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Foreign gene expression and induction of antibody response by recombinant fowl adenovirus-9-based vectors with exogenous promoters

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ABSTRACT

Fowl adenoviruses (FAdVs) are promising vectors for poultry vaccines and gene therapy. The commonly used human cytomegalovirus (CMV) promoter in recombinant FAdV-9 viruses (recFAdV-9s) leads to foreign gene expression that elicits an antibody response. Despite its strength, studies have shown that the CMV promoter is prone to silencing by methylation hampering the *in vivo* application of vectors containing this promoter. Therefore, to improve our virus vector system and circumvent potential limitations of silencing, we engineered recFAdV-9s with foreign gene expression cassettes carrying the CMV enhancer/chicken β-actin (CAG) or the human elongation factor 1 alpha (EF1α) promoters with or without the post-transcriptional regulatory element of woodchuck hepatitis virus (WPRE). Chicken hepatoma cells (CH-SAH) infected with recFAdV-9s carrying either CAG or EF1α promoters expressed higher levels of foreign protein than those infected with recFAdV-9 carrying the CMV promoter. Incorporation of the WPRE element rendered lower gene expression regardless of promoter type. Surprisingly, most chickens inoculated with recFAdV-9 containing the CMV promoter had the highest antibody response to foreign protein compared to other promoters. Our findings suggest the importance of promoter selection for candidate virus vector vaccines based on humoral immune response rather than foreign protein expression levels *in vitro*.

1. Introduction

Fowl adenoviruses (FAdVs) are distributed worldwide and some are associated with poultry diseases [1]. Previous studies have demonstrated their potential as vaccine vectors and gene delivery tools [2,3]. Our laboratory identified non-essential genomic regions of fowl adenovirus 9 (FAdV-9) for *in vitro* replication [4–6]. One of these regions is located at the left end of the genome at nucleotides (nts) 491–2782 [5]. This region consists of 6 open reading frames (ORFs O, 1, 1A, 1B, 1C and 2) with unclear functions in virus replication. Recently, we showed that one of these ORFs, ORF1 (a functional viral dUTPase), stimulates expression of type I interferon and other cytokines in addition to its role in viral DNA replication [7]. FAdV-9Δ4-EGFP is a virus derived from a deletion mutant virus FAdV-9Δ4 lacking the nts 491–2782 region and carrying the enhanced-green fluorescence protein (EGFP) expression cassette with the EGFP gene under the control of the human cytomegalovirus (CMV) promoter. FAdV-9Δ4-EGFP replicates at wild-type levels *in vitro* but deficiently *in vivo* [8,9]. Avian and mammalian cells infected with FAdV-9Δ4-EGFP express EGFP [8], suggesting that the CMV promoter is functional in various cell types. Importantly, chickens inoculated with FAdV-9Δ4-EGFP mount an antibody (Ab) response to EGFP [9], establishing the potential of FAdV-9Δ4 as a vaccine platform. Though CMV is a strong promoter traditionally used in many gene delivery systems including virus vectors [10], its strength could be affected by adjacent DNA sequences, methylation or cell type [11–14]. Alternate promoters such as CMV enhancer/chicken β-actin (CAG) and the human elongation factor 1 alpha (EF1α) are also used, though subject to the same limitations as reported for CMV [15,16]. Therefore, comparative studies on these promoters are necessary to improve the efficacy of FAdV-9-based vaccines in terms of antibody response to the transgene product. We generated replication competent recombinant FAdV-9 viruses (recFAdV-9s) with expression cassettes

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containing the EGFP gene under the control of CMV, CAG, and EF1α promoters with or without the presence of the posttranscriptional regulatory element of woodchuck hepatitis virus (WPRE). The effects of these elements on EGFP expression and antibody response are reported.

2. Materials and methods

2.1. EGFP expression in transiently transfected cells

The pCI-Neo vector (Promega) was modified to generate five dual-reporter expression constructs. The expression cassette was introduced into the multiple cloning site of pCI-Neo, with EGFP under the control of the CMV, CAG, EF1α, chicken β-actin or fowlpox virus L2R promoters. The neomycin resistance gene of the modified pCI-Neo was replaced with firefly luciferase (luc) under the control of the SV40 promoter to generate pCMV-EGFP-Luc, pCAG-EGFP-Luc, pEF1α-EGFP- Luc, pβ-actin-EGFP-Luc and pL2R-EGFP-Luc. Luciferase expression was used as a reference for normalization of fluorescence values and thus rule out sample-to-sample variations [17]. The WPRE element was incorporated between the stop codon and polyadenylation signal to generate another five constructs: pCMV-EGFP-WPRE-Luc, pCAG-EGFP-WPRE-Luc, pEF1α-EGFP-WPRE-Luc, pβ-actin-WPRE-Luc and pL2R-EGFP-Luc.

CH-SAH cells were transfected with dual-expression constructs and EGFP expression was examined at the indicated time points and normalized with respect to luciferase expression. Fold changes in EGFP expression from all constructs were calculated with respect to those measured for the CMV promoter.

