Establishment of Vero cell RNA polymerase I-driven reverse genetics for *Influenza A virus* and its application for pandemic (H1N1) 2009 influenza virus vaccine production

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The constant threat of newly emerging influenza viruses with pandemic potential requires the need for prompt vaccine production. Here, we utilized the Vero cell polymerase I (Poll) promoter, rather than the commonly used human Poll promoter, in an established reverse-genetics system to rescue viable influenza viruses in Vero cells, an approved cell line for human vaccine production. The Vero Poll promoter was more efficient in Vero cells and demonstrated enhanced transcription levels and virus rescue rates commensurate with that of the human RNA Poll promoter in 293T cells. These results appeared to be associated with more efficient generation of A(H1N1)pdm09- and H5N1-derived vaccine seed viruses in Vero cells, whilst the rescue rates in 293T cells were comparable. Our study provides an alternative means for improving vaccine preparation by using a novel reverse-genetics system for generating influenza A viruses.

Annualy, an estimated 20,000-36,000 deaths associated with seasonal influenza are caused by widespread outbreaks in the USA (Thompson et al., 2003). In addition, a novel influenza virus to which the majority of the human population was immunologically naive began circulating in April 2009 and spread rapidly worldwide (Itoh et al., 2009). The World Health Organization (WHO) immediately declared a pandemic for the first time in the 21st century, and many countries began to prepare influenza vaccines to prevent virus dissemination. Finally, the causative virus was controlled, and the pandemic status was lifted by the WHO in August 2010. We are now in the post-pandemic period, meaning that the H1N1 virus will act as a seasonal influenza virus and will continue to circulate for years to come (WHO, 2010).

For the prompt production of influenza vaccines with appropriate antigenic variants [haemagglutinin (HA) and neuraminidase (NA) proteins], the seed viruses are typically generated using a reverse-genetics (RG) method in the backbone of the A/Puerto Rico/8/34 (PR8; H1N1) virus in Vero cells, an approved cell line for human vaccine production by the WHO (WHO, 1998, 2005). The Vero cell line is the continuous cell line most widely accepted by regulatory authorities and has been used for over 30 years for the production of polio and rabies vaccines (Frazzati-Gallina et al., 2001; Montagnon et al., 1984). The recent licensure of a Vero cell-derived live virus vaccine (ACAM200, smallpox vaccine) has coincided with heightened interest in the development of new viral vaccines, from live-attenuated to inactivated whole-virus vaccines, against influenza viruses (Barrett et al., 2009; Keitel et al., 2009). However, generating a vaccine seed virus, such as the 2009 pandemic H1N1 [A(H1N1)pdm09] influenza virus, in a Vero cell line is not optimal because of the low DNA transfection efficiency (Neumann et al., 2005) and perhaps because of the lower specificity of the human RNA polymerase I (Poll) promoter in the RG system. Based on the need for improved virus rescue efficiency in Vero cells, we developed a modified RG system driven by a Vero cell-derived RNA Poll promoter instead of the human RNA Poll promoter.

Eukaryotic rDNA contains 18S, 5.8S and 28S rRNA genes as clusters of head-to-tail repeats. The transcribed units are separated by intergenic spacers, which include the RNA Poll promoter and other transcription-related sequence elements found in most species. A core promoter located in the region ~50 bp upstream of the initiation site (+1) is necessary, and an additional region 150–200 bp upstream of +1 plays a key role in transcription (Paule & White, 2000). To screen the Vero cell-derived RNA Poll promoter region, PCR fragments of the peripheral regions of the Poll promoter were amplified based on the sequence of the complete human rDNA repeating unit (GenBank accession no. U13369.1) (Murakami et al., 2008). Several sequences corresponding to hypothetical promoter regions were identified (97–99% nucleotide sequence identity) and...
aligned with the human RNA PolI promoter region and the *Macaca mulatta* unplaced genomic scaffold (Mmul_051212, GenBank accession no. NW_001149581.1) (bearing 79–80.7 and 91–92.3 % nucleotide sequence identities, respectively) to compare the homology of the transcription initiation sites (Fig. 1a, b). Although the Vero cell line was derived from the kidney cells of an African green monkey, sequence information that matches this cell line is unavailable in GenBank. Therefore, we compared our sequences with the non-human primate (*M. mulatta*) that showed the highest identity. The result of the comparison of the putative Vero cell PolI promoter region showed 92.3 % and 80.7 % nucleotide identity with the *M. mulatta* and human RNA PolI promoter sequences, respectively (Fig. 1c). Two nucleotide differences were observed around the transcription initiation site of the Vero promoter (one deletion and one difference at nt −2 and +8), compared with the human promoter (Fig. 1a, b).

