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Improved dual promotor-driven reverse genetics system for influenza viruses

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Reverse genetic systems for influenza A virus (IAV) allow the generation of genetically manipulated infectious virus from a set of transfected plasmid DNAs encoding the eight genomic viral RNA segments (vRNA). For this purpose, cDNAs representing these eight vRNA segments are cloned into specific plasmid vectors that allow the generation of vRNA-like transcripts using polymerase I (Pol I). In addition, these plasmids support the transcription of viral mRNA by polymerase II (Pol II), leading to the expression of viral protein(s) encoded by the respective transcripts. In an effort to develop this system further, we constructed the bi-directional vector pMP*ccd*B. It is based on pHW2000 (Hoffmann et al., 2000b) but contains additionally (i) the *ccd*B gene whose expression is lethal for most *Escherichia coli* strains and therefore used as a negative selection marker and (ii) more efficient *AarI* cloning sites that flank the *ccd*B gene on either side. Furthermore, we used a modified one-step restriction/ligation protocol to insert the desired cDNA into the respective pMP*ccd*B vector DNA. Both the use of a negative selection marker and an improved cloning protocol were shown to facilitate the generation of genetically engineered IAV as illustrated in this study by the cloning and rescue of the 2009 pandemic isolate A/Giessen/6/2009 (Gi-H1N1).

1. Introduction

Reverse genetic systems for influenza A viruses (IAVs) have become an important technology for the generation of recombinant IAVs from cloned cDNA derived from viral genomic RNA segments (Fodor et al., 1999; Neumann et al., 1999). These systems are now widely used to generate recombinant or reassortant viruses for vaccine production and are excellent tools for the characterization and/or prediction of biological properties of viruses containing specific genetic changes. For example, reverse genetic studies provided important information for assessing the potential threat caused by the pandemic H1N1 (2009) virus (Sakabe et al., 2011; Ye et al., 2010; Zhao et al., 2011). Although the worldwide pandemic caused by the 2009 H1N1 virus came to an end in October 2010 and effective vaccines are now available, the virus remains a potential threat. It is likely to circulate for a few more years and, thus, the emergence of new variants with unexpected properties cannot be excluded (WHO, 2010).

The IAV genome consists of eight single-stranded negative-sense RNA segments (Bouvier and Palese, 2008). The viral RNA (vRNA) is associated with the nucleoprotein (NP) and three polymerase subunits (PB1, PB2, and PA), thus forming the viral ribonucleoprotein (vRNP) complex, which is the minimal replication/transcription unit of the virion (Honda and Ishihama, 1997). While four viral RNA segments (PB2, HA, NP and NA) contain a single open reading frame (ORF) from which the respective proteins are expressed, the other four RNA segments (PB1, PA, M and NS) comprise two ORFs from which the following virus proteins are expressed: PB1 and PB1-F2 (PB1), PA and PA-X (PA), M1 and M2 (M), NS1 and NEP/NS2 (NS) (Chen et al., 2001; Jagger et al., 2012; Lamb and Horvath, 1991). The functional ORFs present on the eight segments are flanked by 3′- and 5′-terminal non-coding regions (NCRs) which play an important role in IAV replication. The NCRs contain 12 and 13 highly conserved terminal nucleotides at the 3′ and 5′ ends, respectively (Desselberger et al., 1980) and are generally used to design universal primers for full-length amplification of the eight IAV vRNAs (Hoffmann et al., 2001).

Over the past few years, several efficient systems for the generation of recombinant IAV have been developed. In the unidirectional system, the cloned IAV cDNAs are preceded by a human

Abbreviations: CEF, Chicken embryo fibroblast; BGH, Bovine growth hormone; rg, recombinant generated; Pdm, Pandemic; FFU, Focus-forming units.

