Reverse genetics approach towards understanding pathogenesis of H5N1 Hong Kong influenza A virus infection

Masato Hatta¹, Gabriele Neumann¹ and Yoshihiro Kawaoka^{1,2*}

¹Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, WI, USA

²Institute of Medical Science, University of Tokyo, Japan

In 1990, Palese and colleagues established a method (reverse genetics) that allowed one to generate influenza virus containing a gene segment derived from cloned cDNA. Although this method contributed tremendously to our understanding of influenza pathogenesis, the requirement of helper viruses limited its use in many experimental settings. Recently, we and others established systems for the generation of influenza viruses entirely from cloned cDNAs. These systems require only DNA cloning and transfection techniques, and can therefore be easily implemented by laboratories working in the fields of molecular biology and virology. Thus, for the first time, a system is now available that allows highly efficient generation of influenza virus without technical limitations. Using this technology, we generated the same strain of H5N1 influenza viruses that caused an outbreak in Hong Kong in 1997, killing six people.

Keywords: reverse genetics; influenza virus; virulence

1. INTRODUCTION

Reverse genetics of negative-sense RNA viruses is defined as the generation of viruses from cloned cDNAs. To artificially generate negative-sense RNA viruses, functional ribonucleoprotein (RNP) complexes are required. This task had been accomplished for non-segmented, negative-sense RNA viruses of both the Rhabdo- and Paramyxoviridae families. Schnell *et al.* (1994) reported the generation of infectious rabies virus from cloned DNA. Plasmids encoding the full-length viral genome and the nucleoprotein (NP) and polymerase proteins, all under the control of the T7 RNA polymerase promoter, were transfected into cells that had been infected with recombinant vaccinia virus expressing T7 RNA polymerase. Since then, similar systems have been described for viruses of all genera of the Rhabdo- and Paramyxoviridae families (Baron & Barnett 1997; Buchholz *et al.* 1999; Clarke *et al.* 2000; Collins *et al.* 1995; Durbin *et al.* 1997; He *et al.* 1997; Hoffman & Banerjee 1997; Jin *et al.* 1998; Lawson *et al.* 1995; Peeters *et al.* 1999; Radecke *et al.* 1995; Romer-Oberdorfer *et al.* 1999; Whelan *et al.* 1995). The genomes of segmented, negative-sense RNA viruses had long been refractory to generation from cloned cDNAs, probably because multiple viral RNPs (vRNPs) have to be provided. Following the approach outlined by Schnell *et al.* (1994), Bridgen & Elliott (1996) reported the generation of bunyamwera virus, belonging to the family Bunyaviridae whose genome is composed of three segments; however, the efficiency of virus generation was

low. The generation of influenza A virus is far more challenging since its genome is composed of eight segments of RNA. To generate influenza A virus from cloned cDNAs, all eight viral RNAs (vRNAs) have to be provided in the cell nucleus, together with the three polymerase proteins and the NP. Here, we review influenza reverse genetics and how one can use this system for studying the pathogenesis of the H5N1 influenza A viruses that caused an outbreak among Hong Kong residents in 1997.

2. RECONSTITUTION OF FUNCTIONAL RNP COMPLEXES

The first step towards the generation of influenza virus is the reconstitution of functional RNP complexes. RNP complexes can be assembled from purified NP and polymerase proteins (Szewczyk *et al.* 1988) or isolated from detergent-treated virus (Honda *et al.* 1987). Parvin *et al.* (1989) and Honda *et al.* (1990) were able to transcribe short synthetic RNAs or full-length vRNAs using RNP complexes reconstituted from purified viral proteins.

Because of inherent technical difficulties, the purification of viral proteins and their reassembly with synthetic RNAs to form functional vRNP complexes was not an efficient approach to artificially generate influenza viruses. Nevertheless, these early experiments were the impetus for future studies, demonstrating that the three polymerase proteins and NP are sufficient for transcription and replication of vRNAs.