The human RNA PolI promoter in the plasmids pHW72-EGFP and pHW72-luciferase, which have been used to assess polymerase activities (Hoffmann et al., 2000a; Salomon et al., 2006; Song et al., 2009b), was replaced with the Vero cell RNA PolI promoter (named vpHW72-EGFP and vpHW72-Luci, respectively) to determine whether this promoter functioned as we expected in Vero cells. Based on their high nucleotide sequence identities to the human counterpart, two different-sized promoters (326 bp, nt −1 to −326, and 212 bp, nt −1 to −212) upstream of the transcription initiation site of the Vero promoter were selected and amplified with specific primer pairs designed based on our sequence information, and were exchanged with the human RNA PolI promoter region (225 bp), which was present in the original vectors (Hoffmann et al., 2000b). The ORFs for the viral ribonucleoprotein (vRNP) complex [comprising the polymerase subunits PB2, PB1 and PA and the nucleoprotein (NP)] genes of PR8 virus were additionally cloned into a pcDNA3.1 myc-His (+) vector (Invitrogen). To determine whether the Vero cell-derived RNA PolI promoter could functionally enhance viral RNA synthesis in vitro, two Vero cell-derived RNA PolI promoter plasmids, vpHW72_212-EGFP and vpHW72_326-EGFP, were co-transfected with the vRNP complex plasmids described above into 293T and Vero cells using the jetPEI (Polyplus Transfection) DNA transfection reagent. The pHW72-EGFP plasmid with the vRNP complex plasmids and vpHW72_212-EGFP or vpHW72_326-EGFP alone were used as positive and negative controls, respectively. At 24 h post-transfection (p.t.), transcription efficiency in both cells was observed by fluorescence microscopy (JP/E200POL; Nikon). The expression of GFP driven by the predicted Vero RNA PolI promoter (from both vpHW72_212-EGFP and vpHW72_326-EGFP) was clearly observed in Vero cells (Fig. 2b). Interestingly, the level of GFP expression driven by the Vero RNA PolI promoter in 293T cells was similar to the expression level driven by the human RNA PolI promoter in this cell line (Fig. 2a). vpHW72_212-EGFP transfection alone did not result in visible GFP expression (Fig. 2a, b). These results clearly demonstrated that the Vero RNA PolI promoter regions that we identified in this study were functional in Vero and 293T cells.

To further compare the transcriptional efficacy of the Vero cell-specific RNA PolI promoter with that of the original human RNA PolI promoter system, we used a luciferase reporter gene assay, as described previously (Song et al., 2009b). The vpHW72_212-Luci and vpHW72_326-Luci plasmids containing the Vero RNA PolI promoters or the pHW72-Luci vector alone was transfected along with the PR8 vRNP complex plasmids into 293T and Vero cells. pCMV-β-gal plasmids containing the reporter gene β-galactosidase were used for normalization, and a negative control was included in which only the vpHW72_212-Luci vector was used. After 24 h of incubation, the cells were lysed, and the amount of luciferase in the lysates was measured using a Promega luciferase assay system and standardized to the β-galactosidase expression level. The standardized relative luciferase activities in the 293T lysates exhibited no significant differences among the samples transfected with vectors encoding different promoters (Fig. 2c). However, when luciferase activities were assayed in Vero and COS-1 cells, both of which are African green monkey kidney-derived cell lines, transfection with the vpHW72_212-Luci construct was increased by 1.7-fold ($P<0.05$) and 1.65-fold ($P<0.05$), respectively, relative to the cells transfected with the human RNA PolI promoter-based pHW72-Luci plasmid (Fig. 2d, e). Thus, the Vero RNA PolI promoters we identified, particularly the 212 bp, were more efficient in these non-human primate-derived cell lines and were also able to enhance transcription levels as high as that of the human RNA PolI promoter in 293T cells. For comparison, the vpHW72_326 plasmids also induced elevated polymerase activities in all cell lines but did not reach significant levels ($P>0.05$).

To adapt this new promoter to an established RG rescue system used for influenza A viruses (Paule & White, 2000), all eight viral segments of the PR8 virus were individually cloned into the pHW2000 plasmid vector modified to contain the 212 bp Vero RNA PolI promoter (pPolIv212) instead of the conventional human RNA PolI promoter. In this study, the 212 bp promoter was utilized due to its substantially higher efficiency over the 326 bp promoter in the luciferase activity assay. The eight plasmids, pPolIv212-PB2, -PB1, -PA, -HA, -NP, -NA, -M (matrix) and -NS (non-structural), of PR8 were co-transfected into 293T and Vero cells. In parallel, corresponding pHW2000-PR8 plasmids were also co-transfected in another set of cells to compare the virus rescue efficiencies. At 30 h p.t., 0.5 μg t-1-tosylamido-2-phenyl-ethyl chloromethyl ketone-treated trypsin (Sigma–Aldrich) ml$^{-1}$ was added to the transfected cells. Supernatants of the rescued samples were harvested at 48 and 72 h p.t., and titrations of the rescued viruses were performed in 10-day-old embryonic chicken eggs to determine the 50 % egg infectious dose (EID$_{50}$) ml$^{-1}$. No significant difference in
rescue efficiency was found in 293T cells between the two different promoter-based systems at either time point (Fig. 3a). Viruses were rescued in three of four trials when the human RNA PolI promoter was used in Vero cells; viruses were successfully rescued in all trials when the Vero RNA PolI promoter was used (Fig. 3b). Additionally, the mean viral titre for the viruses produced using the Vero RNA PolI promoter was two to three times higher than that for viruses produced using the human RNA PolI promoter at the two time points of recombinant virus recovery. These results indicated that the pPolIV212-PR8 plasmids, which included the Vero cell-derived RNA PolI promoter, generated the recombinant PR8 virus more efficiently than the standard human RNA PolI system in Vero cells.

