Most influenza virions were globular, only a few were silkiness and their diameters were between 80 nm and 120 nm. Viral internal nuclear material and spine on top of peplos were distinct.

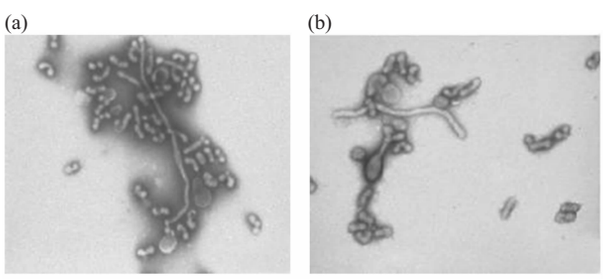

Fig. 3 Identification of the wild-type virus strain A/PR/8/34 and rescued virus by electronic microscope

(a) Wild-type virus strain A/PR/8/34. (b) Rescued virus. (200×)

2.6 Cold-adapted phenotype

As shown in Figure 4 the wild-type virus strain A/New Caledonia/20/99 (H1N1) replicated well at

33℃, 37℃ and 39℃, although a slight reduction in virus titer was observed. However, the titer of the rescued virus at 33℃, 37℃ and 39℃ were 7.6, 5.8 and 3.0 respectively. The plaque forming units of the rescued virus were decreased significantly by increasing temperature (e.g., at 37℃ or 39℃) as compared to the wild-type virus. It showed that the rescued virus was sensitive to temperature and 33℃ was the optimal culture temperature for viral multiplication of the rescued virus.

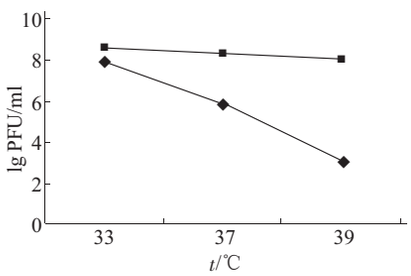

Fig. 4 Virus titer of rescued virus and wild-type virus at 33, 37and 39℃

◆—◆: Rescued virus; ■—■: Wild-type virus.

2.7 CPE

In view of the cold-adapted properties of recombinant virus, 33℃ was optimal for its multiplication after eggs were inoculated with recombinant virus. There is no difference when COS-1 cells were incubated in 33℃, except growth speed was slower than that of at 37℃. The recovery virus was unable to form cytoplasmic effect on Madin-Darby canine kidney (MDCK) cells in the absence of TPCK-trypsin (Figure 5a). However, the cytoplasmic effect was significantly evident in the presence of 1 mg/L TPCK-trypsin at 96 h post-transfection (Figure 5b).

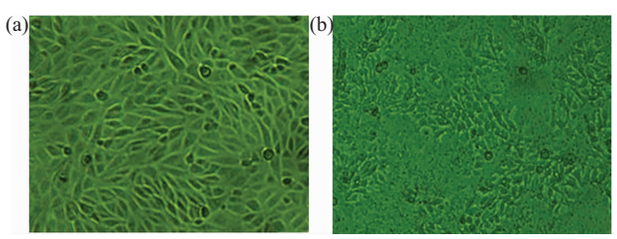

Fig. 5 CPE on MDCK cells at 96 h post-infection with recombinant viruses in presence/absence of TPCK-trypsin (200×)

(a) rMDV-A in absence of TPCK-trypsin: no CPE. (b) rMDV-A in presence of TPCK-trypsin:typical CPE.

3 Discussion

It is important to note that the reverse genetics systems have developed rapidly since late 20th century. To date, these techniques have been used to produce "conventional" vaccine viruses and to design live attenuated vaccines through genetic engineering. In addition, they were used widely in the influenza virus field such as exploring viral gene structure and function, illustrating the mechanism of viral infection and immunity defense, producing vaccine candidates[10,11]. In 1999, Neumann [3] developed initially a system for the generation of influenza A virus by cotransfecting eight plasmids. In China, Chenzf, Lujh and Longjx, *et al*. established the eight-plasmid rescue systems of H1N1, H9N2 and H5N1 subtype influenza virus [12～15]. In this paper, we established a eight-plasmid system for the generation of cold-adapted recombinant H1N1 subtype influenza virus that facilitates the generation of vaccine viruses without the need for time-consuming co-infection and selection procedures.

