Virus Research 197 (2015) 59–66

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Construction and characterization of novel fowlpox virus shuttle vectors

Shouwen Du[a,b,c,1], Cunxia Liu[b,1], Yilong Zhu[b], Yuhang Wang[b], Dayong Ren[b], Maopeng Wang[b], Peng Tan[b], Xiao Li[b], Mingyao Tian[b], Yanfang Zhang[b], Jinze Li[b], Fei Zhao[b], Chang Li[b,c,**], Ningyi Jin[b,c,*]

[a] College of Veterinary Medicine, Jilin University, Changchun 130062, China
[b] Key Laboratory of Jilin Province for Zoonosis Prevention and Control, Institute of Military Veterinary, Academy of Military Medical Sciences, Changchun 130122, China
[c] Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou 225009, China

ARTICLE INFO

Article history:
Received 21 September 2014
Received in revised form
11 December 2014
Accepted 11 December 2014
Available online 18 December 2014

Keywords:
Fowlpox virus 282E4 strain
Shuttle vectors
Morphogenesis
Genetic stability

ABSTRACT

Viral vectors are important vehicles in vaccine research. Avipoxviruses including fowlpox virus (FPV) play major roles in viral vaccine vector development for the prevention and therapy of human and other veterinary diseases due to their immunomodulatory effects and safety profile. Recently, we analyzed the genomic and proteomic backgrounds of the Chinese FPV282E4 strain. Based on analysis of the whole genome of FPV282E4, the FPV150 and FPV193 loci were chosen as insertion sites for foreign genes, and two shuttle vectors with a triple-gene expression cassette were designed and constructed. Homologous recombination between the FPV virus genome and sequences within the shuttle plasmids in infected cells was confirmed. The recombinants were obtained through several rounds of plaque purification using enhanced green fluorescent protein as a reporter and evaluated for the correct expression of foreign genes in vitro using RT-PCR, real-time PCR and Western blotting. Morphogenesis and growth kinetics were assayed via transmission electron microscopy and viral titering, respectively. Results showed that recombinant viruses were generated and correctly expressed foreign genes in CEF, BHK-21 and 293T cells. At least three different exogenous genes could be expressed simultaneously and stably over multiple passages. Additionally, the FPV150 mutation, FPV193 deletion and insertion of foreign genes did not affect the morphogenesis, replication and proliferation of recombinant viruses in cells. Our study contributes to the improvement of FPV vectors for multivalent vaccines.

1. Introduction

Edward Jenner pioneered the field of virology and vaccinology with his remarkable discovery that inoculation with the cowpox virus from lesions of an infected milkmaid conferred immunity to prevent or resist smallpox (Moss, 1996). *Poxviridae* is a large and complex family of DNA viruses, which share a conserved genetic structure. They have linear, double-stranded DNA genomes, which range widely in size, varying from 135 kb to greater than 300 kb. Thus, poxvirus-based vectors have a sizeable cloning capacity to accept large inserts of foreign genes (Barrett and McFadden, 2008). Moreover, they replicate and undergo morphogenesis in the cytoplasm of infected cells, thereby avoiding the risk of mutagenesis by integration or random insertion of viral genes into the infected cell genome (Roberts and Smith, 2008). Vaccinia virus is an attenuated virus capable of replicating in human and other mammalian cells, but it has toxic side effects especially in severely immunocompromised patients. To circumvent this problem, an attenuated vaccinia virus with deletion of some host range or virulence-related genes was developed for high-risk individuals (Sanchez-Sampedro et al., 2013; Wang et al., 2012).

Poxviruses that have been developed as vectors for vaccines or gene therapies in clinical and veterinary applications include modified vaccinia virus strains such as modified vaccinia Ankara (MVA) and NYVAC, as well as avipoxviruses (APVs) such as fowlpox

* Corresponding author at: Liuying West Road 666, Room 423, Jingyue Economic & Technological Development Zone, Changchun, Jilin 130122, China. Tel.: +86 0431 86985929; fax: +86 0431 86985861.
** Corresponding author at: Liuying West Road 666, Room 429, Jingyue Economic & Technological Development Zone, Changchun, Jilin 130122, China. Tel.: +86 0431 86985923; fax: +86 0431 87985923.
E-mail addresses: lichang78@163.com (C. Li), ningyij@hotmail.com (N. Jin).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.virusres.2014.12.015
0168-1702/

Table 1
Primers used in PCR and real-time PCR assays.