3. HELPER VIRUS-DEPENDENT REVERSE GENETICS

Enami *et al.* (1990) were the first to generate influenza virus containing an artificially produced gene. The

*Author and address for correspondence: Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, 2015 Linden Drive, Madison, WI 53706, USA (kawaoka@svm.vetmed.wisc.edu).

neuraminidase (NA) protein of A/WSN/33 virus confers trypsin-independent virus growth in MDBK cells. The authors transfected *in vitro*-generated A/WSN/33 NA vRNPs into cells that had been infected with a virus containing an NA from a trypsin-dependent strain. Thus, in the absence of trypsin, virus containing the A/WSN/33 NA gene segment was selected. Since this success, a number of viruses containing mutations in different gene segments (i.e. subunit PB2 of the polymerase protein, haemagglutinin (HA), NP, NA, matrix (M) and non-structural (NS)) have been produced. However, because of the requirement for helper virus, and thus the difficulty of selecting virus containing a transfected gene, this method had limitations.

4. GENERATION OF INFLUENZA A VIRUS ENTIRELY FROM CLONED cDNAs

A decade after the generation of influenza A virus containing a single gene derived from cloned cDNA, we (Neumann *et al.* 1999), and shortly thereafter Fodor *et al.* (1999), succeeded in synthesizing influenza A virus entirely from cloned cDNAs. We cloned cDNAs encoding all eight vRNAs of A/WSN/33 virus between the human RNA polymerase I promoter and mouse RNA polymerase I terminator (Neumann *et al.* 1999). Transfection of the resulting plasmids into 293T cells yields vRNA synthesized by cellular RNA polymerase I. Cotransfection of protein-expression plasmids for all viral structural proteins yields 8×10^7 infectious viruses ml⁻¹ supernatant, one of the most efficient among reverse genetics systems. Infection with helper virus is no longer required, circumventing the need for cumbersome selection of transfectant viruses. Thus, for the first time, researchers have available a system for designing influenza virus. The system is simple, since it only requires DNA cloning, purification, and transfection techniques—methods that are well established in molecular biology and virology laboratories.

5. USE OF REVERSE GENETICS FOR PATHOGENESIS STUDIES OF HONG KONG H5N1 INFECTION

In 1997, H5N1 influenza virus was directly transmitted to humans from birds, infecting a total of 18 people, six of whom died (Claas *et al.* 1998; Subbarao *et al.* 1998). Although all of the H5N1 isolates from humans have a series of basic amino acids at the HA cleavage site (a known structural feature for high virulence in birds), they are divided into two groups based on virulence in mice (Gao *et al.* 1999; Katz *et al.* 2000). To determine the genetic factors responsible for the difference in virulence between highly pathogenic A/Hong Kong/483/97 (HK483) and low pathogenic A/Hong Kong/486/97 (HK486) viruses, we attempted to generate the HK483 and HK486 viruses entirely from cloned cDNA using plasmid-based reverse genetics (Neumann *et al.* 1999). We constructed plasmids that contain cDNA for the full-length vRNA of HK483 and HK486 viruses between RNA polymerase I promoter and terminator (referred to as PolI plasmids) and then transfected 293T cells with four protein-expression plasmids of A/WSN/33 virus and eight PolI plasmids for the production of vRNA segments that encoded HK483 or HK486 viral genes. The HK483

and HK486 transfectant viruses, designated HK483RG and HK486RG, were generated and inoculated into MDCK cells to produce virus stocks. Using this system, we made a series of reassortant viruses between HK483 and HK486 and tested their virulence in mice.

The results indicated that amino acid 627 of the PB2 protein is important for expressing virulence in these two viruses (Hatta *et al.* 2001). There are eight amino-acid differences between the PB2 proteins of HK483 and HK486 viruses. Further studies may pinpoint amino-acid residues responsible for the difference in virulence between these two viruses and reveal the molecular mechanism by which avian influenza viruses exhibit high virulence in mammals.

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