Firstly, bi-directional transcription/expression pHW2000 was modified in order to improve rescue efficiency and virus titer. We cloned the eight viral cDNA of influenza virus strain containing six internal gene fragments derived from A/AA/6/60 and two plasmids representing the cDNA of the HA and NA segments of 2006～2007 circulating influenza A virus. Different recombinant virus was produced by co-transfecting COS-1 cells with the eight plasmids of 7+1 and 6+2 combinations. The results showed that the six cold-adapted viral internal backbones could work functionally and synergicly, meanwhile, the recombinant virus yields of the embryonated chicken eggs were between 1∶512 and 1∶1 024 infectious viruses per milliliter in the first passage. Even after four passages, the virus titers did not decrease (datum don′t list in this paper). These maybe related to properties of clone vector and exterior gene of different origin. Relatively higher titer is an important factor for developing vaccine candidates, because it can reduce cost and preserve adequate antigen for much longer period of immunity and protection[16].

The viral replication cycle involves a complex interaction between the viral proteins each other and cellular factors. Thus, for the generation of infectious virus, the plasmid-driven synthesis of viral molecules should provide optimal concentrations of viral proteins for the initiation of the replication cycle and for the formation of virus-like particles [17]. Although the eight-plasmid system proved to be efficient, it might be

possible to further increase the production of virus. At present, we are able to generate infectious viral particles from about 10^5 transfected cells. By increasing transfection efficiencies and optimizing the ratio of plasmids and transfection reagent it might be possible to obtain higher recoveries of virus. In addition, the eight-plasmid system use the species-specific human RNA polymerase Ⅰ promoter, which necessitates the use of cells from primate origin [18,19]. Therefore, the use of a cell line, such as Cos-1 cell, might be an important factor for successful virus rescue.

In summary, we have rescued a cold-adapted recombinant H1N1 subtype influenza A virus by co-transfecting eight transcription plasmids entirely from cDNA. The development of cold-adapted rescue system opens the way for the study of mechanism infection and immunity of different aspects of influenza virus. In addition, it allows full manipulation of the genome of the virus, which may result in the development of new vaccine strains for human influenza.

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2006～2007 年流行流感病毒减毒疫苗株的构建 *

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摘要　选择冷适应、温度敏感、减毒的 A/Ann Arbor/6/60 (H2N2)流感病毒株作为骨架病毒，对其 6 个内部基因片段进行了全基因合成，同时人工引入 5 个氨基酸突变(PB1-391E, 581G, 661T, PB2-265S, NP-34G)．HA 和 NA 来源于 2006～2007 当年流行株 A/New Caledonia/20/99 (H1N1)．8 个基因片段通过与改造后的转录载体 pAD3000 连接，构建 8 个基因的拯救载体，经测序获得序列准确的拯救质粒：pMDV-A-PB2、pMDV-A-PB1、pMDV-A-PA、pMDV-A-NP、pMDV-A-M、pMDV-A-NS、pMDV-A-HA、pMDV-A-NA．6 质粒与当年流行株的表面基因 HA 和 NA 进行“6+2”组合的病毒拯救，8 个重组质粒共转染 COS-1 细胞，成功拯救出了具有血凝性的冷适应减毒的重组 A 型人流感病毒．鸡胚尿囊液中重组病毒的血凝效价为 $1∶2^9$～$1∶2^{10}$．构建的 A/AA/6/60 6 个内部基因的病毒骨架拯救系统，为深入研究冷适应减毒人流感病毒的基因功能和新型疫苗研发奠定了基础．

关键词　流感病毒，反向遗传学，8 质粒拯救系统，重组流感病毒
学科分类号　R183.3，R392.1

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