Gene	Primer sequence	Size of product	Description
FPV150L[a]	Forward: 5′-ggacgcgtattgattcacaccgtattacagagg-3′ Reverse: 5′-ggctgcagagcaaatagatgggaacgcgctgat-3′	443 bp	To generate left arm of FPV150 insertion sites
FPV150R[a]	Forward: 5′-cgccgcgggaaaatagaagatgtatggcacgat-3′ Reverse: 5′-cgcccgggttctcctaataagttacaccgtttg-3′	589 bp	To generate right arm of FPV150 insertion sites
FPV193L[a]	Forward: 5′-ggacgcgtataatagtttctcctcactta-3′ Reverse: 5′-ggctgcagttagtatatgctgttatatctaaag-3′	405 bp	To generate left arm of FPV193 insertion sites
FPV193R[a]	Forward: 5′-cgccgcggataagatataaaaaaataaaatgga-3′ Reverse: 5′-cgcccgggcttatcatcatcgtatattctttct-3′	758 bp	To generate right arm of FPV193 insertion sites
FPV150[a]	Forward: 5′-ggacgcgtattgattcacaccgtattacagagg-3′ Reverse: 5′-cgcccgggttctcctaataagttacaccgtttg-3′	1006 bp	To identify recombinant virus rFPV150 or rFPV193
FPV193[a]	Forward: 5′-gctaatagacaaataaaagagtg-3′ Reverse: 5′-agaaggtaatagtaacaaaacata-3′	981 bp	
EGFP[a]	Forward: 5′-atcgatcgatggtgagcaagggcgaggag-3′ Reverse: 5′-gcgtcgacttacttgtacagctcgtccatg-3′	720 bp	
EGFP[b]	Forward: 5′-catcttcttcaaaggacgacg-3′ Reverse: 5′-tgaagtcgatgcccttcag-3′	101 bp	For quantitation of gene expression
P4b[a]	Forward: 5′-ggacgcgtcagcaggtgctaaacaacaa-3′ Reverse: 5′-ggctgcagcggtagcttaacgaagaata-3′	578 bp	For positive control of FPV282E4
β-actin[b]	Forward: 5′-gagaccttcaacaccccagc-3′ Reverse: 5′-atgtcacgcacaatttctc-3′	263 bp	For internal control for real-time PCR

[a] PCR primer.
[b] Real-time PCR primer.

virus (FPV) or canarypox virus (Remy-Ziller et al., 2014; Teigler et al., 2014). APVs including FPV exhibit strict host range restriction to avian species and undergo abortive infection in mammalian cells (Taylor and Paoletti, 1988; Taylor et al., 1992; Weli and Tryland, 2011). However, when used as vaccine vectors, they can correctly express foreign genes in mammalian cells and elicit long-lasting immune responses in immunized hosts (Franchini et al., 2004; Skinner et al., 2005; Srinivasan et al., 2006). These characteristics and their ability to accommodate multiple gene inserts make APVs promising gene therapy and vaccine vectors for diseases caused by pathogenic microorganisms and cancers. Among them, FPV is of great interest as a vehicle, and various exogenous genes including antigens, costimulatory molecules and cytokines transferred by this vector have been shown to be expressed in infected cells (Solomon et al., 2005; Srinivasan et al., 2006; Weli and Tryland, 2011). FPV-based recombinants have been evaluated as vaccine vectors against certain infectious diseases in animal models and clinical trials (Emery et al., 2005; Kaufman et al., 2014). Since the 1990s, our laboratory has carried out numerous studies on FPV vectors and their applications for vaccines, including FPV genomic cleavage map analysis, transfer plasmid vectors and gene expression, and immune responses to recombinants with antigen genes in *mice*, *chickens*, *pigs* or *monkeys* (Jiang et al., 2005; Li et al., 2006, 2012; Ma et al., 2008; Mingxiao et al., 2006).

The genomic background of FPV282E4 was analyzed in our laboratory previously, and a series of transfer plasmids such as pUTA2, pUTAL and pTKE3 were constructed to prepare the recombinant FPV (rFPV). However, some problems still remain in practice, such as low recombination efficiency and a long screening period for recombinant viruses. Therefore, we parsed the whole-genome sequence of the FPV282E4 strain (unpublished data) in order to choose suitable insertion sites for vector design. The present study focuses on the creation of FPV transfer vectors and their applications in generating multivalent vaccines displaying multiple antigen genes.

2. Materials and methods

2.1. Cells, virus strains and plasmids

Primary chicken embryo fibroblasts (CEF) from specific-pathogen-free (SPF) chickens were maintained in Dulbecco's modified Eagle medium (DMEM, HyClone, China) supplemented with 10% (v/v) fetal bovine serum (FBS, HyClone, USA). The 282E4 strain of FPV (FPV282E4) is an attenuated vaccine produced by the Animal Pharmaceutical Factory of Nanjing (Nanjing, China). FPV was prepared and cultured in CEF cells in DMEM supplemented with 10% FBS. The plasmids pV-BFP, pRFP and pV-EGFP were stored in our laboratory. Blue fluorescent protein (BFP) and red fluorescent protein (RFP) were synthesized according to sequences reported in the NCBI database, NM_202581.2 and HQ148301.1, respectively. The enhanced green fluorescent protein (EGFP) came from pEGFP-C1 (Clontech).

2.2. Selection and design of insertion sites for foreign genes

To construct a rFPV virus with exogenous genes, the FPV150 (130,366–131,016 bp, ortholog of vaccinia virus thymidylate kinase gene) and FPV193 (186,335–188,722 bp, ortholog of canarypox virus 134 coding variola B22R-like protein) were selected as insertion sites based on results of the whole-genome sequence analysis of the FPV282E4 strain (unpublished data). The FPV DNA sequences FPV150L/150R and FPV193L/193R were then amplified with their specific primers (Table 1), synthesized as recombinant arms and cloned into the pMD18-T Simple vector (Takara) as backbone plasmids designated as pFPV150 and pFPV193 (Fig. 1), respectively.

2.3. Design and synthesis of a triple-gene expression cassette

To facilitate the insertion of multiple foreign genes into the FPV genome, a new expression cassette containing three independent genes was designed based the on vaccinia virus early/late promoter ($P_{E/L}$), terminator T_5NT, multi-cloning sites (MCS)