To use the Vero RNA PolI promoter-driven system to produce A(H1N1)pdm09 vaccine seed viruses, which are increasingly necessary for the rapid development of appropriate vaccines for pandemics, we cloned the HA...
and NA genes of A/Korea/CJ01/09 (CJ01, H1N1) (Song et al., 2010) into a pPollv212 plasmid vector (pPollv212-CJ01HA and -CJ01NA), and virus rescue was performed in the backbone of the pPollv212-PR8 plasmids in Vero cells. As a positive control, the CJ01 pHW2000-HA and pHW2000-NA genes were also rescued in the background of the pHW2000-PR8 plasmid for comparison. Virus was successfully recovered in two of four trials using the human RNA PolI promoter (pHW2000-PR8 plasmids). However, viruses were rescued in three of four trials using the Vero RNA PolI promoter (pPollv212-PR8 plasmids), and the rescued viruses had nearly two-fold higher titres (Fig. 3c).

In a comparable manner, a recombinant H5N1 pPollv212-DW149 virus bearing the HA and NA of A/environment/Korea/W149/2006 (H5N1) (Song et al., 2009a) was also cumulatively recovered in three of four trials (Fig. 3d). Taken together, these data led us to conclude that the A(H1N1)pdm09/PR8 and H5N1/PR8 viruses could be generated more efficiently by the Vero RNA PolI-driven promoter RG system than by the corresponding human-derived promoter in Vero cells.

Recently, the need for rapid production of vaccines against novel and highly pathogenic influenza viruses has increased following the first pandemic of the 21st century. Numerous studies have focused on the development of alternative virus-generating systems or on improving the efficiency of virus rescue in a short time frame (de Wit et al., 2007; Horimoto et al., 2007; Massin et al., 2005; Murakami et al., 2008; Neumann et al., 2005). Vero cells have been approved for the production of human vaccines consisting...
of whole viruses and can be scaled up to 6000 l without loss of cell viability or virus productivity due to the robust nature of this system (Kistner et al., 2007). In addition to Vero cells, a number of other cells have recently been approved for human vaccine production, such as Madin–Darby canine kidney (MDCK) and chicken embryonic fibroblast (CEF) cell lines. Accordingly, modified RG systems that use the chicken and canine RNA PolI promoters have also been established to generate influenza virus vaccines in CEF and MDCK cells (Massin et al., 2005; Murakami et al., 2008). Although the Vero cell line has been approved for the production of many human vaccines, including influenza vaccines (Brands et al., 1999; Kistner et al., 1998), the RG system based on the human RNA PolI promoter does not work efficiently in Vero cells. Although transfection inefficiency could be attributed to this, the low sequence identity (80.7 % nucleotide identity) between the human and Vero RNA PolI promoters shown here might also contribute to the impaired virus rescue, possibly by affecting the specificity of the human RNA PolI in Vero cells. Our results indicate that the Vero cell RNA PolI-based system is more efficient than the human RNA PolI-based system in Vero cell lines, whereas comparable in vitro transcription and virus rescue

Fig. 3. Comparison of the virus rescue efficiency between the human and Vero RNA PolI promoter systems in 293T and Vero cells. (a, b) Virus rescue efficiency of the PR8 virus in 293T (a) and Vero (b) cells. Each of the eight pHW2000-PR8 or pPolIv212-PR8 plasmids was transfected into 293T or Vero cells, and the supernatant of each well was harvested at 48 and 72 h p.t. for propagation. (c, d) Rescue efficiency of the pandemic 2009/PR8 (c) and highly pathogenic avian influenza H5N1/PR8 vaccine seed virus (d) in Vero cells. Plasmids containing the HA and NA genes from A/Korea/CJ01/09 or A/environment/Korea/W149/2006, and the internal genes of PR8 virus in the pHW2000 or pPolIv212 vector were transfected into Vero cells, and the supernatants were harvested at 48 and 72 h p.t. The results of individual rescue trials are presented in (b)–(d). All the collected samples were titrated in 10-day-old embryonated chicken eggs, and expressed as EID50 ml⁻¹. Horizontal dash lines indicate limit of virus detection (2.5e⁻¹ EID50/ml).
efficiency was observed in 293T cells. Therefore, these data indicate that this new system could also be a useful tool for research purposes. Application of the Vero RNA PolI promoter-driven RG system in Vero cells may improve or provide an alternative means for the prompt production of seasonal, pre-pandemic and pandemic influenza vaccines.

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References


