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Comparative transcriptional activity of five promoters in BAC-cloned MDV for the expression of the hemagglutinin gene of H9N2 avian influenza virus

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A B S T R A C T

On the basis of recent studies, much attention has been given to recombinant MDV (rMDV)-based vaccines. During the construction of rMDV, the activity of promoters to transcribe foreign genes is one of the major factors that can affect protective efficacy. To investigate the transcription activity and efficacy of five different promoters, the advantage of an existing rMDV BAC infectious clone that had been previously constructed was used to construct rMDVs. The expression cassette of the hemagglutinin gene (HA) from a low pathogenic avian influenza virus (LPAIV) H9N2 strain was inserted into the US2 region under five selected promoters. These five promoters included three MDV endogenous promoters (the promoter for the gB gene and a bi-directional promoter in both directions for pp38 (ppp38) and 1.8 kb RNA transcripts (p1.8 kb)), and two exogenous promoters (CMV and SV40). Among these five promoters, the CMV promoter demonstrated the highest activity, followed by p1.8 kb and SV40, which had a similar transcriptional activity level. Two of the MDV endogenous promoters showed much lower transcriptional activities, particularly the promoter ppp38, which had the lowest activity. The results of the in vivo experiment proved that none of the three recombinant viruses of rGX-CMV-HA, rGX-SV40-HA and rGX-p1.8kb-HA provided protection in SPF chickens. Chickens vaccinated with rGX-pPP38-HA induced 50% and rGX-gB-HA induced 25% protection against the challenge with H9N2, respectively.

1. Introduction

Within the last two decades, recombinant virus vector has been a popular tool in the development of vaccines. Infectious Laryngotracheitis virus (ILTV), Fowlpox virus (FPV), Newcastle disease virus (NDV) and Marek's disease virus (MDV) have been commonly used as vectors to construct recombination viruses expressing other foreign genes (Tsukamoto et al., 1999; Swayne et al., 2000; Lüschow et al., 2001; Qiao et al., 2003; Veits et al., 2006; Pavlova et al., 2009). Among these viruses, MDV of different serotypes has been the most useful vector for polyvalent vaccines (Heckert et al., 1996; Reddy et al., 1996; Sakaguchi et al., 1998), and one recombinant HVT (Herpesvirus of turkeys) expressing the Infectious Busal Disease virus (IBDV) VP2 gene is licensed and available commercially (Darteil et al., 1995; Tsukamoto et al., 2002; Bublot et al., 2007). The advantages of using MDV as vectors include the following: (a) recombinant viral vaccines have the potential to induce lifetime protection against Marek's Disease (MD) with just one vaccination; (b) these viruses have a natural host that is limited to avian species, and both humoral (Sonoda et al., 2000) and cellular immune responses against pathogens can be induced in animals (Sonoda et al., 1996); (c) MDV has a large genome, which enables the simultaneous expression of multiple foreign antigens; and (d) different techniques for generating recombinant MDV infectious clones have been well established (Sakaguchi et al., 1994; Sonoda et al., 2000). Recombinant MDV has been successfully developed in all three serotypes. Many of these rMDV clones have been used to express genes from avian influenza viruses (AIVs) to provide protection against both MDV and AIV (Gao et al., 2011; Li et al., 2011a; Cui et al., 2013).

Recently, it was reported that serotype 1 MDV (MDV-1)-vectored vaccine expressing Avian influenza (AI) virus H5 hemagglutinin induced better protection compared to HVT-vectored AI vaccine. Several MDV1-vectored recombinant vaccines

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expressing different foreign genes were generated and reported. Cui et al. (2013) generated an MDV-1(MDV-814 strain)-based recombinant virus that could express H5N2-HA. This virus induced 80% protection against highly pathogenic avian influenza virus (HPAIV), which is better than the protection provided by HVT (66.7%).

There are mainly two methods that are commonly used to construct recombination viruses. One is homologous recombination in eukaryotes (Darteil et al., 1995). The foreign gene expression cassette was flanked with homology arms of the recombinant site of MDV using molecular cloning technology. Next, the constructed plasmid containing the foreign gene expression cassette and homology arms was transfected into CEF cells, which were infected with MDV or co-transfected with BAC-cloned MDV into CEF cells. The recombinant viruses were then detected and purified with IFA using a monoclonal antibody. The other strategy is based on the bacterial artificial chromosome (BAC) system in a prokaryotic cell using the Red/ET recombinant system. Extracts from recombinant plasmids are transfected into eukaryotes to rescue the recombinant viruses (Petherbridge et al., 2003; Baigent et al., 2006). The genomes of MDVs are approximately 140–180 kb, and many regions are non-essential for virus replication in cell culture, which can be selected for the insertion and expression of foreign antigens (Cantello et al., 1991; Sakaguchi et al., 1993; Parcells et al., 1994, 1995; Sonoda et al., 1996). To construct recombinant MDVs and to express foreign antigens as protective vaccines, the insertion sites and the selection of promoters are two major factors that can influence protective efficacy. For example, it was reported that the expression of HA of H5N1-avian influenza virus (AIV) at the US2 insertion site of HVT induced better protection compared to the US10 insertion site, and the plaque formation and growth kinetics were also different in chicken embryo fibroblasts (CEFs) infected with the two recombination viruses, respectively (Gao et al., 2011). With respect to promoters, Tsukamoto et al. (2002) have reported two HVT recombinants (rHVT-cmvVP2 and rHVT-pecVP2) expressing IBDV-VP2 under the control of a human cytomegalovirus (CMV) promoter and CMV/β-actin chimera promoter (Pec promoter). rHVT-pecVP2 expressed approximately four times more VP2 antigen than rHVT-cmvVP2. Moreover, rHVT-pecVP2 provided complete protection, whereas rHVT-cmvVP2 only induced 58% protection (Tsukamoto et al., 2002).