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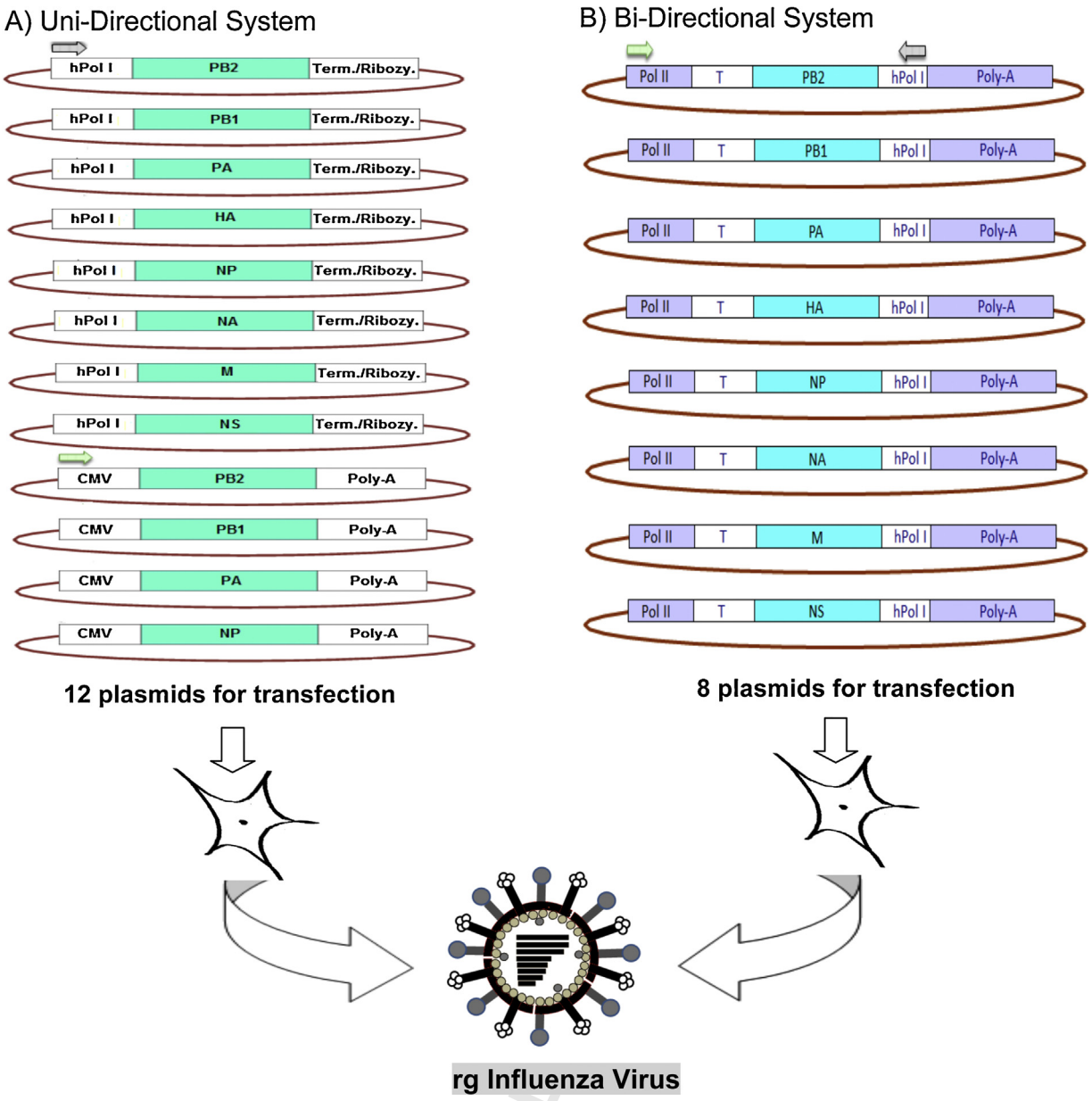

Fig. 1. Schematic representation of the plasmid-driven reverse genetics system for the rescue of influenza A viruses. (A) The uni-directional reverse genetics system in which eight Pol I-driven plasmids for the generation of the vRNA segments are transfected along with four Pol II-driven plasmids expressing PB2, PB1, PA and NP. (B) The dual promotor reverse genetics system in which eight Pol I–Pol II-driven plasmids for the generation of the vRNA and mRNA are transfected to rescue recombinant influenza A virus (rg Influenza Virus).

RNA polymerase I (Pol I) promoter that drives the synthesis of non-capped and non-polyadenylated vRNA-like transcripts with a defined 5′ end. To generate viral RNAs with defined 3′ ends, a hepatitis delta virus ribozyme sequence (Fodor et al., 1999; Pleschka et al., 1996) or a mouse Pol I-terminator (Neumann et al., 1999) is used. To rescue IAV with the uni-directional system, a total of eight plasmids required to generate all the vRNA segments are transfected along with four helper plasmids expressing PB2, PB1, PA and NP, which are essential for vRNP complex formation (Fodor et al., 1999; Neumann et al., 1999; Pleschka et al., 1996) (Fig. 1A). More recently, a bi-directional system was developed in which the number of plasmids to be transfected was reduced from 12 to 8. The latter system employs the human Pol I-promoter/murine Pol I-terminator for vRNA synthesis and a cytomegalovirus (CMV) Pol II-promoter/BGH polyadenylation site (PA-site) for viral mRNA synthesis (Hoffmann et al., 2000a,b) (Fig. 1B). To produce recombinant IAV for human vaccine production, a limited number of mammalian cell lines, such as Vero-, CEF- and MDCK cells, are recommended for transfection (Massin et al., 2005; Murakami et al., 2008; Ozaki et al., 2004). The rescue efficiency of these cell lines using 8 uni-directional plasmids along with 4 helper plasmids or 8 bi-directional plasmids is limited due to the relatively low efficiency of the simultaneous transfection of 8–12 plasmids and the host specificity of the human Pol I-promoter (Murakami et al., 2008; Neumann et al., 2005). To overcome this obstacle, multiple cloning site vectors have been developed that are suitable to insert the cDNAs of all eight viral segments into just one Pol I-driven vector or into two independent Pol I-driven vectors, one of which containing the HA- and NA-segments and the other containing the remaining six vRNA segments. These multi-segment-containing plasmids are then transfected along with the helper plasmids (Neumann et al., 2005). In another approach, bi-directional IAV reverse genetic systems based on either the canine Pol I-promoter (to be used in MDCK cells) (Murakami et al., 2008) or chicken Pol I-promoter (to

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