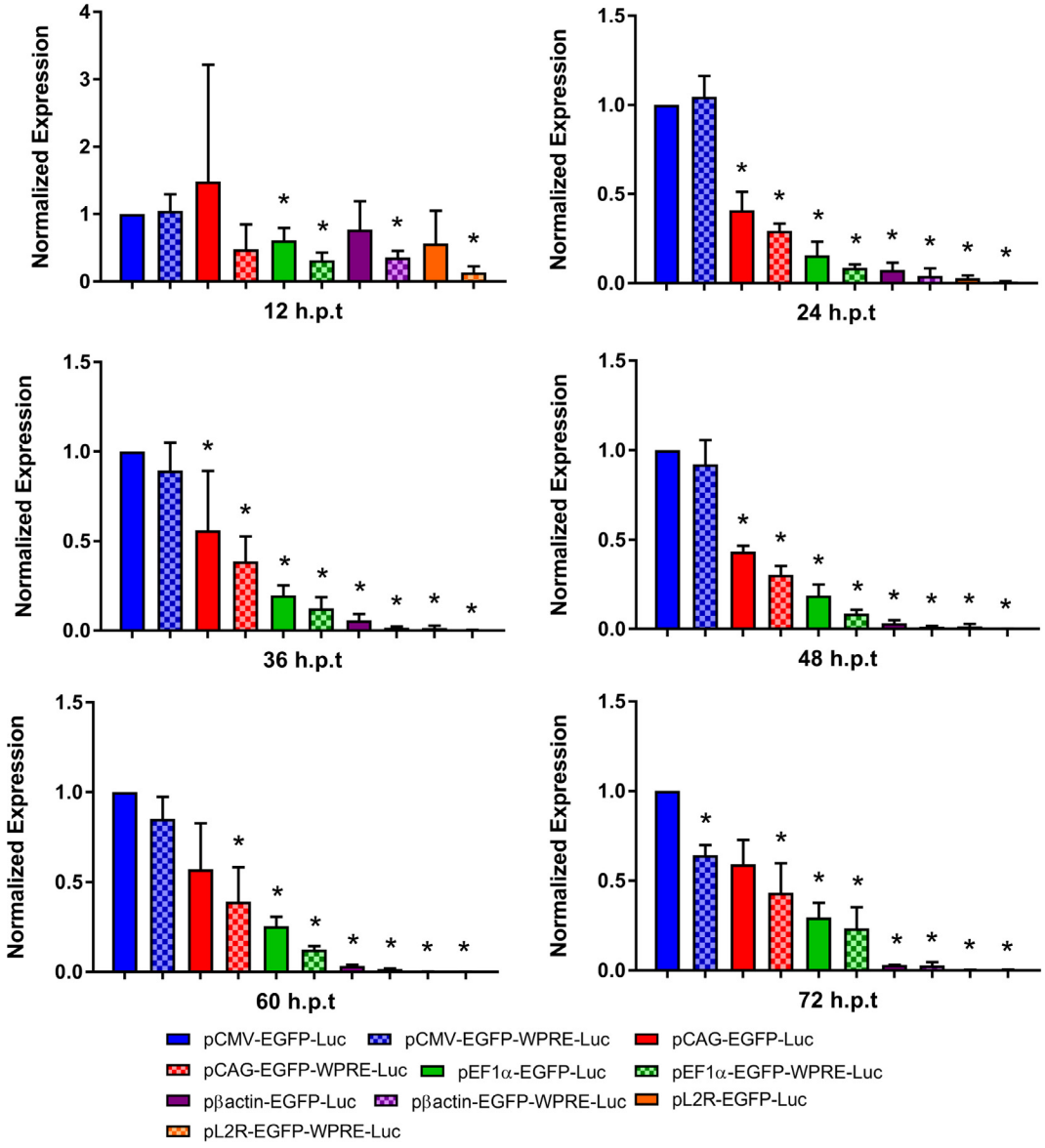

Fig. 1. EGFP expression in transiently transfected CH-SAH cells. CH-SAH cells were seeded in 35 mm dishes (1.8×10^6 cells/plate) and transfected with 2 μg of plasmid DNA. Transfected cells were washed, trypsinized, and resuspended in PBS at different hours post-transfection (h.p.t.). After three freeze-thaw cycles, samples were centrifuged and the protein concentration was determined. EGFP fluorescence was measured in a microplate reader at 480 and 528 nm excitation and emission wavelengths, respectively. Luciferase activity was measured using a Pierce Firefly Luciferase Glow Assay kit. The level of EGFP expression was then measured relative to that of luciferase to account for sample-to-sample variation. The dual-expression (fluorescence/luminescence) of each construct was normalized to the expression level of pCMV-EGFP-Luc (normalized to 1.0) at each time-point. The *t*-test was used to determine the significant difference of EGFP florescence compared to pCMV-EGFP-Luc, indicated by an asterisk (*), where $P < 0.05$. The error bars represent the standard deviation from three independent experiments.

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