In this study, a Meq-deleted MDV-1 strain GX0101ΔMeq (Li et al., 2011b) was used as a vector to express the HA gene of H9N2 of AIV under the control of five different promoters inserted into the same site. The transcription and expression levels of the HA gene were compared in cell cultures. And the protective efficacy of the five recombinant viruses against challenge with the H9N2 virus in chickens was also investigated. In addition to the CMV, the SV40 and MDV-1 gB promoters, a MDV-1's own bi-directional promoter in both directions (for pp38 and 1.8 kb RNA transcripts, respectively) (Shigekane et al., 1999; Ding et al., 2006) were also used and compared for their transcriptional activity in recombinant MDV-1.

2. Materials and methods

2.1. Viruses and promoters

The infectious BAC clone of recombinant MDV named GX0101ΔMeq was previously constructed by Li et al. (2011b). The complete coding region of the H9N2-HA gene was amplified from the LG1 strain. Chicken embryonic fibroblast cells (CEFs) were prepared from 10-day-old specific-pathogen-free (SPF) embryonic chicken eggs, which were used to transfect eukaryotic plasmids and to culture recombinants. The gB promoter, ppp38 and p1.8 kb, were cloned from the parental virus GX0101ΔMeq. The CMV promoter was cloned from pcDNA3.1 (Invitrogen, California, USA), and SV40 was obtained from pLenti/V5 (Invitrogen, California, USA). All of the recombination plasmids were constructed in the host *E. coli* EL250.

2.2. Primers

These recombination plasmids were constructed in prokaryotic cells, and recombinant viruses were rescued in eukaryotic cells. Different methods were used to detect these recombinants. The primer pairs used in this study are provided in Table 1.

2.3. Construction and confirmation of different expression cassettes in eukaryotic expression vectors using indirect immunofluorescence assays

Using the primer pair P1, which contains an Nhe I restriction site (underlined), and P2 containing a BamH I restriction site (underlined) as shown in Table 1, the complete coding region of the AIV-HA gene was amplified using RT-PCR from the H9N2-AIV viral RNA genome and then cloned into a eukaryotic expression vector pcDNA3.1, which contained the CMV promoter. The H9N2-CMV-HA expression cassette of 2.6 kb was then cloned into the PMD18-T vector with the primer pair P3, which contains Xba I/Apa I restriction sites (underlined) and P4. The completed plasmid was called T-CMV-HA. The SV40 promoter, gB promoter, ppp38 and p1.8 kb were amplified using PCR, and the CMV promoter was replaced using the Apa I/Nhe I restriction sites to construct various expression cassettes with different promoters, which were named T-SV40-HA, T-gB-HA, T-ppp38-HA and T-p1.8kb-HA, respectively.

To confirm the expression level of AIV-HA in each recombinant plasmid, 0.8 μg of recombinant plasmid (i.e., T-CMV-HA, T-SV40-HA or T-gB-HA) was used to transfect chicken embryonic fibroblast cells (CEFs) according to the instruction manual of Lipofectamine™ 2000 transfection reagent (Invitrogen, California, USA). It has been previously demonstrated by Ding et al. (2006) that expression of the pp38 and pp24 genes is essential transcription factor when foreign genes are expressed under the bi-directional promoters. Thus, the T-ppp38-HA or T-p1.8kb-HA plasmid was co-transfected with pBudCE4.1-pp38-pp24 (Jiang et al., 2005). The PMD18-T vector was used as a negative control. Transfected cells were then incubated in an incubator at 37 °C and 5% CO_2. Fresh culture medium was used to replace the transfection mix 6 h post-transfection, and cells were further incubated for 2 more days and evaluated using an indirect immunofluorescence assay (IFA). The wells were washed 3 times with phosphate-buffered saline (PBS) and fixed with ethanol for 15 min. The polyclonal anti-H9N2-HA chicken antiserum was prepared from SPF chicken vaccinated with H9N2 inactivated vaccine. Next, 1:100 diluted anti serum was applied as the primary antibody, and fluorescein isothiocyanate (FITC)-labeled goat anti-chicken immunoglobulin G (IgG, 1:300) was used to detect the positive signals. The results were analyzed using a fluorescence microscope.

2.4. Construction of infectious rMDVs expressing H9N2-HA driven by different promoters

To construct recombinant MDV carrying the HA gene expression cassette under a CMV promoter, rGX-cmv-HA, the Kan^+ expression cassette was first replicated from the pKD13 plasmid and cloned into the T-CMV-HA expression plasmid using Xba I and Apa I restriction sites (underlined) contained in the pair of primers F(Kan^+) and R(Kan^+), resulting in T-Kan^+-CMV-HA. To generate recombinant MDV carrying Kan^+-CMV-HA, P5 and P6 primers were designed to amplify the Kan^+-CMV-HA cassette flanked with 50-bp homology